**University of Alberta**

# NON-DIGESTIBLE FIBERS AND DIETARY INTERVENTIONS: ALTERNATIVE APPROACHES FOR TREATMENT OF INFLAMMATORY BOWEL DISEASE

by

PETYA TOSHEVA KOLEVA

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Dedicated to my beloved parents

Stoyanka and Tosho Kolevi

***Посветена на обичните ми родители Стоянка и Тошо Колеви***

**Abstract**

Crohn's disease (CD) and ulcerative colitis (UC), collectively called inflammatory bowel disease (IBD), are associated with chronic intestinal inflammation, affecting approximately 0.7 % of Canadians. Intestinal microbial dysbiosis have been related to IBD. Therapies that manipulate gut microbiota including interventions with non-digestible fermentable carbohydrates, present a promising treatment. This thesis aimed to test the hypotheses: non-digestible fermentable fibers prevent colitis development in experimental and human IBD, and bacterial fermentation products, mucosal integrity and gut associated lymphoid tissue may control microbiota of the host.

The anti-inflammatory properties of β-fructans mixture, fructo-oligosaccharides (FOS) plus inulin, were verified in a clinical trial with patients with mild to moderate active UC. Patients received 7.5 g or 15 g daily oral β-fructans for 9 weeks. Improvements in UC patients treated with β-fructans were associated with shifts in the composition of intestinal microbiota and improved butyrate formation.

The effects of the short-chain FOS versus the long-chain inulin were compared in an animal study of IBD, using HLA-B27 transgenic rats. The results demonstrated that FOS and inulin differ in colitis reduction and also vary in their effects on the intestinal microbiota in HLA-B27 transgenic rats. The protective effects of FOS in this animal model were associated with an increased abundance of bifidobacteria in intestinal microbiota.

A second animal study aimed to compare FOS and isomalto-oligosaccharides, with different structure, in combination with two different diets on inflammation

and intestinal microbiota composition in experimental IBD. The protective effects of fibers on colitis development depended on the diet background. The specific cecal microbiota changes were not associated with colitis reduction. Inflammation was positively correlated to protein fermentation in the colon but was negatively associated with carbohydrate fermentation.

The last study aimed to investigate if dysregulated mechanisms of intestinal epithelial barrier in IBD may contribute to microbial dysbiosis. Expression of genes, involved in barrier function, was studied in samples obtained from UC and CD patients. Gene expression of mucins and anti-microbial peptides was associated with IBD disease type and these findings confirm and extend previous work that epithelial barrier dysfunction may contribute to microbiota dysbiosis in IBD.

## Preface

This thesis has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta.

Chapter 1 is a literature review and my contribution was compiling all relevant information regarding the role of dietary fibers and diet interventions in inflammatory bowel disease.

A version of Chapter 2 has been published as: Koleva, P., Valcheva, R., Sun, X., Gänzle, M. and Dieleman, L. (2013) 'Inulin and fructo-oligosaccharides have divergent effects on colitis and commensal microbiota in HLA-B27 transgenic rats.' British Journal of Nutrition, 108:1633 – 1643. My contribution was in performing all laboratory techniques except histology scoring (performed by Dr. Dieleman), data analysis and interpretation. I also wrote the first draft of the manuscript for comments by co-authors.

Chapter 3 was submitted for publishing to the Journal of Nutrition as: Koleva, P., Ketabi, A., Valcheva, R., Gänzle, M. and Dieleman, L. 'Chemically defined diet alters the protective properties of fructo-oligosaccharides and isomalto-oligosaccharides in HLA-B27 transgenic rats.' My contribution was in performing the laboratory techniques and bioinformatics analysis of the data, as well as writing the first draft of the manuscript. Ali Ketabi contributed to this study by conducting and completing the experiments with the rat chow diet.

Chapter 4 contains experimental work performed by Dr. Rosica Valcheva under the supervision of Prof. Leo Dieleman and Prof. Michael Gänzle. My contributions to this study are involved in pyroseqeuncing and analysis of

sequencing data. A version of this chapter is in preparation for submission to PLOS ONE as: Valcheva, R., Koleva, P., Martínez, I., Walter, J., Gänzle, M., and Dieleman, L. 'Specific microbiota changes and butyrate production are associated with reduction of inflammation in presence of β-fructans.' Dr. Valcheva wrote the first draft of the manuscript. I contributed in the writing with a section from Materials and Methods regarding the performing of the pyrosequencing and data analysis.

A version of Chapetr 5 is in preparation for submission to Journal of Infection and Immunity. Dr. Wine's laboratory from the Department of Pediatrics at the University of Alberta, contributed to this study with the isolation of *E. coli* strains from biopsy samples obtained from non-IBD subjects and CD patients. My contribtuons to this chapter include the conduction of the virulence facors screening, gene expression, data analysis. I also wrote the first draft of the manuscript.

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Before I finish, I would like to express my deepest gratitude to my parents, Stoyanka and Tosho Kolevi, for their unconditional love and support. They were always inspiring and encouraging me to pursue my goals throughout my whole life.

Petya Koleva

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## II. List of Abbreviations

| | |
|---|---|
| CD | Crohn's disease |
| CEACAM6 | Carcinembryogenic antigen cell adhesion molecule 6 |
| Df | Degree of freedom |
| DGGE | Denaturing gradient gel electrophoresis |
| DNA | Deoxyribonucleic acid |
| FDP | Degree of polymerisation |
| DSS | Dextran sodium sulphate |
| ELISA | Enzyme-linked immunosorbent assay |
| FOS | Fructo-oligosaccharides |
| GC | Gas chromatography |
| GGS | Gross gut score |
| GI | Gastrointestinal |
| HLA | Human leukocyte antigen |
| HPLC | High performance liquid chromatography |
| IBD | Inflammatory bowel disease |
| IBS | Irritable bowel syndrom |
| IL-1β | Interleukin 1β |
| IL-23R | Interleukin 23 receptor |
| NSAID | Non-steroidal anti-inflammatory drug |
| PCA | Principle component analysis |
| PCR | Polymerase chain reaction |
| PUFA | Polyunsaturated fatty acids |

| | |
|---|---|
| qPCR | Quantitative polymerase chain reaction |
| rRNA | Ribosomal ribonucleic acid |
| SCFA | Short-chain fatty acids |
| TNF-α | Tumor necrosis factor alpha |
| UC | Ulcerative colitis |

# The Role of Intestinal Microbiota and Dietary Interventions in Inflammatory Bowel Disease: A Literature Review

## 1.1. Inflammatory bowel disease

The inflammatory bowel disease (IBD) is a group of remitting and relapsing disorders characterized by chronic inflammation within the gastrointestinal tract (Kaser *et al*., 2010). The incidence and prevalence of IBD is increasing worldwide (Cosnes *et al*., 2011), with the highest rates observed in Europe and North America (Cosnes *et al*., 2011; Molodecky *et al*., 2012). The incidence of IBD has increased in developing countries and is associated with industrialization and urbanization (Molodecky *et al*., 2012). This is also demonstrated with the increased incidence of migrants who moved from areas with low disease prevalence to countries with westernized style of life (Jayanthi *et al*., 1992). Canada is one of the countries with the highest incidence and prevalence of IBD, where approximately 0.7 % of the population is affected (www.ccfc.ca). Worldwide, about 25 % of new cases of IBD are identified during childhood and young adulthood, where some studies indicate peak incidence of diagnosis in the second and third decades (Molodecky *et al*., 2012). A second peak in latter decades of life has been reported with occurrence of IBD at 60-70 years (Cosnes *et al*., 2011).

There are two main clinically defined phenotypes of IBD, Crohn's disease (CD) and ulcerative colitis (UC), which vary from each other in features and location of inflammation, and profile of complications. UC is characterized by mucosal and continuous inflammation that is limited to the colon, and extends proximally from the rectum (Head *et al*., 2003). Clinical symptoms typical for UC involve severe diarrhea, blood loss, and loss of peristaltic function (Head *et al*., 2003). In more

severe cases, complications of the disease can lead to toxic megacolon and perforations. In contrast, CD can affect any part of the gastrointestinal tract (GI) (i.e. from mouth to anus), but terminal ileum, colon and peri-anal areas are more commonly involved (Hart and Ng, 2011). Inflammation is granulomatous and transmural, affecting all layers of the gut wall. Submucosal fibrosis, granuloma and fistula formations, intestinal strictures, and abscesses are common complications for CD, but not typical for UC (Head *et al*., 2003). Clinical symptoms of CD include diarrhea, abdominal pain, weight loss, anemia and poor nutrient absorption, narrowing of the gut lumen (Hart and Ng, 2011). Despite the differences between these two conditions, there are some overlapping clinical and pathological features that suggest similar origin of the disease (Hart and Ng, 2011; Head *et al*., 2003). Although the exact cause of IBD is not fully understood, the complex interaction of genetic susceptibility, dysregulated immune response, environmental triggers and microbiota are believed to contribute to the disease pathogenesis of IBD.

Linkage analysis and genome-wide association studies have proposed that genetic predisposition is important in determining IBD susceptibility. Seventy one IBD susceptibility loci have been associated uniquely with CD, 47 have been confirmed in UC alone, and 21 IBD susceptibility loci are common to both conditions (Lees *et al*., 2011). The multiple genes associated with IBD are involved in different pathways maintaining host intestinal homeostasis such as: genes included in cellular innate immunity and autophagy; defective bacterial killing; IL-23R/$T_H$17 signalling; imperfect epithelial barrier function; and genes

important for the adaptive immune response (van Limbergen *et al*., 2007; Lees *et al*., 2011).

Along with genetic predisposition, environmental triggers are also required and contribute to the development of IBD. Established factors that are associated with increased or decreased risk of IBD involve the composition of intestinal microbiota, diet, appendectomy, cigarette smoking, stress, formula-fed versus breast feeding, airborne pollutants, use of non-steroidal anti-inflammatory drugs (NSAIDs) and oral contraceptives (Cabre and Domenech, 2012; Ananthakrishnan, 2013). Smoking is one of the most widely studied environmental factors affecting the pathogenesis of IBD, with opposite effects in CD and UC. Active smoking is a risk factor for postoperative recurrence in CD, whereas it has a protective effect in UC, suggesting a reduced risk of colectomy (Mahid *et al*., 2006). Appendectomy, if it is performed in children before the age of 10, is also known to have a protective role in UC (Koutroubakis *et al*., 2002). Medications, such as NSAIDs and oral contraceptives, may increase the risk of CD or UC, but further research is required in order to define their role in the pathogenesis of IBD (Ananthakrishnan, 2013). Airborne pollutants and stress are other environmental factors that have been associated as causative components for disease development in IBD (van Eeden *et al.*, 2001; Lerebours *et al*., 2007). The role of intestinal microbiota and diet in IBD triggering and progression has been also extensively studied, and detailed discussion for those environmental factors is provided below.

## 1.2. Intestinal microbiota in the pathogenesis of inflammatory bowel disease

### 1.2.1. Composition and function of intestinal microbiota in healthy individuals

The human body is colonized by a huge number of bacteria, archaea, viruses and eukaryotic microorganisms, where the gastrointestinal (GI) tract is the most heavily inhabited organ (Ley *et al*., 2006a). The human gut microbiota consists of approximately $10^{12}$ bacteria and more than 1000 prevalent species (Marchesi, 2010). The gut bacteria are termed 'gut microbiome' and collectively contain about 100 times more genes than the human genome (Qin *et al*., 2010). The products of those genes are involved in breaking down dietary fibers, amino acids, drugs, and producing methane and/or vitamins (Arumugam *et al*., 2011). Studies have been focused on the intestinal prokaryotic microbiota for the last few decades, since the unicellular eukaryotes have very low diversity in the human intestines (Scanlan and Marchesi, 2008).

The gut bacteria comprise a large and dynamic community that is not homogeneous. The diversity and numbers of intestinal microbiota increase from stomach to colon (Hooper *et al*., 2001). The stomach and duodenum contain $10^1$ to $10^3$ bacterial cells per gram luminal contents, progressing to $10^4$ to $10^7$ bacteria per gram in jejunum and ileum (O'Hara and Shanahan, 2006). The numbers of bacteria in the large intestine are the highest and reach concentrations of up to $10^{11}$ to $10^{12}$ cells per gram luminal digesta (O'Hara and Shanahan, 2006). The majority of bacteria in the human GI tract belong to two different phyla *Bacteroidetes,* comprising Gram-negative bacteria, and *Firmicutes*, consisting of

Gram-positive microorganisms (Eckburg *et al*., 2005). The rest are classified as bacteria belonging to *Proteobacteria*, *Actinobacteria*, *Verrucobacteria* and *Fusobacteria* (Eckburg *et al*., 2005). About 80 % of intestinal bacteria still cannot be cultured (Eckburg *et al*., 2005). In humans, low diversity of bacterial groups is observed at phylum and genus levels, however it is considerably high at species level (Dethlefsen *et al*., 2007). Moreover, the composition of bacterial species varies greatly between individuals (Guarner and Malagelada, 2003), and it is shaped in response to diet and other environmental factors as well as the host genetic background (Turnbaugh *et al*., 2009). In addition, the gut microbiota are unique in the divergent mammal species, although humans and rodents share similar microbial communities at phylum level, with dominant *Firmicutes* and *Bacteroidetes* (Eckburg *et al*., 2005; Ley *et al*., 2005).

The intestinal microbiota play important functions in epithelial homeostasis, nutrition and immunity including: (i) fermentation of non-digestible dietary fibers; (ii) vitamin production; (iii) protection of the host against invading pathogens; (iv) development and regulation of the immune system; (v) and enteric nerve regulation (Montalto *et al*., 2009; Lee and Mazmanian, 2010; Holmes *et al.,* 2011).

### 1.2.2. Gastrointestinal physiology and intestinal microbial composition in rodents

Rodents have been widely used as animal models of different human diseases, and likewise of IBD, and have greatly improved our understanding of the cause and progression of those disorders. The stomach of rodents is characterized by

glandular and non-glandular parts (Kararli, 1995). The glandular part of the stomach is characterized with a tick wall and is covered with columnar epithelium, occupied by gastric glands, whereas the non-glandular portion of the stomach is lined with stratified squamous epithelium and is used for storage and digestion of food. In contrast, the stomach of humans is of glandular type (Kararli, 1995). Unlike humans who are omnivores and characterized with colonic microbial fermentation, rodents are herbivores and they have well developed cecum where the microbial fermentation occurs (Kararli, 1995). In addition to the physiological differences, the microbial composition of the rodent stomach also varies. Lactobacilli are the dominant bacterial group in this part of the GI tract and they colonize the stratified squamous epithelium in rodents (Walter, 2008). *Lactobacillus* group comprises up to 25 % of the intestinal microbiota of rodents in comparison with lactobacilli in human gut microbiota that are mainly allochthonous members obtained through the food (Walter, 2008). Based on phylum levels, rodents' intestinal microbial compositions are similar by those of humans, with *Firmicutes* and *Bacteroidetes* dominating (Eckburg *et al*., 2005; Ley *et al*., 2005). However, it was reported by Ley and co-authors that 85% of the bacterial taxa in the cecal microbial community of mice represent genera that have not been detected in humans (Ley *et al*., 2005).

### 1.2.3. The intestinal microbiota in inflammatory bowel disease

IBD is one of the several 'modern epidemics' in the western countries, in which pathogenesis has been associated with shifts in the composition of the intestinal microbiota (dysbiosis). Intestinal micro-organisms and their metabolic products

play an important role in the initiation and perpetuation of chronic intestinal inflammation (Farrel and La Mont, 2002). The most convincing evidence has been obtained from some genetically engineered experimental models of IBD that fail to develop chronic colitis under germ-free conditions (Taurog *et al*., 1994; Sartor, 2004). Furthermore, administration of (specific) bacteria into their environment can induce the development of spontaneous colitis (Taurog *et al*., 1994; Rath *et al*., 1996; Rath *et al*., 1999). The role of the microbiota in IBD is further confirmed in human trials, where surgical diversion of the fecal stream can decrease disease activity in CD patients (Fichera *et al*., 2005). Other observations are that inflammation tends to occur in the areas of the GI tract with the highest number of luminal bacteria (Thompson-Chagoyan *et al*., 2005), whereas broad spectrum antibiotics can shift the intestinal microbiota and have therapeutic roles in UC and CD (Peppercorn, 1997; Casellas *et al*., 1998).

There are several hypotheses that have been proposed to explain the role of intestinal bacteria in the pathogenesis of IBD: (i) IBD is triggered by an unidentified infectious agent within the intestine (Sibartie *et al*., 2010); (ii) changes in the composition and/or function of the intestinal bacteria can initiate chronic inflammation (Strober *et al*., 2007); (iii) the defective epithelial barrier function contributes to the disease pathogenesis (Strober *et al*., 2007); (iv) IBD is characterized by an abnormal mucosal immune response towards intestinal microbiota, leading to loss of tolerance (Strober *et al*., 2007). These theories are not mutually exclusive; instead a combination of many host and microbial factors lead to the pathogenesis of IBD. Evidence shows that a constant bacterial stimulus

is necessary to initiate and perpetuate inflammatory process (Aranda *et al*., 1997), but an abnormal mucosal immune response and defects in the epithelial barrier function are also needed to facilitate this process (Strober *et al*., 2007). Improving our understanding how the intestinal microbiota function, of how they interact with the host immune response, and how they are influenced by the host genetic background will perhaps allow us to fully understand the pathogenesis of IBD and possibly provide new therapeutic targets for a better treatment.

## 1.3. Diet and inflammatory bowel disease

### 1.3.1. Impact of diet in shaping the intestinal microbiota

Diet is another environmental factor that plays an important role in shaping the functional properties and composition of the intestinal microbiota (Xu *et al*., 2013). In general, the gut microbiota is relatively stable throughout the life span of humans, however, alteration in diet may influence the microbial community composition and metabolic activity leading to biological changes in the host (Kau *et al*., 2011). The effect of diet on the compositional structure of the intestinal microbiota has been observed during the primary colonization of human gut at birth (Fallani *et al*., 2011). Feces collected from breast-fed infants have higher levels of *Bifidobacterium* spp., whereas those collected from formula-fed infants are dominated by *Bacteroides* spp. and *Clostridium* cluster XIVa (Fallani *et al*., 2011). More animal fat and sugar, and less vegetables and fiber are typical characteristics of the modern Western diet compared to the diet in developing countries (Devereux, 2006). Humanized gnotobiotic mice were fed a diet low in

fat and rich in plant polysaccharides, and when their diet was switched to a Western diet, it modulated their microbial composition and activity (Turnbaugh *et al*., 2009a). Their gut microbiota was characterized with increased abundance of bacteria belonging to *Firmicutes* phylum and a decrease of those belonging to *Bacteroidetes* (Turnbaugh *et al*., 2009a). Striking differences have been reported in the luminal microbiota composition of rural African children on a diet high in carbohydrate and low in animal protein compared to European children on typical Western diet high in animal fat and proteins (De Filipo *et al*., 2010). The children from the African cohort at Burkina Faso had higher levels of *Bacteroidetes* and a lower proportion of *Firmicutes* than the Europeans (De Filipo *et al*., 2010). Furthermore, the African children were found to contain unique bacterial species belonging to *Prevotella* and *Xylanibacter* genera, encoding genes required for the hydrolysis of complex plant polysaccharides, such as cellulose and xylan. The effect of diet on the composition of the intestinal bacteria has been shown in a human study, where 17 healthy volunteers have been fed 4 controlled diets as follows: basal diet; a low-phytochemical diet; enriched with refined grains without fruits or vegetables diet; and a high cruciferous vegetable diet (Li *et al*., 2009). The authors observed that the fecal bacterial composition was modified by the diet of enriched cruciferous vegetables and that the change of the bacterial community was individual-specific. It is also well known that dietary fibers can modulate the profile of the gut microbiota (Tuohy *et al*., 2001; Vulevic *et al*., 2008). Furthermore, it was reported in many studies that the inulin-type fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) have the potential to

stimulate beneficial bacteria in the GI tract, such as lactobacilli or bifidobacteria, or both (Sartor, 2004; Vulevic *et al*., 2008).

Diet influences not only the compositional structure of the intestinal bacteria, but also their metabolic activity. Changes in the production of colonic SCFA have been observed in obese individuals in response to alterations in their diet (Duncan *et al*. 2007). Moreover, the study of the European and African children discussed above reported significantly lower concentrations of SCFA in European children compared to the African cohort (De Filipo *et al*., 2010). Shifts in bacterial composition due to changes in food intake have been in conjunction with significant alterations in bacterial secretion systems, protein export and lipoic acid levels in a human trial with long-term-dietary intervention (Wu *et al*., 2011). Additionally, diets enriched in proteins are able to enhance the activity of bacterial enzymes such as β-glucoronidase, azoreductase and nitroreductase (Brown *et al*., 2012). Overall, diet has an essential role in shaping the gut microbiota composition and their metabolic activity. By altering key bacterial populations it may disrupt the healthy microbiota and transform it into a disease-inducing entity that may contribute to the development of inflammatory responses (Brown *et al*., 2012).

### 1.3.2. Role of diet in the pathogenesis of inflammatory bowel disease

Dietary factors are the second most abundant luminal antigens after bacterial antigens and several lines of evidence suggest that they play a crucial role in the pathogenesis of IBD. Diet may influence intestinal inflammation through different mechanisms involving a direct antigen effect, alteration of host gene expression,

effects on gut permeability, and modulation of inflammatory mediators (Amre *et al*., 2007; Goodman and Gordon, 2010). In addition, diet may also influence inflammation indirectly via impact on gut microbiota (Dore and Corthier, 2010). Thus, the role of diet as a risk factor of developing IBD has been widely investigated in a large number of observational, case-control and prospective cohort studies. Many studies have suggested that dietary components, such as high intake of refined sugars, starch, a high caloric diet and processed fat are associated with an increased risk of IBD, CD in particular (Sakamoto *et al*., 2005; Amre *et al*., 2007). Conversely, increased consumption of dietary fiber, including fruits and vegetables, has been demonstrated to decrease the risk of developing IBD (Halfvarson *et al*., 2006; Amre *et al*., 2007), in fact some of the fermentable fibers have been regarded as protective dietary components against the development of IBD (Cherbut *et al*., 2003; Furrie *et al*., 2005; Hoentjen *et al*., 2005). Overall, a large number of nutrients and foods have been investigated and demonstrated to play a role in the etiology of IBD (Halfvarson *et al*., 2006; Amre *et al*., 2007; Goodman and Gordon, 2010).

## 1.4. Therapeutic approaches in IBD

### 1.4.1. Conventional treatment approaches in IBD

CD and UC are incurable so far, however, the purposes of the current therapeutic strategies are focused inducing and maintaining the disease in remission in order to improve the quality of life of patients. Symptoms of the disease can be controlled with different medications, whereas surgical interventions maybe

required in severe cases and complications. Conventional therapies for IBD involve aminosalicylates, antibiotics, corticosteroids and immunomodulatory agents, such as methotrexate, azathioprine, 6-mercaptopurine (Burger and Travis, 2011), and anti-TNFα agents, where the choice of therapeutic strategy depends on disease severity, patient acceptability and mode of drug delivery.

### 1.4.2. Alternative therapeutic manipulations of the intestinal dysbiosis in IBD

Intestinal microbiota have a symbiotic role with the host and are an important factor in the maintainance of host health. The composition and function of the gut bacteria is altered in IBD and these shifts may take part during the disease progression and maintenance (Engle *et al*., 2002; Farrel and La Mont, 2002; Musso *et al*., 2010; Salonen *et al*., 2010; Karlsson *et al*., 2013). Hence, there is great interest in developing new therapeutic approaches and disease prevention strategies for manipulating the composition of the intestinal microbiota to affect the course of the disease.

Alternative approaches for restoring the intestinal dysbiosis in IBD can be achieved through several mechanisms: (i) antimicrobial treatments; (ii) probiotics; (iii) fecal transplantation; or (iv) interventions with dietary fibers/diets. The use of antibiotics remains one of the most effective tools of controlling infectious diseases. Even though no specific infectious agent has been identified as the cause of IBD, antibiotics are often used as an initial treatment approach for this GI disorder (Sartor, 2004). It is believed that antibiotics can control symptoms of IBD by directly influencing intestinal bacteria and repressing gut associated

immune response (Sartor, 2004). Furthermore, antibiotic treatments can be effective as long-term therapy in some IBD patients, in particular in CD patients with fistulas and recurrent abscesses (Rahimi *et al*., 2006) as well as to prevent postoperative CD recurrence. Probiotics have been defined by WHO/FAO as "live micro-organisms which, when administered in adequate amounts confer a health benefit on the host" and are also a potential way to manipulate intestinal dysbiosis (http://www.fda.gov). It is believed that probiotics achieve their beneficial effects through the following mechanisms: competing with pathogenic bacteria; improving epithelial and mucosal barrier function; and altering immunoregulation (Sartor, 2004). Several probiotic interventions have shown promise as therapeutic options in UC (Rembacken *et al*., 1999; Kruis *et al*., 2001), however results of probiotic trials with CD patients have been disappointing (Parntera *et al*., 2002; Sartor, 2004). The fecal transplantation of intestinal microbiota from a healthy donor to restore the gut microbiota of a diseased individual has recently been used for the management of IBD (Anderson *et al*., 2012). Fecal microbiota transplantation can be an effective and safe alternative treatment for IBD (Anderson *et al*., 2012), however, the evidence comes from case reports. Well-designed controlled trials are required in order to confirm the positive outcomes of these case reports. Dietary fibers and diet also have great potential to manipulate intestinal microbial dysbiosis in IBD (Sartor, 2004; Dore and Corthier, 2010), and their role as alternative therapeutic options is discussed in detail below.

### 1.4.3. Non-digestible fermentable fibers and inflammatory bowel disease

Non-digestible dietary fibers that are fermented by intestinal microbiota are widely emerging as promising dietary therapeutic approaches in IBD to reduce chronic intestinal inflammation. Dietary fibers are an important component of the human diet. These are a complex group of substances consisting of resistant starch, non-starch polysaccharides, plant cell-wall polysaccharides (cellulose and hemicellulose), oligosaccharides, pectins, gums, and lignin (Flint *et al*., 2008). In terms of their physiological and nutritional role, dietary fibers can be divided into soluble fibers, which are easily fermentable by intestinal bacteria, and insoluble fibers - barely fermentable carbohydrates. Furthermore, dietary fibers can be classified as digestible and non-digestible. The digestible carbohydrates are hydrolized by the enzymes of the host gastrointestinal system and the monomers resulting from this hydrolysis are absorbed in the small intestines. On the other hand, non-digestible fibers cannot be broken down by the mammalian enzymes and reach the large intestine mainly intact. The gut microbiota play a crucial role in metabolizing the non-digestible oligosaccharides and polysaccharides in the colon (Gill *et al*., 2006). The primary difference between oligosaccharides and polysaccharides is their degree of polymerisation (DP) (Cummings and Stephen, 2007). Besides their chain length, the classification of dietary fibers also depends on their structural monomers and type of linkages (α or β) (Cummins and Stepeh, 2007).

The beneficial effects of intestinal microbiota on the host are mainly mediated by their metabolites. Dietary fibers are fermented by gut bacteria to SCFA, where acetate, propionate and butyrate are the major end-products (Flint *et al*., 2008). The host epithelial cells transport the SCFA either by passive diffusion or with the help of membrane-bound transporters such as monocarboxylic acid transporters (MCT1) (Saksena *et al*., 2009) and solute carrier family 5 member (SLC5A8) (Gopal *et al*., 2004). SCFA play a key role in the colonic epithelial homeostasis by helping to maintain the intestinal barrier function, stimulating water and NaCl absorption, regulating epithelial proliferation, modulating immune response, decreasing oxidative DNA damage, and helping to prevent colonic cancer (Xu *et al*., 2013). Diet and dietary fibers can modulate the intestinal bacterial composition and their metabolic profiles. African children consuming diets with high fiber content, had significantly higher SCFA concentration compared to European children (De Filippo *et al*., 2010). Experimental and clinical studies have also shown that low concentrations of SCFA are associated with IBD (Galvez *et al*., 2005).

There is evidence from animal models that dietary fibers may provide a therapeutic option in the treatment of IBD. Lactulose and goat milk oligosaccharides are fibers that mediate reduction of dextran sulfate sodium (DSS)-induced colitis in Wistar rats (Rumi *et al*., 2004). Treatment with a mixture of inulin and FOS reduced colitis in HLA-B27 transgenic rats (Hoentjen *et al*., 2005). Other studies in rats reported protective effects of inulin on DSS-induced colitis (Videla *et al*., 2001), and protective effects of FOS on trinitrobenzene

sulfonic acid induced colitis (Cherbut *et al*., 2003). Not all studies using fructans have reported positive outcomes. Resistant starch but not FOS, improved DSS-induced colitis in rats (Moreau *et al.,* 2003). However, a direct comparison of dietary fibers differing in their molecular weight and structure in an animal model of colitis has not been reported.

A number of dietary fibers have also been investigated in clinical trials with UC patients. In an open-label trial, germinated barley foodstuff, a nutritional supplement high in glutamine-rich protein and hemi-cellulose-rich fiber, has been shown to reduce both clinical and endoscopical severity in UC with mild to moderate activity (Bamba *et al*., 2002). A symbiotic treatment of inulin plus *Bifidobacterium longum* was effective in terms of clinical improvement in patients with active UC in pilot study (Furrie *et al*., 2005). Although, the colitis-protective effects of non-digestible fermentable carbohydrates are often attributed to bifidobacteria and lactobacilli, other mechanistic explanations are relatively unknown and are part of the current thesis.

## 1.5. Hypothesis and Objectives

This thesis aimed to test two hypotheses that non-digestible fermentable fibers prevent colitis development in experimental and human IBD, and bacterial fermentation products, mucosal integrity and gut associated lymphoid tissue may control microbiota composition of the host. In order to investigate these hypotheses the following objectives were addressed:

- To compare the effects of the β-fructans, long-chain inulin versus short-chain FOS, on the composition of intestinal bacteria and colitis reduction in a preventive study of experimental IBD (Chapter 2).
- To study if feeding a purified AIN-76A diet, containing high sucrose amount, versus a standard rat chow diet, supplemented with or without FOS or isomalto-oligosaccharides (IMO), can modify the effects of these fibers on colitis development and intestinal microbiota in HLA-B27 transgenic rats (Chapter 3).
- To study if a combination of dietary β-fructans (fructo-oligosaccharides (FOS) : inulin /1:1/) can reduce inflammation in patients with mild to moderate UC (Chapter 4).
- To compare virulence factors of *Escherichia coli* strains isolated from IBD and non-IBD subjects, and investigate their association with dysregulated intestinal barrier function in IBD (Chapter 5).

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# Inulin and Fructo-oligosaccharides Have Divergent Effects on Colitis and Commensal Microbiota in HLA-B27 Transgenic Rats

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## 2.1. Introduction

Inflammatory bowel disease (IBD), encompassing ulcerative colitis (UC) and Crohn's disease (CD), is a group of chronic intestinal disorders associated with uncontrolled inflammation within the gastrointestinal tract (Hanauer, 2006). The pathogenesis of IBD is attributed to an interaction of genetic, immune and environmental factors (Hanauer, 2006). Intestinal micro-organisms and their products play an important role in the initiation and perpetuation of chronic intestinal inflammation (Farrell and Mont, 2002). Inflammation occurs in areas with the highest number of luminal bacteria (Thompson-Chagoyan *et al*., 2005). Broad spectrum antibiotics (Rath *et al*., 1996) and surgical diversion of the fecal stream can prevent disease recurrence in Crohn's disease (Fichera *et al*., 2005). Moreover, microbial imbalance, called 'dysbiosis', is observed in IBD patients (Frank *et al*., 2007). IBD patients have greater numbers of mucosa-associated intestinal bacteria compared to non-IBD controls (Swidsinski *et al*., 2002). A reduced abundance of *Bacteroidetes* and *Firmicutes* species and an increase of virulent *Escherichia coli* has been observed in the mucosa-associated microbiota of IBD patients (Swidsinski et al., 2002; Baumgart *et al*., 2007; Sokol *et al*., 2008; Thomazini *et al.,* 2011). The role of microbiota in IBD was further confirmed in animal models for IBD. HLA-B27/human β2-microglobulin transgenic rats raised under specific pathogen-free environment spontaneously develop chronic colitis mimicking IBD, and arthritis. These transgenic rats raised in germ-free conditions fail to develop inflammation, but introduction of (specific) bacteria induces colitis (Rath *et al*., 1996; Rath HC, Ikeda *et al*., 1999; Taurog *et al.,* 1994). Similarly,

other rodent models of colitis remain disease-free in the absence of bacteria (Taurog *et al.,* 1994).

Modification of the gastrointestinal microbiota may restore the balance of the host bacteria and reduce inflammation. Non-digestible carbohydrates such as β-fructans stimulate the growth of specific resident bacteria in the gut and thus allow manipulation of host microbiota. Inulin and fructo-oligosaccharides (FOS) are linear β(2→1) linked fructans. Inulin has a degree of polymerisation (DP) between 10 and 60, whereas FOS has a DP varying between 2 and 10. Dietary inulin and FOS are not hydrolysed by mammalian enzymes, but are readily fermented by the bacterial community in the cecum and colon, and favour the growth of intestinal bifidobacteria (Taurog *et al*., 1994; Sartor, 2004). These β-fructans occur at high levels in plants such as chicory, asparagus, leek, onion, garlic and Jerusalem artichoke, and are also produced by *Lactobacillus* spp. in cereal fermentations (Roberfroid *et al*., 1998; Rossi *et al.,* 2005). Studies in animal models for IBD indicate that a combination of inulin and FOS reduce chronic intestinal inflammation. Treatment of HLA-B27 transgenic rats with inulin and FOS reduced inflammation in conjunction with an increase of cecal *Lactobacillus* spp. and *Bifidobacterium* spp. (Campbell *et al*., 1997; Tieking *et al*., 2005). Inulin and FOS alone or in combination with two strains of *Bifidobacterium infantis* also reduced inflammation in dextran sodium sulphate (DSS)-induced colitis in rats (Osman *et al*., 2006). However, FOS exacerbated DSS-induced colitis in mice fed a purified diet (Goto *et al.,* 2010). Small clinical studies in patients with active UC reported reduced colonic inflammation after

oral therapy with *Bifidobacterium longum* in combination with the dietary fibers inulin and FOS (Furrie *et al*., 2005), whereas a reduction of the inflammatory fecal marker calprotectin was reported in active UC patients administered inulin plus FOS (Casellas *et al*., 2007). However, the use of FOS plus inulin mixture was ineffective in a large randomised, placebo-controlled trial in patients with mild-to-moderately active Crohn's disease (Benjamin *et al*., 2011). Whereas studies in animal models and some of the clinical trials indicate that prebiotics show promise in the treatment or prevention of IBD, the divergent outcomes of studies clearly demonstrate that additional data on the mechanisms of action of non-digestible carbohydrates are required before these can be recognised as a valid tool in the management of IBD.

Inulin and FOS differ in their effects on cecal, colonic and fecal microbiota of rats that were colonised with human microbiota (Kleessen *et al*., 2001). Likewise, dietary β-glucans differing in their molecular weight exerted different effects on the intestinal microbiota in pigs (Metzler-Zebeli *et al*., 2010). However, past studies on dietary intervention to prevent colitis in rodent models provided only a partial characterisation of intestinal microbiota (Hoentjen *et al*., 2005; Osman *et al*., 2006). Moreover, no studies have been performed using β-fructans with different degree of polymerision in a well-validated, spontaneous colitis model. It was therefore the aim of the present study to compare the effect of inulin or FOS on intestinal inflammation and to determine how changes in the profile of cecal and fecal microbiota correlate with colitis reduction in HLA-B27 transgenic rats, the model in which this fiber mixture was previously shown to be beneficial.

Inflammation was assessed by a validated gross gut score (GGS), histology score, and by quantification of pro-inflammatory mucosal IL1-β concentration.

## 2.2. Materials and Methods

### 2.2.1. Experimental design and sample collection

HLA-B27 transgenic rats, a validated colitis model, were used in this study. Animals were randomly assigned to three different treatment groups: (1) commercial standard diet as a control (5053 PicoLabw Rodent Diet 20; Lab Diet, Inc.); (2) FOS (average DP 4, Orafti P-95; Raffinerie Tirlemontoise); (3) inulin (average DP 25, Orafti HP). Then, 8 g/kg body weight of the respective fructans were mixed with the standard diet, a dose previously found to be optimal for its colitis-reducing effects (Dmytrash *et al*., 2006). Feed was provided *ad libitum* and the average feed consumption of the rats was 20 g of standard diet/d. The body weight of the animals was measured every 2 weeks and the fructan addition to the diet was adjusted according to the body weight. The number of animals included in the study was as follows: control group – thirteen females and nine males, FOS group – three females and nine males and inulin group – seven females and eight males. Treatment started at 4 weeks of age (before colitis occurred) and continued until 16 weeks of age. Fecal samples were collected from each group at 4 and 16 weeks of age. All rats were euthanised at 16 weeks of age. At necropsy, cecal and colonic tissues and their contents were taken for histology, quantification of mucosal IL-1β, as well as for microbiota analysis. All samples were immediately frozen and stored at -80°C. Animal trials were approved by the University of

Alberta Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines.

### 2.2.2. Assessment of treatment effects on inflammation

Cecal and colon tissues were fixed and stained with haematoxylin and eosin, as previously described (Rath *et al*., 1996). Blinded evaluation of cecal and colonic microscopic inflammation was performed using a validated histology score ranging from 0 to 4 (Rath *et al*., 1996). Histology score included the following parameters: (1) number of inflammatory and goblet cells, (2) mucosal thickening, (3) infiltration of submucosal cells and (4) destruction of the architecture of the intestinal epithelium. The degree of macroscopic inflammation was further assessed using a validated GGS based on a scale from 0 to 4, as previously described (Rath *et al*., 1996). Cecal inflammation evaluated by GGS included the criteria (1) number of cecal nodules, (2) severity of mesenteric contractions, (3) severity of adhesions and (4) extent of cecal wall thickening. The mucosal IL1-β concentration in the cecal and colonic homogenates was quantified by a rat-specific IL1-β ELISA (Rath *et al*., 1999), using a commercial DuoSet ELISA Development System kit (R&D Systems, Inc.). Recombinant rat IL-1β 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 normalisation of the targeted cytokine measurement and presented as mg IL-1β/g protein.

### 2.2.3. Genomic DNA extraction and quantitative PCR analysis

Bacterial DNA was extracted from the cecal and fecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, Inc.). Quantity and quality of DNA were checked on a NanoDrop spectrophotometer system ND-1000, version 3.3.0 (Thermo Fisher Scientific, Inc.). Before PCR analysis, samples were diluted to contain comparable DNA concentrations.

Quantitative PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). The primers and probes based on 16S rRNA gene sequences were chosen to target total bacteria, *Bacteroides-Prevotella-Porphyromonas* spp., the dominant group of Gram-negative bacteria, *Clostridium* clusters IV and XIVa, the main Gram-positive bacterial groups in intestinal microbiota, as well as clostridial clusters I and XI, which include pathogens (Table 2-1). Genes encoding *Clostridium difficile* toxin B and *Clostridium perfringens* α toxin were quantified by qPCR to specifically identify toxin-producing organisms in these clusters (Table 2-1). Microbiota analysis further included the *Enterobacteriaceae* family, which also includes pathogens, and bacteria known to have beneficial effect on the host such as *Bifidobacterium* spp. and *Lactobacillus-Pediococcus-Leuconostoc-Weissella* spp. (*Lactobacillus* group; Table 2-1). Samples were analysed in duplicate in MicroAmp Fast Optical ninety-six-well reaction plates capped with MicroAmp Optical Adhesive Film (Applied Biosystems). The PCR reaction mixtures consisted of 12.5 ml Fast SYBR Green Master Mix (Applied Biosystems), 0.4 mM of each primer (Table 2-1), 2 ml of template DNA of cecal or fecal samples and sterile Milli-Q water to final volume

of 25ml. The cycling programme was as follows: initial denaturation at 95ºC for 5 min; forty cycles of 95ºC for 15 s, primer annealing at the optimal temperatures (Table 2-1) for 30 s, and extension at 72ºC for 30 s. Melting curves were obtained by a stepwise increase of the temperature from 60 to 95ºC (at 10 s/0·5ºC). Melting-curve data were analysed to verify amplification of the correct targeted PCR products. For quantification with the gene-specific primers and the group-specific primers for *Clostridium* cluster XI, TaqMan assays were performed. Amplifications were carried out in a total volume of 25 ml, containing 12.5ml TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), 0.4 mM of each primer (Table 2-1), 1ml of TaqMan probe (100 nM), 2 ml of template DNA of cecal or fecal samples and 7.5ml sterile Milli-Q water. These amplification conditions were as follows: one cycle at 50ºC for 2 min; one cycle at 95ºC for 10 min; forty-five cycles of 95ºC for 20 s, primer annealing at the optimal temperatures (Table 1) for 30 s, and extension at 72ºC for 45 s. Fluorescent emission was measured at the extension step for the SYBR Green assays and at the primer annealing step for TaqMan assays. For generation of standard curves, 10-fold serial dilutions of purified and quantified PCR products were used. The standard curves of the individual qPCR assays were obtained by PCR using primers listed in Table 1 and genomic DNA isolated from *Clostridium perfringens* ATCC 13124, *Clostridium difficile* 75, or DNA extracted from the fecal samples. Individual reactions of the standard curves were run in duplicate on each plate for the respective bacterial group. The detection limit was $10^2$ copy

numbers/g cecal content or feces for the group-specific primers and primers for *C. difficile* toxin B and *C. perfringens* α toxin.

### 2.2.4. Analysis of fecal microbiota using PCR-denaturing gradient gel electrophoresis

A total of four sets of group-specific primers targeting regions of the bacterial 16S rRNA gene were used to investigate the diversity of *Lactobacillus* group, *Bifidobacterium* spp., *Bacteroides* group and *Clostridium* cluster IV by PCR-DGGE. PCR specific for bifidobacteria was performed using primers g-Bifid F and g-Bifid R-GC (Table 2-1) in a Gene Amp PCR System 9700 (Applied Biosystems). To assess diversity in the *Lactobacillus* group, *Bacteroides* group, and *Clostridium* cluster IV in fecal microbiota by DGGE, the respective group-specific primer sets were employed at the appropriate annealing temperature (Table 2-1). PCR products were then used as templates in a second PCR, performed with the same primer pairs with attached GC clamp to the 5' end of the reverse primer (Table 2-1). Amplicons were checked by electrophoresis in 2% (w/v) agarose gel before DGGE analysis. PCR fragments were analysed by DGGE with a DCode Universal Mutation Detection System (Bio-Rad) on a 6% (w/v) polyacrylamide gel (37.5:1 acrylamide–bisacrylamide). *Bifidobacterium*-specific amplicons were separated in 40–70% denaturing gradient (100% corresponds to 7 M-urea and 40% (w/v) formamide). Amplicons obtained with primers targeting *Lactobacillus* and *Bacteroides* groups, and *Clostridium* cluster IV were separated in a denaturing gradient from 30% to 55 %. Electrophoresis was performed in buffer containing 40 mM-Tris, 20 mM-acetic acid, 1 mM-

EDTA at a constant voltage of 130 V and a temperature of 60ºC for 4 h. Gels were stained with SYBR Safe 1 X solution (Invitrogen) for 1 h and 30 min and photographed by UV transillumination. DGGE profiles were compared using Bionumerics software (version 4.01, Applied Maths) and similarities were expressed based on Pearson correlation coefficients. All gels were normalised using a reference sample with bands distributed throughout the whole gel.

### 2.2.5. DNA sequencing

A total of five samples were randomly chosen and amplified in duplicates with group-specific primers for bifidobacteria (Table 2-1). Samples were analysed by DGGE, and the bands obtained with primers g-Bifid F and g-Bifid R-GC were sequenced by service of Macrogen. Sequences were compared to those in the Ribosomal Database Project (rdp.cme.msu.edu/seqmatch). The GenBank accession numbers for the sequences are HQ283419, HQ283420, HQ283421, HQ283422 and HQ268606.

**Table 2-1.** Primers and PCR conditions.

| Target group/gene* | Oligonucleotide sequence (5′→ 3′) | Tm (ºC) | Reference or source |
|---|---|---|---|
| Total bacteria (200 bp) | F: CGGYCCAGACTCCTACGGG<br>R: TTACCGCGGCTGCTGGCAC | 63 | Lee *et al*., 1996 |
| *Lactobacillus* group (341 bp) | F: AGCAGTAGGGAATCTTCCA | 63 | Walter *et al*., 2001 |
| | R: CACCGCTACACATGGAG | | Heilig *et al*., 2002 |
| | GC clamp- CACCGCTACACATGGAG** | | This study |
| *Bacteroides* group (140 bp) | F: GGTGTCGGCTTAAGTGCCAT<br>R: CGGAYGTAAGGGCCGTGC | 60 | Rinttila *et al*., 2004 |
| | GC clamp- CGGAYGTAAGGGCCGTGC** | | This study |
| *Enterobacteriaceae* family (195 bp) | F: CATTGACGTTACCCGCAGAAGAAGC<br>R: CTCTACGAGACTCAAGCTTGC | 63 | Bartosch *et al*., 2004 |
| *Bifidobacterium* spp. (243bp) | F: TCGCGTCYGGTGTGAAAG<br>R: CCACATCCAGCRTCCAC | 60 | Rinttila *et al*., 2004 |
| (596 bp) | F: CTCCTGGAAACGGGTGG<br>GC clamp- GGTGTTCTTCCCGATATCTACA† | 65 | Matsuki *et al*., 2004 |
| *Clostridium* cluster I (120 bp) | F: ATGCAAGTCGAGCGAKG<br>R: TATGCGGTATTAATCTYCCTTT | 58 | Rinttila *et al*., 2004 |
| *Clostridium* cluster IV (239 bp) | F: GCACAAGCAGTGGAGT<br>R: CTTCCTCCGTTTTGTCAA | 60 | Matsuki *et al*., 2002 |
| | GC clamp- CTTCCTCCGTTTTGTCAA* | | This study |
| *Clostridium* cluster XIVa (438-441 bp) | F: AAATGACGGTACCTGACTAA<br>R: CTTTGAGTTTCATTCTTGCGAA | 58 | Matsuki *et al*., 2004 |
| *Clostridium* cluster XI (139 bp) | F: ACGCTACTTGAGGAGGA<br>R: GAGCCGTAGCCTTTCACT<br>FAM-GTGCCAGCAGCCGCGGTAATACG-BHQ | 58 | Song *et al*., 2004 |
| *Clostridium perfringens* α toxin (109 bp) | F: GCTAATGTTACTGCCGTTGA<br>R: CCTCATTAGTTTTGCAACC<br>6FAM-GCGCAGGACATGTTAAGTTTG-TAMRA | 55 | Messelhäusser *et al*., 2007 |
| *Clostridium difficile* toxin B (177 bp) | F: GAAAGTCCAAGTTTACGCTCAAT<br>R: GCTGCACCTAAACTTACACCA<br>FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA | 58 | van den Berg *et al*., 2006 |

Tm, melting temperature; F, forward; R, reverse; *Product size in breackets (bp)
**GC clamp sequence –
CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG
†GC clamp sequence –
CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC

### 2.2.6. Analysis of SCFA

Cecal contents and feces (100 mg) were mixed with 300 ml water, vortexed vigorously and centrifuged for 15 min at 20 800 g. The supernatant (100 ml) was mixed with 300 ml 7% perchloric acid, incubated at 4ºC overnight and the precipitates were removed by centrifugation for 5 min at 20 800 g. SCFA concentration was determined using HPLC (Agilent 1200 Series; Agilent Technologies, Inc.) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories). The column was eluted with 5 mM $H_2SO_4$ at a temperature of 70ºC and a flow rate of 0.4 ml/min. Detection of the SCFA was achieved employing UV (210 nm) and refractive index detectors. External standards were used to calculate the concentration of the SCFA.

### 2.2.7. Statistical analysis

Data analysis was performed using the mixed procedure (PROC MIXED) of the Statistical Analysis Systems (SAS Institute, Inc.). The differences in fecal microbiota and SCFA between groups were evaluated employing repeated measures ANOVA. There were two fixed effects – time and treatment, whereas the random variation was individual rats. The variance and covariance associated with the responses over time were assumed by compound symmetry structure. To detect differences between groups, cecal bacterial populations, SCFA and cecal tissue inflammation were analysed using randomised block design, in which treatments were fixed effects and animals were random effects. Degree of freedoms were estimated by the Kenward–Rogers method and the probability of difference was used to test differences between least square means of different

treatments. Results are expressed as least-square means with their standard errors. A P value of 0.05 was considered statistically significant.

Principal component analysis and linear discriminant analysis were performed using JMP software (version 8.0.1, SAS Institute, Inc.). The loading plot is a graphical representation of the amount of variation within the data set and shows the correlation of the individual variables of the first two principal components (PC1 and PC2). The correlations between mucosal inflammation and bacterial populations were assessed by Spearman's correlation test using GraphPad Prism version 5.00 (GraphPad Software).

## 2.3. Results

### 2.3.1. Effect of inulin and fructo-oligosaccharides on chronic intestinal inflammation

The evaluation of mucosal IL-1β concentration and histology scores indicated that the cecum was more inflamed in comparison with colonic tissue in rats on a control diet (Figure 2-1 A–C). Histology scores and GGS showed that inulin and FOS reduced inflammation in the cecum and colon (Figure. 2-1 A and B). The concentration of the proinflammatory cytokine IL-1β was decreased in cecal tissue of animals treated with inulin ($P<0.024$) or FOS ($P<0.001$) vs. control rats, further confirming a protective effect on intestinal inflammation by β-fructans treatment (Figure 2-1 C). However, GGS and histology scores indicated that the intestinal inflammation in FOS-treated rats was less severe compared to inulin-treated animals (Figure 2-1 A and B).

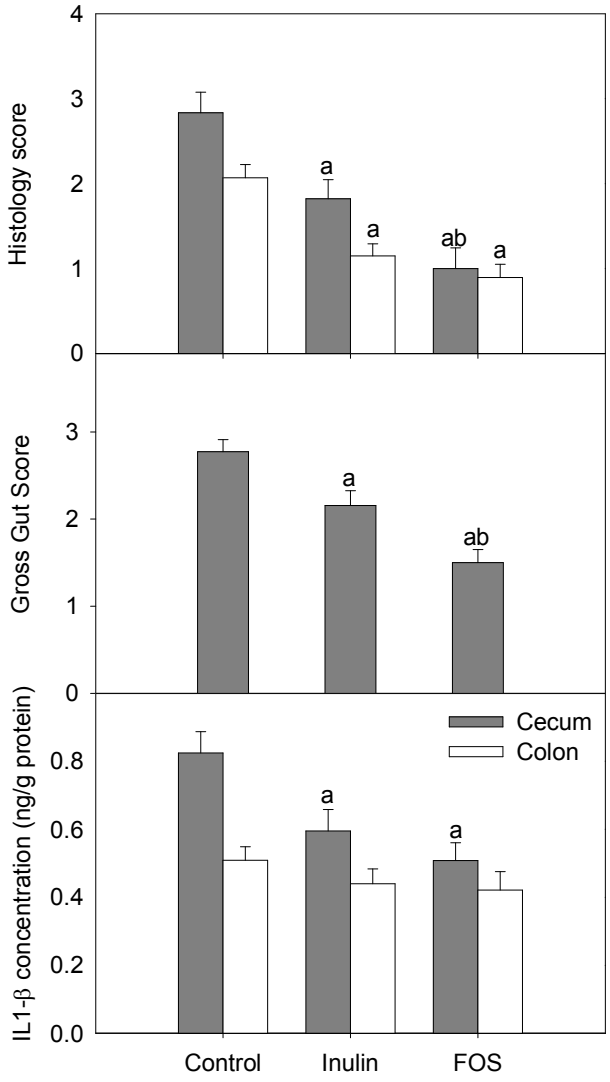


**Figure 2-1.** (A) Histology score, (B) gross gut score (GGS) and (C) IL-1β concentration of cecal (■) and colonic (□) tissue samples collected from HLA-B27 transgenic rats. The rats were either treated with inulin or fructo-oligosaccharides (FOS), or not. Values are least-square means, with their standard errors represented by vertical bars. [a,b,c]Least-square mean values (obtained from the same tissue) with unlike letters were significantly different ($P<0.05$).

### 2.3.2. Quantification of bacterial populations by quantitative PCR

To determine the dietary impact of the different treatments on cecal and fecal microbiota of HLA-B27 transgenic rats, qPCR was performed (Figures 2-2 and 2-3). Fecal and cecal samples were analysed to allow the comparison of samples from the same animal over time (fecal samples, Figure 2-2) as well as the analysis from the intestinal site with the highest degree of inflammation (cecal samples, Figure 2-3). For fecal microbiota, treatment with both fructans decreased the numbers of total bacteria and the *Bacteroides* group. Dietary intervention with fructans reduced *Clostridium* cluster XI and cluster XIVa by more than one log (Figure 2-2). FOS treatment increased the numbers of bifidobacteria and *Clostridium* cluster I ($P<0.001$); however, FOS-fed animals also showed significantly decreased copy numbers of *Enterobacteriaceae* ($P<0.023$) and *C. difficile* toxin B ($P<0.001$). Inulin specifically mediated the reduction of the *Lactobacillus* group ($P<0.03$) and clostridial cluster IV ($P<0.001$). Animals harboured high copy numbers of 16S rRNA genes of organisms belonging to the clostridial cluster I. This cluster comprises commensal fibrolytic and butyrate-producing bacteria as well as toxinogenic species such as *C. perfringens* (McLane *et al*., 2006). The low abundance of gene copy numbers of *C. perfringens* α toxin indicates very low numbers of toxinogenic *C. perfringens*. However, gene copy numbers of *C. difficile* toxin B were equivalent to the copy numbers of 16S rRNA genes of *Clostridium* cluster XI, which demonstrated that *C. difficile* was the main representative of that clostridial cluster (Figures 2-2 and 2-3).

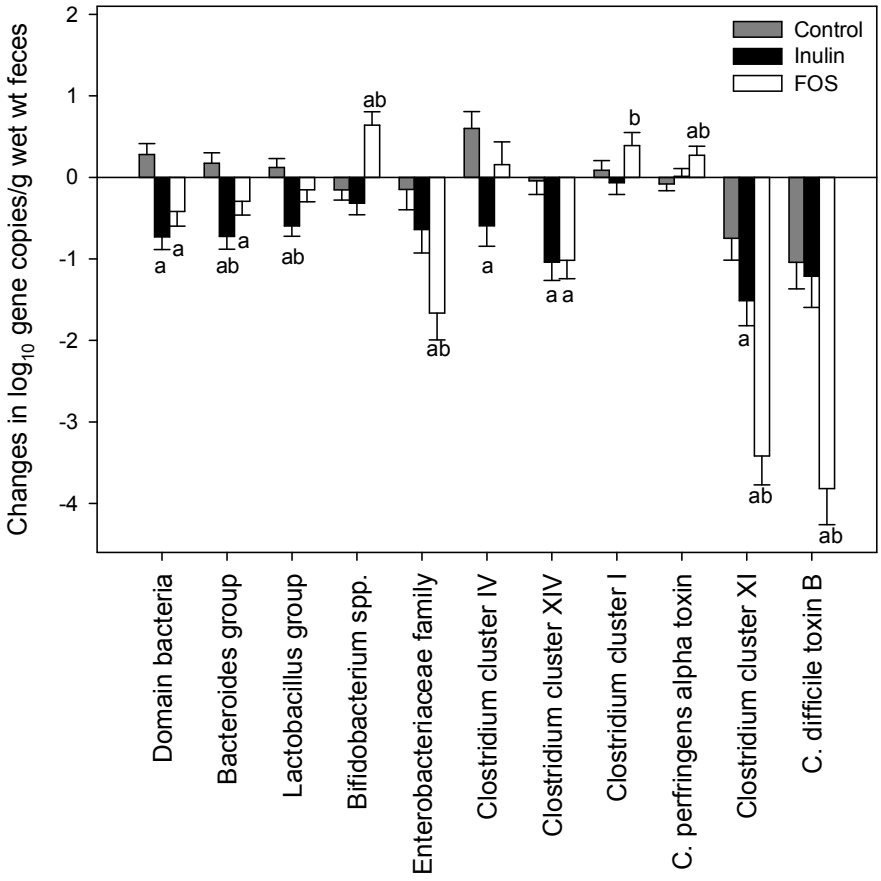


**Figure 2-2.** Quantification of bacterial populations of fecal samples ((▒) control; (■) inulin; (□) fructo-oligosaccharides) collected from HLA-B27 transgenic rats before and at the end of the fiber treatments. Values are change in log10 copy numbers of 16S rDNA (week 16 - week 4) least-square means, with their standard errors represented by vertical bars. [a,b,c]Least-square mean values (obtained with the same primer pairs) with unlike letters were significantly different ($P<0.05$).

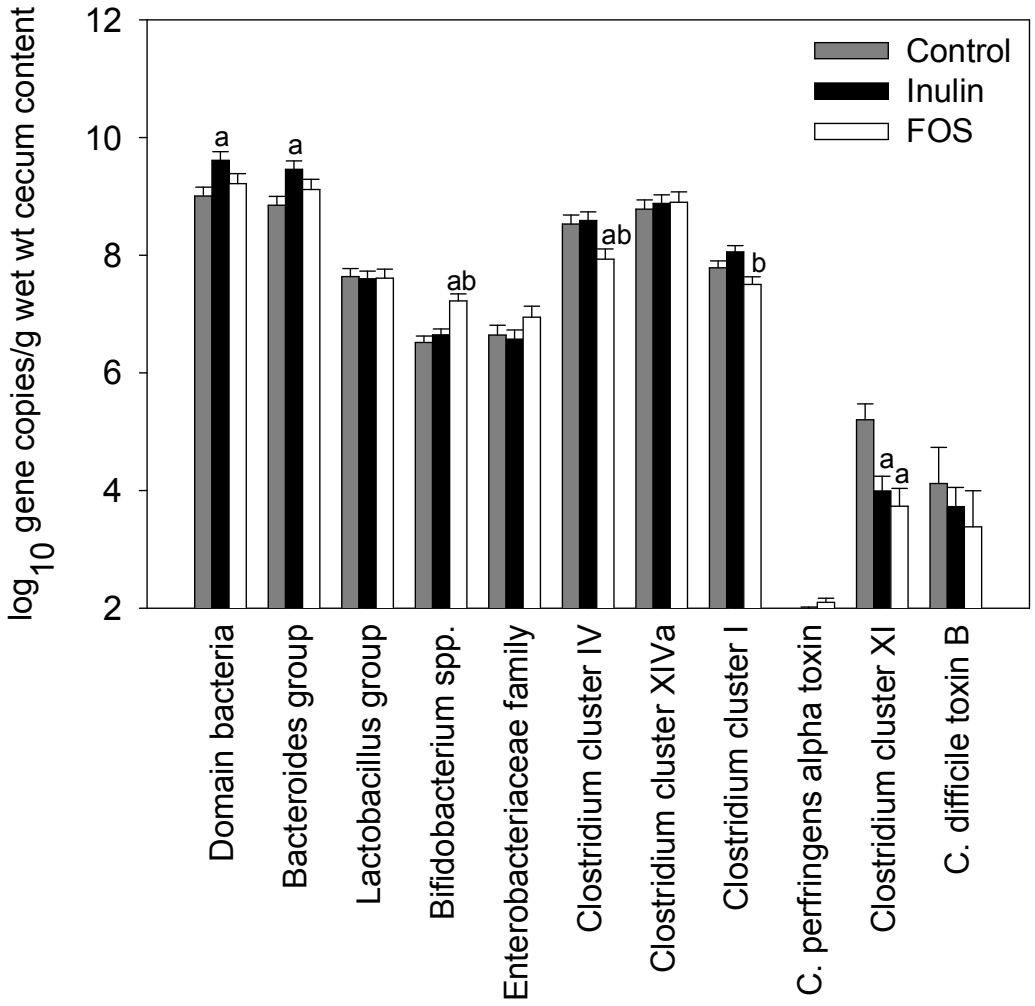


**Figure 2-3.** Quantitative PCR analysis of bacterial populations of cecal samples ((▒) control; (■), inulin; (□), fructo-oligosaccharides) collected from HLA-B27 transgenic rats at 16 weeks of age. Values are least-square means, with their standard errors represented by vertical bars. [a,b,c]Least-square mean values (obtained with the same primer pairs) with unlike letters were significantly different ($P<0.05$).

The effects of inulin and FOS treatments on the cecal microbiota were less pronounced compared to their effects on the fecal microbiota (Figure 2-3). Compared to cecal samples from control animals, inulin increased the numbers of 16S rRNA gene copies of total bacteria ($P<0.006$) and organisms from the *Bacteroides-Prevotella-Porphyromonas* group ($P<0.008$), but reduced organisms in *Clostridium* cluster XI ($P<0.001$) (Figure 2-3). FOS treatment increased the numbers of cecal bifidobacteria by one log ($P<0.001$), whereas *Clostridium* cluster IV ($P<0.015$) and *Clostridium* cluster XI ($P<0.001$) were decreased versus rats in the control group. The numbers of cecal bifidobacteria and organisms belonging to clostridial clusters I and IV in FOS-treated animals were also significantly different in comparison to rats treated with inulin.

### 2.3.3. PCR-denaturing gradient gel electrophoresis profiles

To determine whether quantitative changes in intestinal microbiota were accompanied by qualitative changes, PCR-DGGE analysis was performed using primers targeting four phylogenic groups whose abundance was altered by dietary intervention with fructans. The cluster analysis for DGGE profiles obtained with group-specific primers targeting the *Bacteroides* group is shown in Figure 2-4. DGGE patterns were separated into two main clusters. The upper cluster mainly consisted of inulin- and FOS-treated animals, and the lower cluster contained mainly control animals (Figure 2-4). This result indicates that diet-induced changes in the abundance were indeed associated with qualitative changes in composition of bacterial taxa in the *Bacteroides* group. However, patterns generated with primers specific for the *Lactobacillus* group and the clostridial

cluster IV did not segregate according to the different diets (Figure 2-4). Amplicons obtained with primers specific for *Bifidobacterium* spp. migrated as a single fragment on DGGE gels, indicating the presence of one *Bifidobacterium* species only in each animal (data not shown). Sequence analysis of five amplicons (one FOS-fed animal, two animals each from the inulin and control groups) was performed to identify bifidobacteria on species level; four sequences matched *Bifidobacterium animalis* with 99% identity; one sequence matched *Bifidobacterium pseudolongum* (99% identity).

### 2.3.4. Cecal and fecal SCFA

SCFA composition in the fecal and cecal samples collected from HLA-B27 transgenic rats at the end point of carbohydrate treatments (16 weeks) did not differ significantly between the diets (data not shown). The total SCFA concentration of the fecal samples collected at 4 and 16 weeks of age was 54.8±8.4 and 68±5.8 μmol/g, respectively, for the control group, 45.8±9.4 and 78.5±6.4 μmol/g, respectively, for the inulin group, and 46.7±8.7 and 77.2±7.4 μmol/g, respectively, for the FOS group. The increase in total SCFA over the treatment period was significant for inulin- and FOS treated animals ($P<0.023$ and $P<0.018$, respectively), but not for the control group. Acetate was the major product (about 70 % of the total SCFA); fecal acetate significantly increased with age for all groups (Supplementary Figure 2-1).

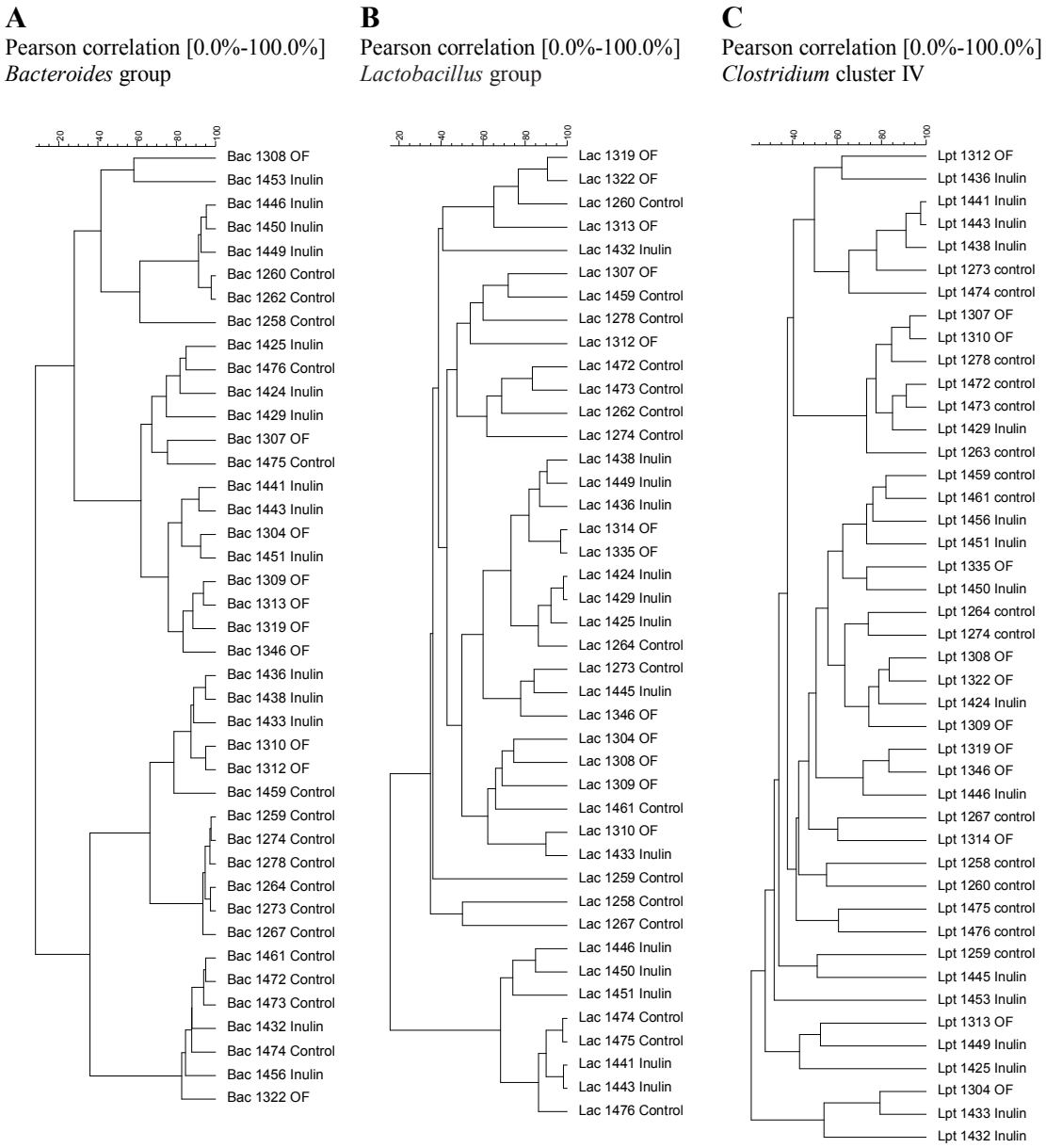


**Figure 2-4.** Pearson correlation coefficient of DGGE profiles of fecal communities of the *Bacteroides-Prevotella-Porphyromonas* group (A), *Lactobacillus-Pediococcus-Leuconostoc-Weissella* group (B) and *Clostridium* cluster IV (C). DNA extracted from fecal samples collected at the end point (16 weeks of age) was used as a template.

### 2.3.5. Correlations among bacterial populations, SCFA and degree of inflammation for cecum and feces

Correlations among the individual variables of intestinal bacterial populations, SCFA, and the degree of cecal inflammation were initially performed by PC analysis. The animals in the three treatment groups clustered separately in the linear discriminant analysis, indicating a differential effect of the two fructans on intestinal microbiota and intestinal inflammation (data not shown). Loading plots are depicted in Figure 2-5 (A; cecum samples) and Figure 2-5 (B; fecal samples) to indicate correlations between variables. In the cecal samples, a cluster located on the upper left quadrant included *Clostridium* cluster XI, IL-1β concentration, GGS, histology score, propionate and butyrate (Figure 2-5A). *Bifidobacterium* spp., *Enterobacteriaceae*, *C. perfringens* α toxin, acetate and total SCFA formed a cluster opposite to the first one, indicating a negative correlation. A third cluster containing total bacteria, *Bacteroides* and *Lactobacillus* groups and clostridial clusters I, IV, cluster XIVa was located in the upper right quadrant of the loading plot (Figure 2-5A). For fecal samples (Figure 2-5B), *Bifidobacterium* spp., *Clostridium* cluster I and *C. perfringens* α toxin, as well as acetate, propionate, butyrate and total SCFA formed a cluster negatively influenced by PC2. *Clostridium* cluster XI and *C. difficile* toxin B positively correlated with inflammation markers such as GGS, histology score and IL-1β concentration; and together formed a second cluster positively influenced by PC2 and negatively related to the first cluster. Total bacteria, the *Bacteroides* and *Lactobacillus*

groups, and the clostridial clusters IV and XIVa were separated as a third cluster, which was positively correlated mainly to PC1.

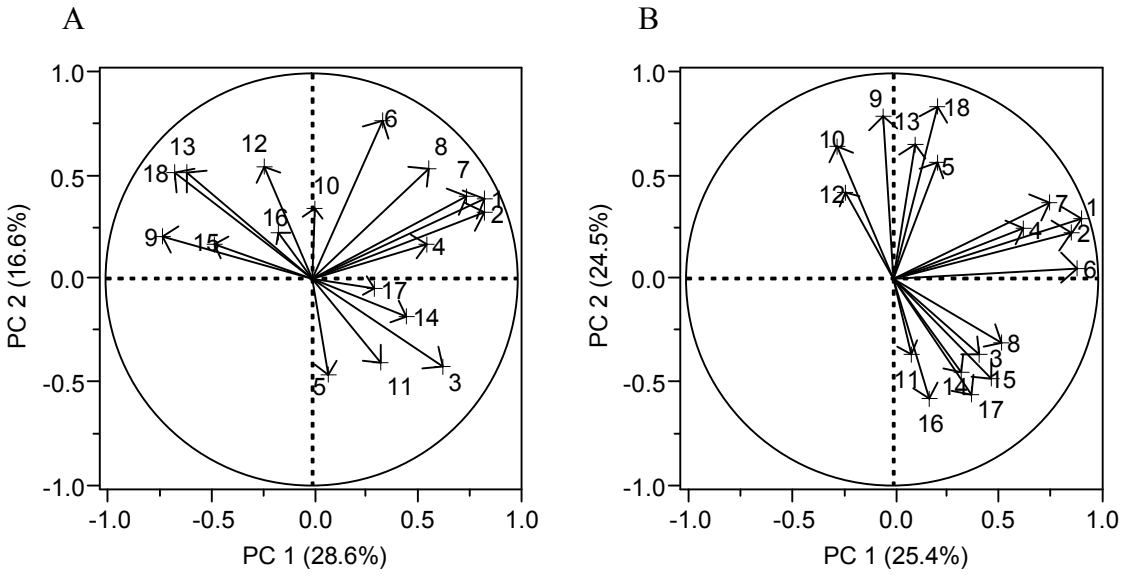


**Figure 2-5.** Loading plots of the first two principal components (PC1 and PC2) for (A) cecum and (B) feces show correlations among copy numbers for bacterial groups, SCFA, gross gut score (GGS) and IL-1β concentration. 1 – total bacteria; 2 – *Bacteroides-Prevotella-Porphyromonas* group; 3 – *Bifidobacterium* spp.; 4 – *Lactobacillus-Pediococcus-Leuconostoc-Weissella* group; 5 – *Enterobacteriaceae* family; 6 – *Clostridium* cluster IV; 7 – *Clostridium* cluster XIVa; 8 – *Clostridium* cluster I; 9 – *Clostridium* cluster XI; 10 – *Clostridium difficile* toxin B; 11 – *Clostridium perfringens* α toxin; 12 – IL-1β concentration; 13 – GGS; 14 – acetate; 15 – propionate; 16 – butyrate; 17 – total SCFA; 18 – histology score.

The associations between the variables were further analysed by Spearman's correlation analysis to determine which bacterial groups or metabolites were associated with chronic intestinal inflammation (Table 2-2). The analysis revealed significant negative correlations of *Bifidobacterium* spp. with inflammation markers both in cecum and feces. However, *Clostridium* cluster XI and genes coding for toxin B of *C. difficile* were positively correlated with inflammation (Table 2-2). Surprisingly, the correlations between *Clostridium* cluster XIVa and GGS ($r = 0.29$ and $P<0.044$) and histology score ($r= 0.39$ and $P<0.006$) were positive.

**Table 2-2.** Correlations (r) between abundance in bacterial groups and mucosal inflammation indicated by IL-1β concentration, gross gut score (GGS) and histology score in cecal or fecal samples.

| Bacterial groups | IL1-β concentration (ng/g protein) | Gross gut score (GGS) | Histology score |
|---|---|---|---|
| Cecum | | | |
| *Bifidobacterium* spp. | -0.18 (0.278) | **-0.44 (0.002)** | **-0.60 (0.001)** |
| *Clostridium* cluster XI | 0.22 (0.127) | **0.53 (0.001)** | **0.59 (0.001)** |
| Feces | | | |
| *Bifidobacterium* spp. | -0.15 (0.299) | -0.33 (0.019) | -0.25 (0.079) |
| *Clostridium* cluster XIVa | 0.04 (0.809) | 0.29 (0.044) | **0.39 (0.006)** |
| *Clostridium* cluster XI | 0.29 (0.041) | **0.42 (0.002)** | **0.64 (0.001)** |
| *Clostridium difficile* toxin B | **0.39 (0.041)** | **0.42 (0.003)** | **0.43 (0.002)** |

Correlations are assessed by Spearman's correlation test. Coefficients with $P<0.05$ and r of $\geq 0.35$ are indicated in bold face type.

## 2.4. Discussion

Dietary interventions with non-digestible fermentable carbohydrates such as inulin and FOS present a potential tool for the prevention or treatment of IBD. An understanding of their protective mechanisms of action, which probably involves an altered composition and activity of intestinal microbiota, is required in order to determine chain length, structure, and linkages type of non-digestible carbohydrates that effectively improve colitis. To our knowledge, this is the first study to determine whether dietary inulin and FOS, differing in their DP, have divergent effects on the composition of intestinal microbiota in a well-validated colitis model. Different from intervention studies using healthy animals (Kleessen *et al*., 2001), intestinal microbiota in colitis models are also affected by inflammation (Andoh *et al*., 2007). In our study design, fecal microbiota were analysed over time to evaluate the effect of dietary intervention and inflammation; cecal microbiota were analysed at the time of killing of the animals to obtain insight into the composition of microbiota in the intestinal compartment exhibiting the highest degree of inflammation.

Treatment with a mixture of inulin and FOS reduced colitis in HLA-B27 transgenic rats (Hoentjen *et al*., 2005). Other studies in rats reported protective effects of β-fructans inulin on DSS-induced colitis (Videla *et al*., 2001), and trinitrobenzene sulphonic acid-induced colitis (Cherbut *et al.*, 2003; Lara-Villoslada *et al*., 2006). However, not all studies using fructans have reported positive outcomes (Moreau *et al*., 2003). A direct comparison of two β-fructans differing in their molecular weight has not been reported in a colitis model. Both

fructans had anti-inflammatory effects, but the effects of FOS on cecal inflammation were more pronounced compared to inulin.

Prevention of chronic inflammation was previously associated with increased numbers of intestinal lactobacilli and bifidobacteria (Hoentjen *et al*., 2005). The present study confirmed that the abundance of bifidobacteria was consistently and negatively correlated with chronic intestinal inflammation. FOS but not inulin increased the numbers of cecal and fecal bifidobacteria. FOS did not alter the diversity of bifidobacteria; *B. animalis* was predominant in all animals. This divergent effect on the abundance of bifidobacteria corresponded to the differential effect of inulin and FOS on chronic intestinal inflammation and probably relates to the preferential metabolism of FOS by most Bifidobacterium spp. A majority of bifidobacteria metabolise FOS by oligosaccharide transport and hydrolysis by intracellular β-fructofuranosidases (Rossi *et al*., 2005; van den Broek *et al*., 2008). Only few bifidobacteria possess extracellular enzymes to degrade polymeric β-fructans such as inulin (van den Broek *et al*., 2008; Korakli *et al*., 2002).

Intestinal bacteria that are more abundant than bifidobacteria may also contribute to protection. FOS also increased the cecal abundance of the *Bacteroides* group. DGGE showed that this group, which ferments a wide range of non-digestible carbohydrates, is qualitatively influenced by inulin and FOS. The *Bacteroides* group plays a controversial role in IBD. *Bacteroides vulgatus* induced colitis in gnotobiotic HLA-B27 transgenic rats (Rath *et al*., 1996; Rath *et al*., 1999), and were found more frequently in colonic biopsies taken from UC patients compared

to healthy subjects (Matsuda *et al*., 2000; Lucke *et al*., 2006). However, other strains of *B. vulgatus* protected against *E. coli*-induced colitis in gnotobiotic IL-2-deficient mice (Waidmann *et al.,* 2003). A diet rich in resistant starch that is associated with a high fecal concentration of SCFA and a reduced risk of IBD also resulted in a high abundance of organisms in the *Bacteroides* group (de Filippo *et al*., 2010).

The abundance of *Enterobacteriaceae* in other colitis models positively correlated with host-mediated inflammation (Lupp *et al.,* 2007); and bacteria of the *Enterobacteriaceae* family such as pathogenic *E. coli* frequently are more abundant in IBD patients in comparison to healthy individuals (Baumgart *et al.,* 2007; Thomazini *et al.,* 2011; Andoh *et al.,* 2007). However, HLA-B27 transgenic rats mono-associated with *E. coli* did not develop colitis (Rath *et al.,* 1999) and *E. coli* Nissle 1917 even maintained remission in patients with UC (Kruis *et al*., 1997), indicating that some *Enterobacteriaceae* are protective. In this study, PC and correlation analyses of intestinal microbiota and inflammation markers did not reveal a consistent association of *Enterobacteriaceae* with inflammation or protection.

Chronic intestinal inflammation consistently correlated with the abundance of *Clostridium* cluster XI as well as the abundance of genes encoding for the *C. difficile* toxin B. Fructans substantially decreased the gene copy numbers of the *Clostridium* cluster XI in fecal microbiota. This decrease was matched by a reduction of gene copy numbers of the *C. difficile* toxin B. *C. difficile* is the major cause of nosocomial diarrhoea, and growth and toxin production of *C. difficile* is

favoured by dysbiosis (Heinlen and Ballard, 2010). Patients with UC are prone to infections with *C. difficile*, which further increases morbidity and even mortality in these patients receiving immunosuppressive drugs (Musa *et al*., 2010; DuPont and DuPont, 2011). Independent of any colitis reducing effect of dietary β-fructans in patients, a reduction of *C. difficile* overgrowth thus justifies their therapeutic use.

SCFA – acetate, propionate and butyrate – are the major end-products of intestinal fermentation of β-fructans (Cummings and Macfarlane, 1997). Lactate formation by lactobacilli and bifidobacteria increase SCFA through cross-feeding of butyrate-producing bacteria (Louis *et al.,* 2007). Butyrate is the main fuel for colonocytes and also promotes the down-regulation of pro-inflammatory cascades in experimental and human IBD (Videla *et al*., 2001). The protective effects of bifidobacteria in an animal model for enteric infection were recently attributed to acetate formation (Fukuda *et al*., 2011). The protective effect of a non-purified diet in DSS-induced colitis in mice when compared to a purified diet also correlated to high intestinal concentration of SCFA (Goto *et al*., 2010). Stimulation of SCFA production by β-fructans may thus contribute to their anti-inflammatory effect. In this study, PC analysis indicated a negative correlation between fecal SCFA and inflammation markers. However, more than 90% of SCFA produced in the intestine are absorbed in the cecal and colonic epithelial cells (Regmi *et al*., 2011; Topping and Clifton, 2001). Therefore, SCFA levels in the feces of rats are a poor indicator of intestinal carbohydrate fermentation (Hoentjen *et al*., 2005; Kleessen *et al*., 2001; Regmi *et al*., 2011).

In summary, despite the wide use of FOS and inulin as functional food ingredients and their well-studied prebiotic activity, very little is known about the relationship between their DP and their effect on intestinal microbiota or host health (Kleessen *et al*., 2001). Our results demonstrate that FOS and inulin differ in colitis reduction and also vary in their effects on the intestinal microbiota in HLA-B27 transgenic rats. The abundance of *Bifidobacterium* spp. and *Clostridium* cluster XI correlated negatively and positively, respectively, to chronic intestinal inflammation. The protective effects of intestinal microbiota in IBD may relate to the formation of SCFA in the large intestine; however, additional studies are required to confirm a protective role of SCFA in experimental colitis and in clinical studies.

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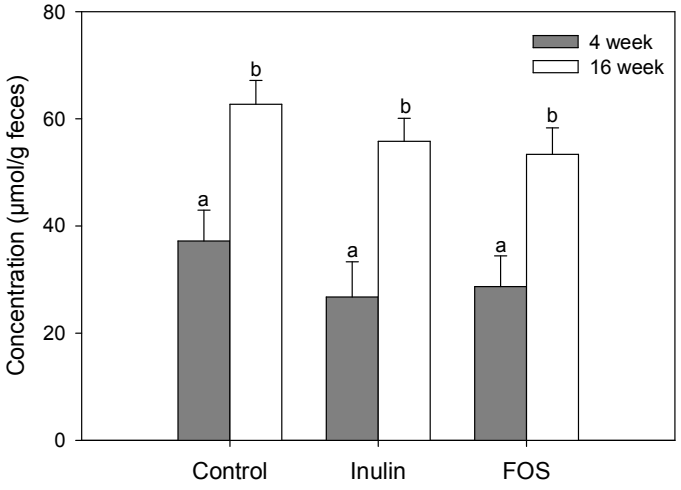
## 2.6. Supplementary Material

**Supplementary Figure 2-1.** Concentration of actetate in fecal samples collected from HLA-B27 transgenic rats before (4 weeks) and at the end (16 weeks) of dietary intervention with inulin or FOS. Results are presented as least-square mean ± SEM, and [a,b]means with different letters was considered as statistically significant with $P<0.05$.



# Chemically Defined Diet Alters the Protective Properties of Fructo-Oligosaccharides and Isomalto-Oligosaccharides in HLA-B27 Transgenic Rats

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## 3.1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD), collectively called inflammatory bowel diseases (IBD), are characterized by chronic inflammation of the gut. Intestinal bacteria contribute to initiation and perpetuation of chronic intestinal inflammation (Ferrell and la Mont, 2002). IBD patients exhibit a lower diversity of intestinal bacteria, indicating microbial imbalance or 'dysbiosis' (Marteau, 2009; Frank *et al*., 2011). Inflammation in IBD patients generally occurs in those parts of the gastrointestinal tract with the highest abundance of micro-organisms (Thompson-Chagoyan *et al*., 2005). The composition and activity of intestinal microbiota is modulated by the diet (Serikov *et al*., 2010) and the diet is also thought to play a role in IBD development and progression (Ferguson *et al*., 2007). Diets high in sucrose, refined carbohydrates and ω-6 polyunsaturated fatty acids increase the risk of developing IBD (Hou et al., 2011; Issa *et al.,* 2011). A 'Western' diet high in animal fat, proteins and refined carbohydrates, but low in fruits, vegetables and whole grains was associated with increased incidence and prevalence of Crohn's disease (Amre *et al*., 2007). Dietary fibers, ω-3 polyunsaturated fatty acids or vitamin D attenuated chronic intestinal inflammation in animal studies (Meister and Ghosh, 2005; Koleva *et al*., 2012).

Non-digestible carbohydrates are emerging as potential therapy in IBD. Fructo-oligosaccharides (FOS) are among the best studied compounds. FOS are linear fructosyl-β-(2→1)-(fructosyl)n-β-(2→1)-glucose oligomers with a degree of polymerisation ranging from 3 to 10 (Bomet *et al*., 2002). They are naturally

present in edible plants including chicory, Jerusalem artichokes, and onions, and are also produced by *Lactobacillus* species in cereal fermentations (Bomet *et al*., 2002; Tieking *et al*., 2005). Commercial isomalto-oligosaccharides (IMO) consist predominantly of α-(1→6) linked isomalto-oligosaccharides and α-(1→4) and α-(1→6) linked oligosaccharides of the panose-series (Ketabi *et al*., 2011; Hu *et al*., 2013). IMO are partially digested in the upper gastrointestinal tract. IMO stimulate growth of intestinal lactobacilli in rodents (Ketabi *et al*., 2011); stimulation of intestinal lactobacilli and bifidobacteria by dietary IMO was observed in human studies (Goffin *et al*., 2011; Kohmoto *et al*., 1992). FOS are indigestible and stimulate growth of intestinal bifidobacteria in human microbiota (Bomet *et al*., 2002; Ketabi *et al*., 2011; Roberfroid *et al*., 1998). β-Fructans reduced colitis in HLA-B27 transgenic rats, a validated spontaneous IBD model (Koleva *et al*., 2012; Hoentjen *et al*., 2005). The protective effect of FOS in this model was associated with an increased abundance of bifidobacteria in intestinal microbiota; however, it remains unclear whether the bifidogenic effect mediated colitis reduction (Koleva *et al*., 2012; Hoentjen *et al*., 2005). Past studies employed commercial rat chow with complex composition and with seasonal variations. FOS failed to attenuate inflammation in dextran sodium sulphate (DSS)-induced colitis in rats fed a purified diet (Geier *et al*., 2007). Interestingly, supplementation of a non-purified diet with FOS reduced DSS-induced colitis in mice, whereas FOS supplementation of a purified diet exacerbated colitis (Goto *et al*., 2010).

Despite the increasing body of evidence demonstrating beneficial effects of dietary fiber, including non-digestible oligosaccharides, on intestinal inflammation in animal models of IBD, there are only limited data indicating the interaction of dietary fiber with other dietary components. Therefore, the present study aimed to compare the effects of FOS or IMO on reduction of colitis and changes of intestinal microbiota in HLA-B27 transgenic rats in the presence of either chemically defined diet AIN-76A or a standard rat chow. In order to investigate whether microbiota changes mediated the colitis-modulating effects of these fibers in the presence of these two different diets, we studied the composition of cecal and fecal microbiota and quantified microbial metabolites in association with chronic intestinal inflammation.

## 3.2. Materials and Methods

### 3.2.1. Animals, diets and study design

Animal use was approved by the Animal Care and Use Committee of the University of Alberta and conducted in accordance with the Canadian Council on Animal Care Guidelines. HLA-B27 transgenic rats, a validated animal model mimicking chronic inflammatory disease in human, were used in the study. Rats 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. Animals were fed with a commercial rat chow (5053 PicoLab® Rodent Diet 20, Lab Diet Inc., Leduc, AB, Canada) or the chemically defined diet AIN-76A (Teklad Custom Research Diet, Harland Laboratories, Madison, WI,

USA). Rats were randomly allocated to six treatment groups: two control groups on rat chow or AIN-76A diet; four treatment groups on rat chow or AIN-76A supplemented with 8 g/kg body weight of either FOS (Orafti P-95, Raffinerie Tirlemontoise, Tienen, Belgium); or IMO (BioNeutra, Edmonton, Canada). Four females and four males were assigned to each group. The average feed consumption of the rats was 20 g/d. The body weight of rats was monitored every two weeks and the fiber content of the diet was adjusted accordingly. Treatment started at four weeks of age, before colitis occurred, and continued until 16 weeks of age (Koleva *et al*., 2012). Fecal samples were collected immediately after defecation from each group at 4 and 16 weeks of age. Animals were killed at 16 weeks by $CO_2$ asphyxia. At necropsy, cecal and colonic tissues and digesta were collected for histological examination, quantification of mucosal IL-1β, as well as for microbiota analysis. Feces, tissue samples and cecal contents were immediately frozen and stored at -80ºC.

**Table 3-1.** Composition of experimental diets.

| | AIN-76A | | Rat chow | |
|---|---|---|---|---|
| **Nutrients** | **per 100 g** | **% kcal** | **per 100 g** | **% kcal** |
| Protein | 20.0 | 17.7 | 20.0 | 24.7 |
| Fat | 5.0 | 5.2 | 10.6 | 13.2 |
| Carbohydrate | | 64.9 | | 62.1 |
| Starch | 15.0 | | 33.9 | |
| Saccharides | 50.0 (sucrose only) | | 15.0 | |
| Fiber | 5.0 | | 4.7 | |

### 3.2.2. Histology and enzyme-linked immunosorbent assay (ELISA)

Histological damage of cecum and colon was scored using a validated scale ranging from zero to four as previously described (Rath *et al*., 1996). Samples were scored blindly employing a validated microscopic inflammation scale (Rath *et al*., 1996) using light microscopy. Tissues were examined for the following parameters: (i) presence of epithelial cell exfoliation; (ii) crypt loss; (iii) mucosal thickening and (iv) submucosal cell infiltration. The mucosal interleukin-1β (IL-1β) was quantified in cecal and colonic tissue homogenates using a commercial rat-specific IL-1β ELISA DuoSet ELISA Development System kit (R&D Systems, Inc., Minneapolis, MN, U.S.A.).

### 3.2.3. DNA isolation

Total DNA was isolated from feces and cecal contents with a QIAamp DNA Stool Mini Kit (Qiagen, Inc. Mississauga, ON, Canada). DNA concentration and quality were checked spectrophotometrically using a NanoDrop system ND-1000, version 3.3.0 (Thermo Fisher Scientific, Inc., Ottawa, ON, Canada). Samples were diluted prior to PCR analysis, in order to adjust DNA concentrations to 50 mg/L.

### 3.2.4. Quantitative PCR (qPCR)

Intestinal microbiota were analysed by quantitative PCR using primers, PCR conditions and experimental protocols described previously (Koleva *et al*., 2012). Butyrate producing bacteria were enumerated based on the detection of butyryl-coenzyme A (CoA) transferase (Louis and Flint, 2007) and butyrate-kinase (Louis *et al.,* 2004) genes. Genes encoding for *Clostridium difficile* toxin B (van den Berg *et al*., 2006) and virulence factors of *Escherichia coli* (Han *et al*., 2007)

were also measured. Primer pairs for bacterial groups, genes involved in the butyrate production and toxins are shown in Table 3-2. The copy numbers of virulence factors of *E. coli,* heat-sable (STa and STb) and heat-labile (LT) enterotoxins, and the gene encoding the enteroaggregative heat stable toxin 1 (EAST1), and genes encoding *C. perfringens* α toxin were below the detection limit of $10^4$ gene copies / g in all samples.

### 3.2.5. Analysis of cecal microbiota profiles by PCR-DGGE

The V3 region of 16S rDNA was amplified with the primer pairs HDA1-GC and HDA2 (Table 3-2). Amplicons were used as templates in a second PCR with the same primer pairs. DGGE analysis was performed using DCode Universal Mutation Detection System (Bio-Rad) on a 6 % (w/v) polyacrylamide gel (37.5:1 acrylamide:bisacrylamaide). DGGE gels contained a denaturing gradient from 30 to 55 % of 7 M urea and 40 % (w/v) formamide. Electrophoresis was carried out in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris-base; 20 mM acetic acid; 1 mM EDTA; pH=8.5) for 4 h at a constant voltage of 130 V and a temperature of 60ºC. Staining was done in SYBR Safe 1x solution (Invitrogen) for 1 h and gels were visualized by UV illumination. Profile analysis of DGGE patterns was conducted using BioNumerics software (version 6.01, Applied Maths, Belgium) based on Pearson correlation coefficient (curved based) and unweighted-pair group method using arithmetic averages (UPGMA). A reference sample was run on each gel and used for normalization between the gels.

### 3.2.6. Short-chain fatty acids (SCFA) quantification in cecal contents

For the analysis of SCFA, 0.1 g of cecal content was weighed, mixed with 0.3 mL of internal standard solution containing 25 % phosphoric acid and internal standard (100 mmol/L iso-caproate), and centrifuged at 17 000 g for 10 min. Subsequently, clear supernatants were collected and the concentration of SCFA was determined by a gas chromatography on a Varian 3400 series instrument (Varian, Palo Alto, CA, USA) equipped with a Stabilwax DA column (30 m, 0.53 mm ID, 0.5 μm film thickness) (Restek, Bellefonte, PA, USA). The temperature of the injector was 170ºC and the temperature of the detector was set at 190ºC, and the carrier gas was helium SCFA in feces and cecal digesta were quantified by gas chromatography as previously described (Htoo *et al.*, 2007). The head pressure was 7.5 psi and the split vent flow was fixed at 20 mL/min. The initial temperature of the column was kept at 90ºC for 0.1 min, increased to 170ºC at a rate of 10ºC/min and kept for 2 min. External standards were used to determine the concentration of acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate and results were expressed as μmol SCFA/g weight of cecal content.

**Table 3-2.** Primers used in the study.

| Target bacterial group/gene* | Oligonucleotide sequence (5′→ 3′) | Tm (°C) | Reference or source |
|---|---|---|---|
| Universal primers (200 bp) | HDA1: GC clamp-ACTCCTACGGGAGGCAGCAGT**<br>HDA2: GTATTACCGCGGCTGCTGGCAC | 52 | Walter *et al*., 2000 |
| Total bacteria (200 bp) | F: CGGYCCAGACTCCTACGGG<br>R: TTACCGCGGCTGCTGGCAC | 63 | Lee *et al*., 1996 |
| *Bacteroides* group (140 bp) | F: GGTGTCGGCTTAAGTGCCAT<br>R: CGGAYGTAAGGGCCGTGC | 60 | Rinttila *et al*., 2004 |
| *Lactobacillus* group (341 bp) | F: AGCAGTAGGGAATCTTCCA<br>R: CACCGCTACACATGGAG | 63 | Walter *et al*.,2001<br>Heilig *et al*., 2002 |
| *Bifidobacterium* spp. (243 bp) | F: TCGCGTCYGGTGTGAAAG<br>R: CCACATCCAGCRTCCAC | 60 | Rinttila *et al*., 2004 |
| *Clostridium* cluster I (665 bp) | F: GTGAAATGCGTAGAGATTAGGAA<br>R: GATYYGCGATTACTAGYAACTC | 58 | le Bourhis *et al*., 2005 |
| *Clostridium perfringens* α toxin (109 bp) | F: GCTAATGTTACTGCCGTTGA<br>R: CCTCATTAGTTTTGCAACC<br>6FAM-GCGCAGGACATGTTAAGTTTG-TAMRA | 55 | Messelhäuser *et al*., 2007 |
| *Clostridium* cluster IV (239 bp) | F: GCACAAGCAGTGGAGT<br>R: CTTCCTCCGTTTTGTCAA | 60 | Matsuki *et al*., 2002 |
| *Clostridium* cluster XIVa (438-441 bp) | F: AAATGACGGTACCTGACTAA<br>R: CTTTGAGTTTCATTCTTGCGAA | 58 | Matsuki *et al*., 2004 |
| Butyryl-CoA transferase (530 bp) | F: GCIGAICATTTCACIGGAAYWSITGGCAYATG<br>R: CCTGCCTTTGCAATRTCIACRAANGC | 55 | Louis *et al*., 2007 |
| Butyrate-kinase (301 bp) | F: GTATAGATTACTIRYIATHAAYCCNGG<br>R: CAAGCTCRTCIACIACIACNGGRTCNAC | 55 | Louis *et al*., 2004 |
| *Clostridium* cluster XI (139 bp) | F: ACGCTACTTGAGGAGGA<br>R: GAGCCGTAGCCTTTCACT<br>FAM-GTGCCAGCAGCCGCGGTAATACG-BHQ | 58 | Song *et al*., 2004 |
| *Clostridium difficile* toxin B (177 bp) | F: GAAAGTCCAAGTTTACGCTCAAT<br>R: GCTGCACCTAAACTTACACCA<br>FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA | 58 | van den Berg *et al*., 2006 |
| *Enterobacteriaceae* family (195 bp) | F: CATTGACGTTACCCGCAGAAGAAGC<br>R: CTCTACGAGACTCAAGCTTGC | 63 | Bartosch *et al*., 2004 |
| STa (193 bp) | F: ATGAAAAAGCTAATGTTGGC<br>R: TACAACAAAGTTCACAGCAG | 56 | Han *et al*., 2006 |
| STb (204 bp) | F: AATATCGCATTTCTTCTTGC<br>R: GCATCCTTTTGCTGCAAC | 56 | Han *et al*., 2006 |
| LT (291 bp) | F: CTATTACAGAACTATGTTCGG<br>R: TACTGATTGCCGCAATTG | 56 | Han *et al*., 2006 |
| EAST1 (109 bp) | F: TGCCATCAACACAGTATATCC<br>R: GCGAGTGACGGCTTTGT | 56 | Han *et al*., 2006 |

F, R - forward or reverse primer; Tm – annealing temperature; *Product size (bp)
**GC clamp sequence – CGCCCGGGGCGCGCCCCGTGGCGGGGCGGGGGCGCGCGGGG

### 3.2.7. Statistical analysis

Statistical analysis of data was carried out with the Statistical Analysis Systems (SAS Institute, Inc.). Differences between diets were evaluated by two-way analysis of variance followed by probability of difference test to perform all possible pair-wise comparisons between means of different groups. Bonferroni adjustment of the alpha level was used and probability of $<0.05$ was considered statistically significant. All results were presented as means ± SEM. Spearman's correlation test was employed to check for any correlation between mucosal inflammation and SCFA using GraphPad Prism version 5.00 (GraphPad Software). In addition, linear discriminant analysis and principle component analysis (PCA) were performed using JMP software (version 9.0.1, SAS Institute Inc., NC) to examine correlations between gene copies of bacterial groups, SCFA and intestinal inflammation.

## 3.3. Results

### 3.3.1. Effects of diets and fibers on intestinal inflammation

Supplementation of rat chow with either FOS or IMO significantly reduced cecal and colonic microscopic inflammation compared to control animals (Figure 3-1). In contrast, supplementation of IMO to AIN-76A diet failed to reduce inflammation whereas FOS reduced colonic but not cecal inflammation (Figure 3-1). Reduced inflammation upon supplementation of rat chow with IMO or FOS was associated with reduced concentrations of the pro-inflammatory cytokine IL-

1β (Figure 3-1). FOS or IMO thus reduced intestinal inflammation when supplemented to rat chow but not when added to AIN-76A.

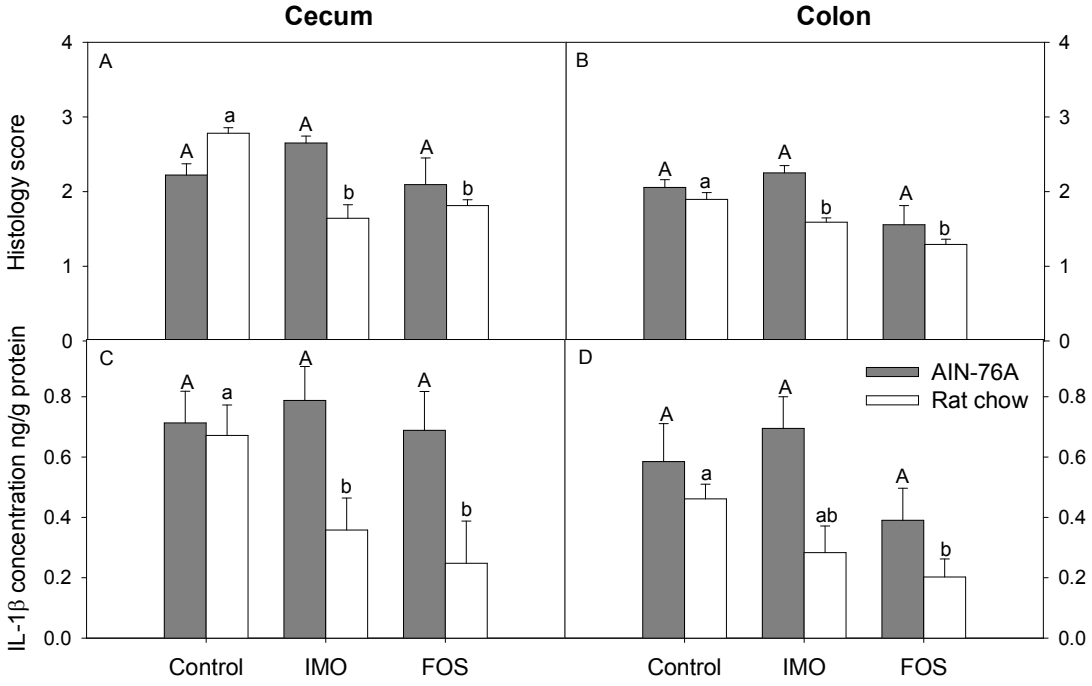


**Figure 3-1.** Effect of diet and fibers on intestinal inflammation. Inflammation was assessed by histological scoring of cecum (panel A) and colon (panel B), and by quantification of the IL-1β in cecal (panel C) and colonic (panel D) tissues collected from HLA-B27 transgenic rats fed AIN-76A or rat chow diet supplemented either with FOS or IMO, or not. Values are expressed as means ± SEM, n=6 and n=8 for rat chow and AIN-76A diet, respectively. Values in the same panel that do not share a common superscript differ significantly ($P< 0.05$, Bonferroni adjustment).

### 3.3.2. Analysis of intestinal microbiota by quantitative PCR

To determine whether the different diets had divergent effects on intestinal microbiota, bacterial groups in cecal digesta were quantified by PCR (Table 3-3). The abundance of *Clostridium* clusters XI and XIVa and genes encoding for butyrate-kinase gene was higher in animals fed rat chow compared to AIN-76A (Table 3-3). *Clostridium difficile* toxin B was detected only in samples collected from animals on rat chow and gene copy numbers of that toxin were equal or higher than the 16S rRNA genes of *Clostridium* cluster XI (Table 3-3). Supplementation with FOS treatment significantly increased copy numbers of *Bifidobacterium* spp. and *Enterobacteriaceae* family; copy numbers of *Clostridium* cluster IV were decreased compared to the control and IMO groups for both diets (Table 3-3). Supplementation of feed with IMO induced only few significant changes of cecal microbiota.

Changes of intestinal microbiota in the same animal over time were assessed by analysis of fecal microbiota (Figure 3-2). All animals fed AIN-76A had significantly higher abundance of the *Bacteroides* group and reduced abundance of lactobacilli and *Clostridium* cluster I when compared to animals on rat chow. Different from cecal samples, *Clostridium* cluster XIVa and copy numbers of butyrate-kinase gene did not differ between the diets and treatment groups (Figure 3-2). The supplementation with FOS significantly increased bifidobacteria, *Enterobacteriaceae* and butyryl-CoA transferase genes and decreased the abundance of the *Clostridium* cluster IV when compared to control and IMO groups on both diets.

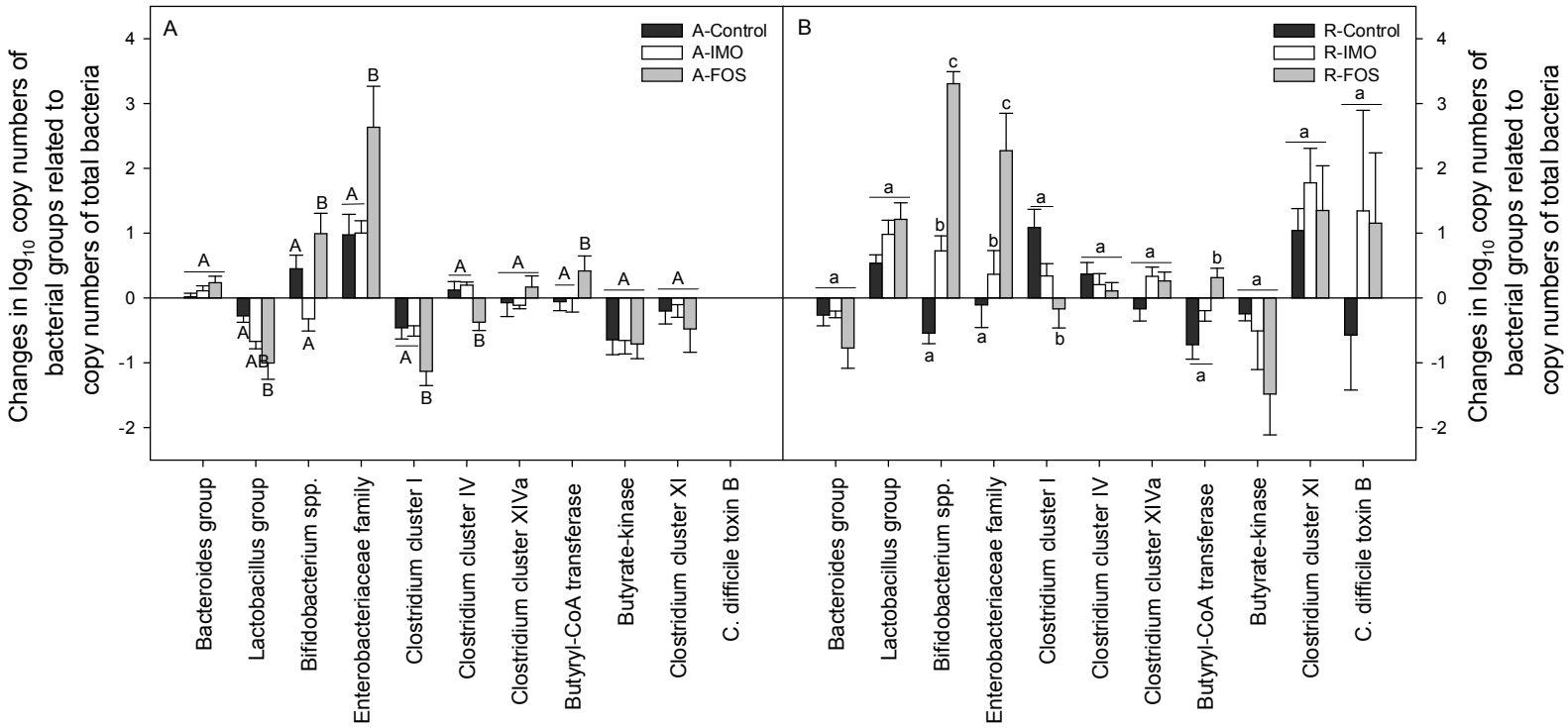


**Figure 3-2.** Effects of diets (A, AIN-76A; B, rat chow) and fibers on changes of fecal bacterial composition (week 16 – week 4) in HLA-B27 transgenic rats, evaluated by qPCR. Vertical bars represent means and standard error of the mean. Values obtained with the same primer pair that do not share a common superscript are significantly different ($P< 0.05$, Bonferroni adjustment).

**Table 3-3.** Quantification of predominant bacterial groups in cecal contents of HLA-B27 transgenic rats on AIN-76A diet or rat chow diet at the end of the experimental treatment period (16 weeks of age).

| Target bacterial group/gene | AIN-76A diet | | | Rat chow diet | | |
|---|---|---|---|---|---|---|
| | Control | IMO | FOS | Control | IMO | FOS |
| *Bacteroides* group | 30.6±3.0$^{A}$ | 29.5±4.0$^{A}$ | 35.9±6.1$^{A}$ | 34.0±5.7$^{a}$ | 39.3±4.0$^{a}$ | 46.6±3.5$^{a}$ |
| *Lactobacillus* group | 1.9±0.8$^{A}$ | 2.5±0.6$^{A}$ | 2.8±1.2$^{A}$ | 9.6±2.9$^{a}$ | 5.1±2.1$^{a}$ | 9.9±5.2$^{a}$ |
| *Bifidobacterium* spp. | 0.1±0.03$^{A}$ | 0.2±0.04$^{A}$ | 2.8±0.7$^{B}$ | 0.04±0.01$^{a}$ | 0.08±0.02$^{a}$ | 3.1±1.0$^{b}$ |
| *Enterobacteriaceae* family | 1.2±1.1$^{A}$ | 0.3±0.2$^{B}$ | 2.7±1.4$^{C}$ | 0.009±0.002$^{a}$ | 0.06±0.03$^{a}$ | 0.7±0.6$^{b}$ |
| *Clostridium* cluster I | 0.4±0.1$^{A}$ | 0.3±0.1$^{A}$ | 0.2±0.1$^{B}$ | 0.2±0.02$^{a}$ | 0.3±0.1$^{a}$ | 0.04±0.01$^{b}$ |
| *Clostridium* cluster IV | 5.7±1.0$^{A}$ | 7.9±1.1$^{A}$ | 0.6±0.1$^{B}$ | 9.2±1.9$^{a}$ | 11.9±5.2$^{a}$ | 2.2±0.6$^{b}$ |
| *Clostridium* cluster XIVa | 4.2±1.0$^{A}$ | 3.0±0.3$^{A}$ | 2.9±0.9$^{A}$ | 12.8±1.7$^{a}$ | 10.3±1.4$^{a}$ | 11.9±2.9$^{a}$ |
| Butyryl-CoA transferase | 0.01±0.003$^{A}$ | 0.02±0.007$^{A}$ | 0.02±0.01$^{A}$ | 0.01±0.003$^{a}$ | 0.004±0.001$^{a}$ | 0.01±0.002$^{a}$ |
| Butyrate-kinase | 0.01±0.002$^{A}$ | 0.01±0.002$^{A}$ | 0.01±0.01$^{A}$ | 0.03±0.01$^{a}$ | 0.03±0.01$^{a}$ | 0.07±0.01$^{b}$ |
| *Clostridium* cluster XI | $0.8\times10^{-5}$ | $0.9\times10^{-5}$ | $0.8\times10^{-5}$ | $0.4\times10^{-4}$ | 0.0004 | 0.001 |
| | $\pm0.2\times10^{-5A}$ | $\pm0.4\times10^{-5A}$ | $\pm0.1\times10^{-5A}$ | $\pm0.3\times10^{-4A}$ | ±0.0003$^{A}$ | ±0.0003$^{B}$ |
| *C. difficile* toxin B | ND** | ND | ND | $0.8\times10^{-4}$ | 0.02 | 0.09 |
| | | | | $\pm0.4\times10^{-4A}$ | ±0.009$^{B}$ | ±0.05$^{B}$ |

*Results are represented as means±SEM and depict the percence of the respective bacterial group/gene related to total bacteria. Values obtained with the same primer pair that do not share a common superscript are significantly different ($P< 0.05$, Bonferroni adjustment).

### 3.3.3. Qualitative analysis of DGGE band profiles of cecal microbiota

Effects of diets and fibers on qualitative changes of the composition of cecal microbiota in HLA-B27 transgenic rats were determined by PCR-DGGE. Cluster analysis revealed that the DGGE profiles were separated into two main clusters (Figure 3-3). Cluster I consisted of FOS- and IMO-fed animals on rat chow diet, which also showed reduced cecal and colonic inflammation. FOS- and IMO-fed animals on rat chow clustered separately, demonstrating differences in cecal microbiota composition between FOS and IMO dietary interventions. Cluster II consisted of inflamed animals and comprised the majority of animals fed AIN-76A (Cluster IIa). Animals fed raw chow without oligosaccharide supplementation formed a distinct group in the Cluster II (Cluster IIb) (Figure 3-3). FOS-fed rats on AIN-76A were spread throughout the whole dendrogram.

### 3.3.4. SCFA composition of cecal contents

To assess the effect of diet and fiber supplementation on production of microbial metabolites, SCFA were quantified in cecal digesta. Digesta from all treatment groups on rat chow had significantly higher total SCFA concentrations (220.4±22.5, 238.5±11.4 and 182.7±16.8 μmol/g, for control, IMO, and FOS groups, respectively) compared rats on AIN-76A with or without FOS (118.3±10.8 and 131.7±9.8 μmol/g, respectively). Supplementation of AIN-76A with IMO resulted in significantly higher SCFA concentrations (194.9±17.1 μmol/g) in comparison with the control and FOS groups ($P$=0.014 and $P$<0.004, respectively) and did not differ from treatment groups on rat chow. The relative concentration of acetate was significantly increased in IMO- and FOS-treated rats

versus control group on rat chow. FOS supplementation increased relative acetate concentrations in rats fed AIN-76A (Figure 3-4). AIN-76A significantly increased proportions of propionate, valerate and branched SCFA compared to the rat chow (Figure 3-4). Relative butyrate levels were higher in animals on rat chow, however the difference was significant only for rat chow without oligosaccharide supplementation (Figure 3-4).

### 3.3.5. Correlation of SCFA concentrations to microbiota and inflammation

Spearman's correlation analysis was performed for cecal parameters to detect associations of bacterial metabolites, the abundance of bacterial groups and intestinal inflammation (Table 3-4). The relative concentration of acetate was negatively correlated to the cecal histology score and the levels of IL-1β. The relative concentrations of propionate and all branched chain fatty acids were positively correlated with inflammation markers (Table 3-4). Butyrate was positively correlated with clostridial clusters IV and XIVa. Moreover, the same clostridial clusters and *Lactobacillus* group showed negative correlations with propionate, iso-butyrate and iso-valerate. The *Bacteroides-Prevotella-Porphyromonas* group was positively associated with acetate and negatively correlated with iso-butyrate and valerate (Table 3-4).

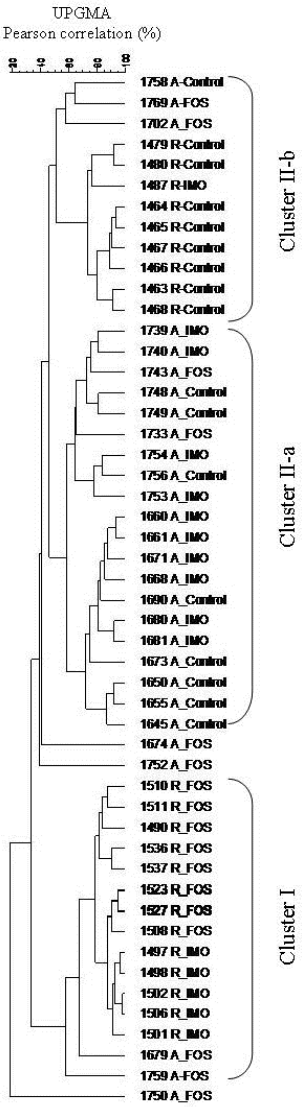


**Figure 3-3.** PCR-DGGE analysis of cecal microbiota of HLA-B27 transgenic rats on AIN-76A diet (indicated with A) or rat chow diet (indicated with R) supplemented either with fructo-oligosaccharides (FOS) or with isomalto-oligosaccharides (IMO), or not (control animals). Cecal contents were collected for DNA extraction at the end point of the fiber treatment (16 weeks ofage). UPGMA arithmetic algorithm based on Pearson correlation coefficient was used to conduct the dendrogram.

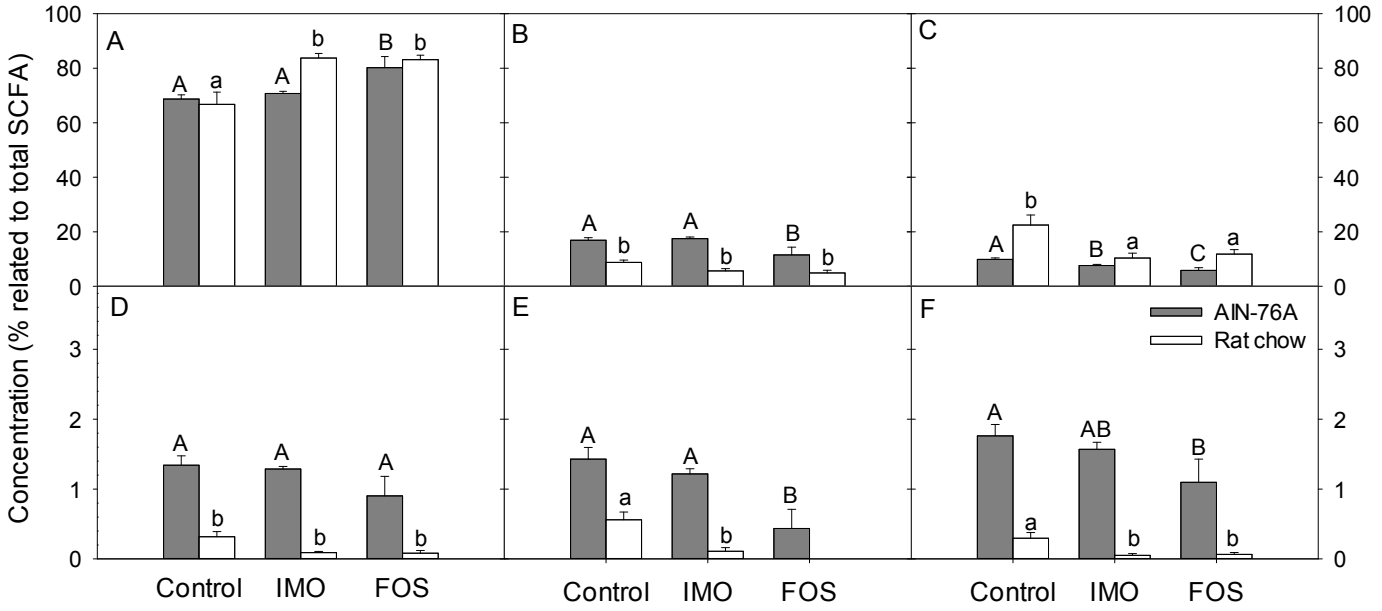


**Figure 3-4.** Effects of diets and fiber additives on SCFA concentration in cecal contents collected at the end point of the fiber treatment of HLA-B27 transgenic rats. Vertical bars show concentration of the respective SCFA related to the concentration of total SCFA and values are expressed as means ± SEM, n=6 and n=8 for rat chow and AIN-76A diet, respectively. Values that do not share a common supperscript differ significantly ($P< 0.05$, Bonferroni adjustment). A – acetate; B – propionate; C – butyrate; D – isobutyrate; E – valerate; F – isovalerate.

**Table 3-4.** Correlations between cecal parameters, such as SCFA, inflammation markers, and copy numbers of predominant bacterial groups.

| SCFA / Bacterial group | Acetate | Propionate | Butyrate | Iso-butyrate | Valerate | Iso-valerate |
|---|---|---|---|---|---|---|
| *Bacteroides* group | **0.44** | -0.34 | -0.04 | -0.33 | **-0.43** | -0.35 |
| | **(0.001)** | (0.016) | (0.792) | (0.019) | **(0.002)** | (0.013) |
| *Lactobacillus* group | 0.26 | **-0.56** | 0.23 | **-0.55** | -0.36 | **-0.54** |
| | (0.07) | **(< 0.001)** | (0.125) | **(< 0.001)** | (0.009) | **(< 0.001)** |
| *Bifidobacterium* spp. | 0.35 | -0.18 | -0.37 | -0.09 | -0.30 | -0.06 |
| | (0.012) | (0.218) | (0.007) | (0.52) | (0.034) | (0.686) |
| *Enterobacteriaceae* family | 0.21 | -0.05 | -0.30 | 0.03 | -0.16 | 0.06 |
| | (0.147) | (0.702) | (0.035) | (0.849) | (0.266) | (0.699) |
| *Clostridium* cluster I | -0.30 | 0.34 | 0.02 | 0.31 | **0.49** | 0.32 |
| | (0.032) | (0.014) | (0.904) | (0.025) | **(< 0.001)** | (0.023) |
| *Clostridium* cluster IV | **-0.44** | 0.23 | **0.41** | 0.22 | **0.42** | 0.19 |
| | **(0.007)** | (0.129) | **(0.003)** | (0.121) | **(0.004)** | (0.191) |
| *Clostridium* cluster XIVa | 0.13 | **-0.47** | **0.57** | **-0.45** | -0.36 | **-0.43** |
| | (0.374) | **(< 0.001)** | **(< 0.001)** | **(0.001)** | (0.01) | **(0.002)** |
| IL-1 β concentration (pg/mg protein) | **-0.44** | **0.47** | -0.1 | **0.54** | **0.45** | **0.54** |
| | **(0.001)** | **(0.004)** | (0.562) | **(0.001)** | **(0.001)** | **(0.001)** |
| Histology score | **-0.56** | **0.44** | 0.06 | **0.48** | **0.40** | **0.43** |
| | **(< 0.001)** | **(0.001)** | (0.686) | **(< 0.001)** | **(0.004)** | **(0.002)** |

*Correlations are assessed by Spearman's correlation test. Coefficients with $P<0.05$ and r of $\geq 0.4$ are indicated in bold face type.

### 3.3.6. Multivariate data analysis

The relationship between dietary intervention and intestinal microbiota was further assessed by linear discriminant analysis (Figure 3-5A and 3-5B). Cecal and fecal samples from FOS-treated rats clustered separately from IMO-treated and control rats for both diets. Animals on rat chow clustered separately from animals on AIN-76A. IMO-treated animals and control animals on AIN-76A clustered together but IMO-treated animals and control animals on rat chow were separated into two clusters. Linear discriminant analysis thus supports the dendrogram obtained by DGGE **(**Figure 3-3**).**

Principle component analysis (PCA) of the abundance of bacterial groups, bacterial metabolites and inflammation markers is shown in Figure 3-6. The loading plot of the first two eigenvalues (PC 1 and PC 2) separates the variables into three main clusters. *Clostridium* cluster I, butyryl-CoA transferase gene, propionate, iso-butyrate, valerate and iso-valerate, together with the inflammation markers, histology score and levels of IL-1β, clustered into one group located on the right quadrant of the graph and were highly influenced by PC 1. A second cluster, containing *Bacteroides* and *Lactobacillus* groups, bifidobacteria, *Enterobacteriaceae* group, butyrate-kinase gene and clostridial cluster XI, was situated opposite to the first cluster, representing a negative correlation. *Clostridium* cluster XIVa, total SCFA and butyrate formed a third cluster located between the two mentioned above, and were influenced positively by PC 2 (Figure 3-6).

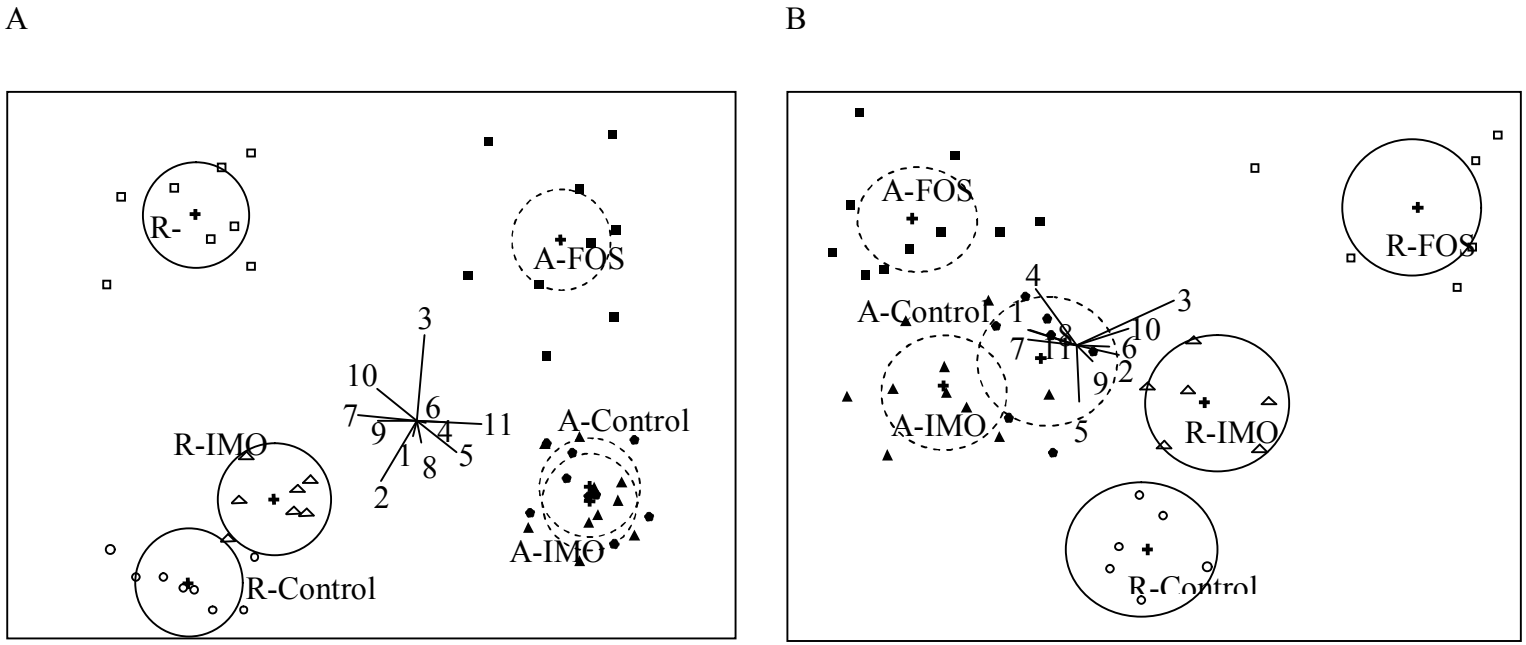


**Figure 3-5.** Linear discriminant analysis for cecal (A) and fecal (B) samples based on gene copy numbers of bacterial groups. HLA-B27 transgenic rats are fed either AIN-76A diet (indicated with A) or rat chow diet (indicated with R) supplemented either with fructo-oligosaccharides (FOS) or with isomalto-oligosaccharides (IMO), or not (control animals) for a period of 12 weeks. Each symbol represents an individual animal. 1 – *Bacteroides* group; 2 – *Lactobacillus* group; 3 – *Bifidobacterium* spp.; 4 – *Enterobacteriaceae* family; 5 – *Clostridium* cluster I; 6 – *Clostridium* cluster IV; 7 – *Clostridium* cluster XIVa; 8 – Butyryl-CoA transferase gene; 9 – Butyrate-kinase gene; 10 – *Clostridium* cluster XI; 11 – *Clostridium difficile* toxin B.

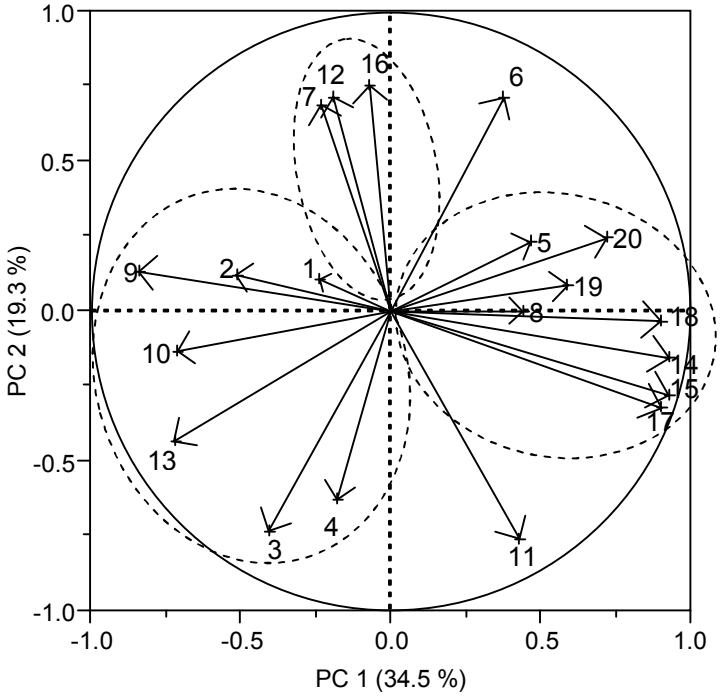


**Figure 3-6.** Principle component analysis (PCA) based on the following variables in cecum: gene copy numbers of bacterial groups; SCFA, histology score and mucosal IL-1β concentration. The loading plot of the first two principle components (PC1 and PC2) depicts the correlations between the different variables. 1 – *Bacteroides* group; 2 – *Lactobacillus* group; 3 – *Bifidobacterium* spp.; 4 – *Enterobacteriaceae* family; 5 – *Clostridium* cluster I; 6 – *Clostridium* cluster IV; 7 – *Clostridium* cluster XIVa; 8 – Butyryl-CoA transferase gene; 9 – Butyrate-kinase gene; 10 – *Clostridium* cluster XI; 11 – *Clostridium difficile* toxin B; 12 – total SCFA; 13 –acetate; 14 – propionate; 15 – iso-butyrate; 16 – butyrate; 17 – iso-valerate; 18 – valerate; 19 – cecal IL-1β concentration; 20 –histology score of cecum.

### 3.4. Discussion

Diet and dietary fibers can restore homeostasis from an intestinal inflammatory state (Issa *et al*., 2011; Meister *et al*., 2005; Koleva *et al*., 2012; Goto *et al*., 2010) and potentially reduce intestinal inflammation in IBD. Animal studies demonstrated that FOS reduced experimental colitis (Koleva *et al*., 2012; Hoentjen *et al*., 2005); however, their beneficial effect depends on the dietary background (Goto *et al*., 2010). Small scale trials in active UC indicated protective effects of FOS administration (Casellas *et al*., 2007) but FOS were not effective in treatment of active Crohn's disease (Benjamin *et al*., 2011). The divergent outcomes of IBD-intervention studies with FOS may reflect the lack of information on their mode of action, and the lack of knowledge on their interaction with other components of the diet.

Beneficial effects of FOS, including their potential benefits in prevention or treatment of IBD, were attributed to their ability to stimulate commensal bifidobacteria (Koleva *et al*., 2012; Roberfroid *et al*. 1998; Hoentjen *et al*., 2005). This study compared the effect of bifidogenic FOS with the effect of IMO, which exhibit no bifidogenic effects in rats (Ketabi *et al*., 2011). Moreover, oligosaccharides were added to conventional rat chow and a purified diet, AIN-76A. This experimental design with different oligosaccharides and diets indicates that the bifidogenic effect of FOS and their protective effect in IBD are independent of each other. To our knowledge, the present study is the first to demonstrate that IMO may reduce chronic intestinal inflammation in a colitis model. In contrast to previous studies in healthy rodents (Ketabi *et al*., 2011),

IMO induced only minor shifts in intestinal microbiota in the rodent colitis model (Table 3-3, Figure 3-2 and 3-5). This discrepancy may reflect the influence of intestinal inflammation on intestinal microbiota (Figure 3-3). However, IMO supplementation significantly increased cecal SCFA levels.

In keeping with the prior observations, dietary supplementation with FOS increased the abundance of intestinal bifidobacteria and *Enterobacteriaceae* (Koleva *et al*., 2011; Roberfroid *et al.*, 1998; Hoentjen *et al*., 2005). This effect was independent of the background diet, but FOS addition to AIN-76A failed to reduce cecal inflammation. Taken together, these results indicate that the specific changes in the composition of intestinal microbiota composition that are induced by FOS do not mediate colitis reduction. High sucrose consumption and a high intake of refined carbohydrates have been associated with an increased risk of IBD (Hou *et al*., 2011; Reif *et al*., 1997). Due to the high sucrose content, the AIN-76A diet is highly digestible and its sole source of fiber is cellulose, which is not fermented by human or rodent gut microbiota (Cummings *et al*., 2001). A comparison of a diet abundant in whole grains versus a diet with high refined grains demonstrated that whole-grains shifted fecal microbiota (Ross *et al*., 2011). A more detailed analysis of the effect of whole grains on human gut microbiota confirmed an increased bacterial diversity, a higher *Firmicutes* / *Bacteroides* ratio, and immunological improvements upon inclusion of whole grains in the diet (Martinez *et al*., 2013). Our observation that rat chow increased the abundance of the *Lactobacillus* group and the *Clostridium* cluster IV when compared to AIN-76A confirms earlier studies related to the effect of whole grains on the

composition of gut microbiota (Ross *et al*., 2011; Martinez *et al*., 2013). The effect of whole grains on gut microbes was attributed to the diverse fiber components in whole grains that are absent in refined grains or purified dietary fiber supplements such as resistant starch or FOS (Martinez *et al*., 2013). Phytochemicals such as phenolic compounds may additionally contribute to the beneficial effects of whole grains on host health. Rat chow consists of natural sources that include fermentable fiber, fatty acids, and phytochemicals, although their composition is poorly defined and may not be consistent over time. FOS exacerbated DSS-induced colitis in mice fed a purified diet but FOS supplementation showed anti-inflammatory effects in mice fed a non-purified diet (Geier *et al*., 2007). Our study in HLA-B27 transgenic rats demonstrated that the effect of the background diet on intestinal microbiota and cecal SCFA formation was greater than the effect of supplementation with IMO or FOS. However, it remains unclear whether the anti-inflammatory effects of FOS and IMO in the rat chow were solely dependent on the amount and diversity of fermentable fibers, or also involved synergistic activity of dietary fibers and phytochemicals, particularly phenolic compounds. Phenolic compounds in apples reduced colitis in the same rat colitis model (Castagnini *et al*., 2009).

Non-digestible carbohydrates are fermented by intestinal bacteria to SCFA in the cecum and colon. Acetate, propionate and butyrate are the major end-products of fermentation (Cummings and Macfarlane, 1997). IBD patients exhibited reduced colonic SCFA concentrations (Huda-Faujan *et al*., 2010). In the present study, the relative concentration of branched chain SCFA in rats fed AIN-76A was more

than twofold higher when compared to rats fed rat chow. Branched chain SCFA are exclusively derived from amino acid fermentation by strict anaerobes (Barker, 1981; Kim *et al*., 2008). In contrast, the absolute cecal SCFA concentrations in animals fed rat chow were higher when compared to animals fed the highly digestible AIN-76A diet. The higher SCFA levels in animals fed rat chow thus reflect enhanced intestinal carbohydrate fermentation based on the higher content of non-digestible but fermentable carbohydrates in rat chow when compared to AIN-76A. Accordingly, total SCFA concentrations, relative butyrate concentrations, and the abundance of *Clostridium* clusters IV and XIVa were highly correlated. The major fiber-fermenting and butyrate-producing bacterial species in the large intestine belong to those two clostridial clusters (Louis and Flint, 2009). The effect of the diet on cecal SCFA formation was also supported by the quantification of genes encoding for butyryl-CoA transferase and butyrate-kinase. Cecal copy numbers of butyrate-kinase were increased in animals on rat chow versus AIN-76A diet. The higher content of non-digestible but fermentable carbohydrates in rat show likely contributed to the protective effect of this diet when combined with oligosaccharide supplementation. Markers of inflammation were negatively correlated to cecal acetate concentrations, and positively correlated to cecal concentrations of branched chain SCFA. PCA indicated a relationship of butyrate-producing bacteria, relative concentration of butyrate, and reduced inflammation in this colitis model. Inflammation thus was consistently associated with reduced carbohydrate fermentation and increased protein fermentation in the large intestine. Intestinal production of linear SCFA,

especially butyrate, from carbohydrates is proposed to exhibit anti-inflammatory effects due to the ability of SCFA to suppress NF-κB reporter activity and the release of pro-inflammatory cytokines (Tedelind *et al*., 2007; Wong *et al*., 2006). Butyrate also plays an important role as major energy source for colonocytes (Cummings, 1997; Donohoe *et al.,* 2011). Intestinal SCFA were suggested as a possible mechanism contributing to the anti-inflammatory effects of dietary fibers in the treatment of IBD (Koleva et al., 2012; Tedelind *et al*., 2007). Remarkably, the protective effect of commensal bifidobacteria was attributed to the production of acetate (Fukuda *et al*., 2011), indicating that SCFA production is a major contributor to improved host health.

In conclusion, FOS and IMO supplementation of rat chow reduced cecal and colonic inflammation in HLA-B27 transgenic rats, but supplementation of the AIN-76A diet with oligosaccharides generally failed to reduce inflammation. Diet, supplementation with oligosaccharides, and inflammation all influenced the composition of intestinal microbiota. PCR-DGGE analysis, which discriminates intestinal microbiota on the species level, clustered the animals on the basis of intestinal inflammation, but protective effects of diet or supplementation with non-digestible oligosaccharides were not related to the stimulation of specific bacterial groups or genera. Interestingly, intestinal inflammation correlated to reduced carbohydrate fermentation in the large intestine and a higher contribution of amino acid fermentation to microbial metabolism in the cecum. This study thus supports earlier observations that successful (dietary) intervention in IBD is associated with increased carbohydrate fermentation in the large intestine, and

increased intestinal formation of straight-chain SCFA (Koleva *et al*., 2012; Geier *et al*., 2007; Wong *et al*., 2006; Cummings, 1997). The role of other components of whole-grain diets, particularly unsaturated fatty acids and phenolic compounds (Meister and Ghosh, 2005; Castagnini *et al.,* 2009) in prevention of IBD remains to be elucidated.

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# Specific Microbiota Changes and Butyrate Production are Associated with Reduction of Inflammation in Presence of β-Fructans

This chapter contains an experimental work performed by Dr. Rosica Valcheva under the supervision of Prof. Leo Dieleman and Prof. Michael Gänzle. My contributions to this study are involved in pyroseqeuncing and analysis of sequencing data. A version of this chapter is in preparation for submission to PLOS ONE as: Valcheva, R., Koleva, P., Martínez, I., Walter, J., Gänzle, M., and Dieleman, L. 'Specific microbiota changes and butyrate production are associated with reduction of inflammation in presence of β -fructans.

## 4.1. Introduction

Crohn's disease (CD) and ulcerative colitis (UC), collectively called inflammatory bowel diseases (IBD), are chronic inflammatory conditions of the intestine. Lesions of the epithelial layer are caused by activated immune cells in response to resident intestinal bacteria in genetically susceptible hosts (Duchmann *et al*., 1995; Rath *et al*., 1996; Sellon *et al*., 1998). To date 75 loci associated with barrier function, IL-23 pathway and IL-10 signaling are linked to UC (Franke *et al*., 2008; Frnake *et al*., 2010; Lees *et al*., 2011). Most susceptibility genes for UC relate to innate immunity and the primary defense against infection, reflecting that bacteria link genetics with environment (Rogler *et al*., 2011). Genetic rodent models also implicate bacteria as causative agents of IBD (Rath *et al*., 1996; Olszak *et al*., 2012). Hense twin pair studies highlight the important role of epigenetics and environment (Orholm *et al*., 2000; Spehlmann *et al*., 2008; Thompson *et al*., 1996).

The microbial community in IBD is disturbed. It is described with reduced diversity and stability both at the luminal and mucosal sites compared to non-IBD controls (Ott *et al*., 2008; Scanlan *et al.*, 2006; Walker *et al*., 2011). While consistent differences within the microbiota associated with CD subtypes have been identified in several studies, particularly a reduction of *Faecalibacterium prausnitzii*, specific microbiota patterns associated with UC have not been identified to date (Willing *et al*., 2009; Joosens *et al*., 2011; Willing *et al*., 2010).

Modulation of the gut microbiota through dietary intervention may reduce disease activity in IBD. Probiotic bacteria have shown efficacy in IBD treatment (Jonkers

*et al*., 2012). Non-digestible carbohydrates may also be protective; however, the assessment of their clinical effectiveness is incomplete. β-(2→1)-Linked fructans at a dose of 15 g/day were beneficial in a small open trial for active CD (Lindsay *et al*., 2006). A randomized placebo-controlled study in inactive and mild to moderate active CD treated with 20 g/day β-fructans for 4 weeks reported significant reduction in disease activity (Joossens *et al*., 2012). However, a randomized placebo-controlled trial involving active CD, only reported no clinical benefit of the same β-fructans despite increased anti-inflammatory response in mucosal samples from the prebiotic treated group (Benjamin *et al*., 2011). Studies in UC patients treated with β-fructans alone or in combination with probiotic bacteria showed reduction in fecal calprotectin (Casellas *et al*., 2007) or β-defensins 2, 3 and 4, TNF-α and IL-1α mRNA expression in colonic mucosa (Furrie *et al*., 2005).

The aim of this study was to identify whether dietary supplementation with β-fructans induces compositional and functional shifts in the fecal and mucosal microbiota that are associated with improvements in intestinal inflammation. Therefore, samples were obtained from patients with mild to moderate active UC that were treated with 7.5 g or 15 g daily oral inulin plus fructo-oligosaccharides (FOS) (1:1) for 9 weeks. The clinical response, the composition of fecal and mucosal microbiota, and colonic butyrate metabolism were analyzed.

## 4.2. Materials and methods

***Astericks (*) indicates contributions by Dr. Rosica Valcheva***

### *4.2.1. Patients, inclusion and exclusion criteria

Patients aged 18 – 65 years, with histological and endoscopic confirmed diagnosis and mild to moderate active UC with clinical score in the range of 3 – 8 on the 12 point Mayo Clinical Score scale (Disease Activity Index [DAI]) (Schroeder *et al*., 1987) were eligible to participate. Inclusion criteria were treatment with stable doses of 5-aminosalicylic acid (5-ASA) for at least 2 weeks, and negative tests for *Clostridium difficile* toxin, ova and parasite cultures in stool specimens. (Supplementary Table 4-1).

Exclusion criteria were: (i) use of oral steroids in the last 4 weeks; (ii) use of antibiotics in the last 2 weeks; (iii) use of immunosuppressive or anti-TNF agent within the last 3 months; or (iv) use of anti-diarrheal drugs in the last 1 week of the screening visit.

Fifty three patients were screened, 29 matched the inclusion and exclusion criteria. Each patient provided written informed consent before participation. The research protocol was approved by Health Research Ethics Board at the University of Alberta.

### *4.2.2. Study design and medication

In the open label pilot study, eligible patients were randomly assigned to one of two tested doses of β-fructans. Patients received either 7.5 g or 15 g (divided in two equal doses) β-fructans orally administered daily for 9 weeks. At screening visit, each patient's demographic characteristics, medical history and current

medications were recorded. Total Mayo score including endoscopic disease assessment of the mucosa was assessed at baseline and at week 9. Biopsy samples (15-20 cm from the anal verge) and fecal specimens for microbiome analysis and fecal calprotectin assay were collected at entry and week 9. Patients experiencing a significant adverse event or deterioration in disease activity were withdrawn from the study and treated with standard therapy.

A preparation of fructooligosaccharides (FOS) and inulin in a ratio of 1:1 was administered; the preparation was provided by Beneo – Orafti Active Food Ingredients (Belgium). FOS and inulin are β-(2→1)-linked polyfructans with a degree of polymerization of 2 – 8 and 2 – 60, respectively.

***4.2.3. Clinical outcome**

The primary end point of clinical response was defined as a decrease in the Mayo score of ≥ 3 plus the total DAI remaining ≥ 3 or entering clinical remission after 9 weeks of treatment (Mayo score < 2). Patients meeting these criteria were defined as responders. The clinical and endoscopic remission as defined in the study protocol was identified by a score of 0 in the rectal bleeding and stool frequency part of the Mayo together with a score of 0 or 1 in the sigmoidoscopic portion of DAI. Patients who did not meet these criteria were defined as non-responders.

***4.2.4. Fecal calprotectin and short chain fatty acids**

The BÜHLMANN Calprotectin ELISA test kit (ALPCO Diagnostics, New Hampshire, USA) was used following the manufacturer's instructions. The results are based on two independent ELISA experiments. Short-chain fatty acids (SCFA) were analyzed as described (Htoo *et al*., 2007).

***4.2.5. Genomic DNA extraction from feces and mucosal biopsies**

DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Mississauga, ON, Canada). Biopsies stored in 1 ml of TN150 buffer (10 mM Tris-HCl, 150 mM NaCl [pH 8.0]) were incubated with SDS (10 % w/v) and proteinase K (20 mg $ml^{-1}$) at 55˚C for 2 h. The cells were then physically disrupted with zirconium beads (diameter 0.1 mm) (500 mg) in a FastPrep-24 (MP Biomedicals, Solon, OH) in 3 cycles of 30-second bead-beating step at 4 m/s speed followed by cooling on ice for 5 min each. Subsequently, the samples were heated at 95˚C for 15 min and further processed according to the kit protocol.

***4.2.6. Total RNA extraction and reverse-transcription PCR**

Total RNA from biopsies was isolated in TRIzol (Invitrogen, Burlington, ON, Canada) using the RNeasy kit (Qiagen). The total RNA concentration of each sample was quantified on NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) and 500 ng of the purified RNA was subjected to reverse transcription using QuantiTect Reverse Transcription kit (Qiagen).

**4.2.7. PCR - denaturing gradient gel electrophoresis (DGGE)**

The diversity of the dominant fecal and mucosa-associated microbiota at baseline and at 9 wk was assessed by PCR-DGGE. Universal primers HDA1-GC and HDA2 (Walter *et al*., 2000) were used to amplify V3 variable region of 16S rRNA gene. Amplicons were analyzed with DGGE (DCode™ Universal Mutation Detection System, Bio-Rad, Hercules, USA) as previously described (Koleva *et al*., 2012). DGGE patterns were compared using BioNumerics software (version

4.01, Applied Maths, St-Martens-Latem, Belgium). Similarities were expressed based on Pearson correlation coefficient.

### *4.2.8. Quantitative PCR

Quantitative PCR (qPCR) was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Each reaction was run in duplicate in 20 µl consisting of 10 µl (2x) QuantiFast SYBR Green master mix (Qiagen), 1 µl (10 µM) of primers, and 1 µl (app. 100 ng) of template DNA or cDNA. Absolute quantification of butyryl-coenzyme A (CoA) CoA transferase (Louis *et al*., 2007) was achieved using standard curve generated by 10-fold serial dilutions of a PCR product with known concentration. Primer specificity was verified by determining the amplicon melting curve. Gene expression of monocarboxylate transporter 1 (MCT1) (Thibault *et al*., 2007) was calculated by the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization of the cDNA levels (Thiele *et al*., 1999). Biopsy samples collected at the entry of the study were used as the calibrator samples. The negative controls contained water or RT negative RNA instead of template DNA.

### 4.2.9. Pyrosequencing and bioinformatic analysis of 16S rRNA tags

Pyrosequencing using Roche 454 GS-FLX Titanium protocol was performed as previously described (Martinez *et al*., 2010). Quality control was performed in Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso *et al*., 2010). The total number of sequences used in the taxonomy based analysis was 198,919 for feces and 369,867 for biopsies for an average number of sequences of

5526 ± 358 and 9247 ± 786 for stool and mucosal samples, respectively. The number of sequences was standardized across the data set and 2000 sequences per sample were randomly chosen for taxonomy-based analysis. Taxonomy assignment at phylum, family and genus levels was performed by CLASSIFIER tool of the Ribosomal Database Project (RDP) (rdp.cme.msu.edu/classifier). Species richness and diversity were estimated by Shannon and Simpson diversity indices and UniFrac, both metrics performed in QIIME.

#### 4.2.10. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). *P* value of <0.05 was considered statistically significant. Data analysis was performed using Statistical Analysis Systems (SAS Institute, Inc., Cary, NC). Differences in the abundance of bacterial taxa between samples taken at 9 week minus the corresponding value at 0 week for high and low dose of fiber treatment were evaluated employing the Mann-Whitney test. Principle component analysis (PCA) was performed using JMP software program (version 8.0.1, SAS Institute, Inc., Cary, NC). Correlations between mucosal inflammation and bacterial populations were assessed by Spearman's correlation test employing GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA).

## 4.3. Results

#### *4.3.1. β-Fructans showed dose dependent clinical efficacy

Of the 29 recruited patients, 24 completed the treatment (15 in the 7.5 g dose group and 9 in the 15 g group). Five patients withdrew from the study due to

incompliance (n = 4) or deterioration of the symptoms (n = 1). Twelve patients responded to β-fructans treatment. β-Fructans showed dose dependent clinical efficacy with 67 % clinical response in 15 g group versus only 33 % in the 7.5 g dose group. At the entry of the study, both populations were with similar Mayo and endoscopy scores (Figure 4-1). β-Fructan intervention resulted in a decrease of Mayo score in both treatment groups, but only the high dose showed a significant decrease in the endoscopic activity (Figure 4-1). A strong correlation between Mayo score changes and fecal calprotectin changes was identified (r = 0.53, $P$ = 0.016), confirming calprotectin as objective marker for UC disease activity. One third of the patients reported increased flatulence and bloating particularly 48 to 72 hours after starting β-fructans intake. Those patients were equally represented in the two dose groups.

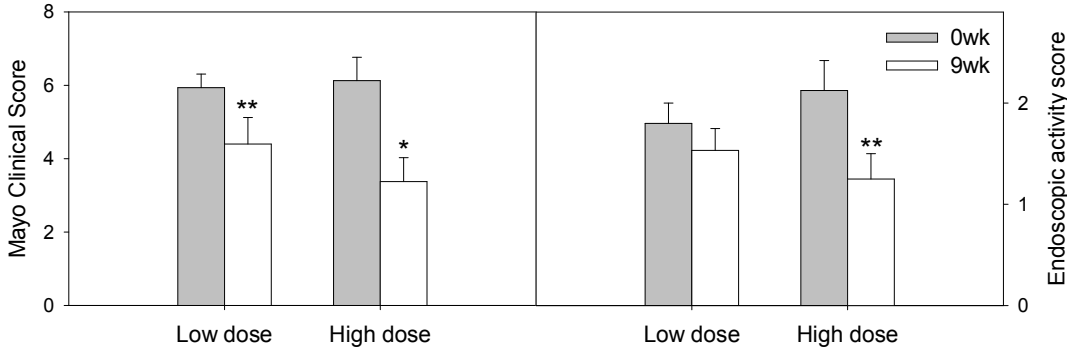


**Figure 4-1.** Mayo Clinical Score and endoscopic activity scores at 0 and 9 weeks for each of the treated groups. Values are shown as vertical bars and are expressed as means and standard error of the mean. Significant differences are indicated with '**' ($P<0.05$).

### 4.3.2. The intestinal microbiota are individual- and site-specific

The intestinal microbiota composition during treatment was qualitatively and quantitatively assessed by DGGE and rDNA-tagged pyrosequencing. Cluster analysis of DGGE patterns from mucosal and fecal microbiota (Figures 4-2A and B) identified no significant difference in the overall microbiome composition before and after β-fructans treatment. This suggested that the effect of the fiber intervention was smaller compared to inter-individual differences.

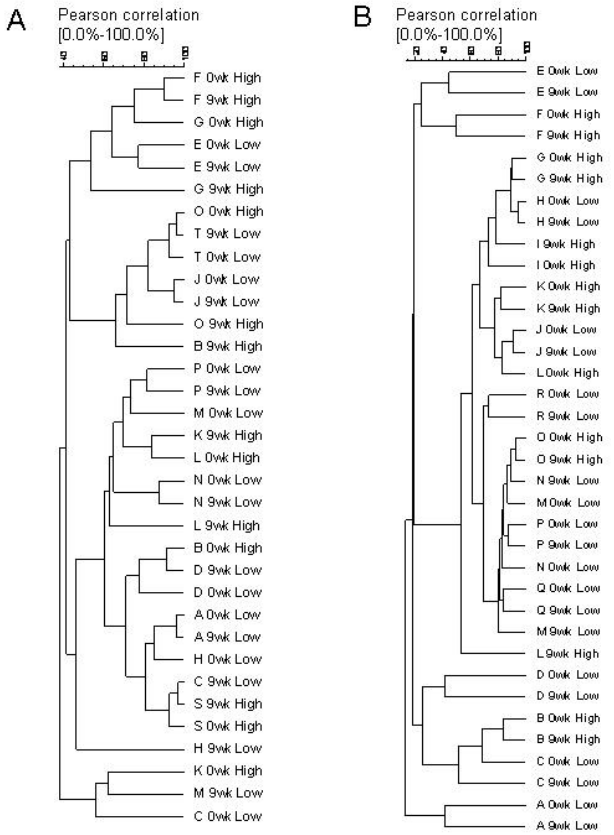


**Figure 4-2.** Cluster dendrogram generated using the Pearson correlation coefficient of the DGGE patterns from 16S rDNA amplicons of total bacterial DNA in biopsy (A) and fecal (B) samples from UC patients treated with two different doses of FOS/inulin.

**Table 4-1.** Bacterial taxa identified by pyrosequencing of 16S rDNA sequence tags from fecal and biopsy samples of UC patients treated with FOS/inulin for 9 weeks. Only bacterial taxa for which significant differences between feces and biopsies were detected are shown.

| Bacterial Taxon | | | Fecal microbiota | | Mucosal microbiota | |
|---|---|---|---|---|---|---|
| **Phylum** | **Family** | **Genus** | **0 week** | **9 week** | **0 week** | **9 week** |
| ***Bacteroidetes*** | | | 36.27±6.24* | 33.26±6.03* | 23.20±2.71 | 11.96±2.29 |
| | *Bacteroidaceae* | *Bacteroides* | 29.43±5.26 | 27.93±5.73* | 18.03±2.27 | 9.14±2.06 |
| ***Firmicutes*** | | | 50.84±5.77 | 56.36±6.42 | 48.31±2.97 | 52.46±3.44 |
| | *Streptococcaceae* | | 0.86 ± 0.46* | 0.18 ± 0.07* | 3.19 ± 0.62 | 4.49 ± 0.76 |
| | | *Streptococcus* | 0.82±0.46 | 0.18±0.07* | 0.48±0.09 | 0.66±0.13 |
| | *Leuconostocaceae* | | 0.06±0.03* | 0.07±0.05* | 14.39±2.68 | 21.36±3.27 |
| | *Enterococcaceae* | | 0.09±0.09* | 0.06±0.05* | 0.49±0.11 | 0.71±0.13 |
| | *Lachnospiraceae* | | 14.90±3.11 | 15.97±2.37 | 15.97±2.66 | 11.21±2.54 |
| | | *Blautia* | 1.56±0.46* | 1.67±0.47 | 3.89±0.70 | 2.68±0.49 |
| | | *Roseburia* | 1.21±0.29 | 1.85±0.63* | 0.80±0.16 | 0.43±0.13 |
| | *Ruminococcaceae* | | 17.48±4.32* | 18.69±4.55* | 5.83±1.05 | 5.11±1.82 |
| | | *Subdoligranulum* | 8.18±4.22* | 4.97±3.43* | 0.58±0.24 | 0.54±0.28 |
| | unclassified *Ruminococcaceae* | | 2.71±0.66* | 3.30±0.66* | 1.29±0.45 | 0.79±0.37 |
| | *Veillonellaceae* | | 7.27±2.51* | 8.01±2.49* | 0.57±0.19 | 0.98±0.52 |
| | | *Dialister* | 5.27±2.18* | 6.59±2.41* | 0.09±0.04 | 0.06±0.03 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Unclassified *Clostridiales* | | 6.82 ± 0.90* | 7.19 ± 1.00* | 3.93 ± 0.75 | 2.78 ± 0.74 |
| ***Proteobacteria*** | | | 4.36±2.06* | 1.25±0.27* | 20.25±4.18 | 25.08±4.55 |
| | *Enterobacteriaceae* | | 3.51 ± 2.06 | 0.64 ± 0.29* | 4.12 ± 0.71 | 3.63 ± 0.65 |
| | | *Citrobacter* | 1.00±1.00 | 0.01±0.01* | 1.33±0.27 | 1.66±0.33 |
| | unclassified *Enterobacteriaceae* | | 0.31±0.22* | 0.07±0.02* | 1.31±0.26 | 1.35±0.23 |
| ***Verrucomicrobia*** | | | 1.13±0.92 | 1.66±0.74* | 0.95±0.84 | 0.04±0.02 |
| | *Verrucomicrobiaceae* | *Akkermansia* | 1.13±0.92 | 1.66±0.74* | 0.95±0.84 | 0.04±0.02 |

Results are presented as means and standard error of the mean. *Means for significantly different abundance ($P<0.05$) in fecal versus biopsy samples.

Both DGGE analysis and rDNA sequencing revealed greater diversity in the mucosa-associated microbiota in UC patients compared to the fecal microbiota. 16S rDNA tags sequencing identified 66 genera from 38 families in the biopsies, in comparison to 42 genera from 27 families in feces (Supplementary Table 4-2 provides an overview on all data). Differences between mucosal and fecal microbiota were observed at phylum, family and genus levels (Table 4-1). At the phylum level, *Proteobacteria* accounted for less than 5 % and more than 20 % of organisms in fecal and mucosal microbiota, respectively. This was due to a higher abundance of *Enterobacteriaceae* and families that were exclusively detected in mucosal samples (Supplementary Table 4-2). In contrast, phylum *Bacteroidetes* was more abundant in fecal microbiota when compared to mucosal microbiota. Strict anaerobic representatives of the phylum *Firmicutes*, including *Veillonellaceae, Lachnospiraceae* and *Ruminococcaceae* were more abundant in fecal microbiota. Meanwhile facultative anaerobes as lactic acid bacteria composed the core of the mucosa-associated *Firmicutes*. In particular, *Leuconostocaceae,* which accounted for less than 1 % of fecal microbiota were more than 10 % of mucosal microbiota. The site specificity of the microbiota consortia was validated by UniFrac Distance Matrix analysis (Supplementary Figure 4-1).

### 4.3.3. Effect of β-fructans on the colonic microbiota in active UC

The effect of β-fructans on colonic microbiota was analyzed in two directions; 1) large-scale shifts across all samples and 2) dose-specific shifts across the samples from each prebiotic dose. The first analysis showed that treatment with β-fructans

induced a significant decrease of *Bacteriodetes* phylum while *Leuconostocaceae* tend to increase in the mucosal samples (Suppl. Table 4-3). In addition, mucosal *Lactobacillus* ssp. and *Oscillobacter* ssp. were altered at the end of the prebiotic intervention (Supplementary Table 4-3).

To identify shifts of the intestinal microbiota in response to β-fructans dose, a comparison between the changes of the fecal and mucosa-associated microbiota in patients treated with low versus high dose was performed. Figure 4-3 depicts changes in abundance at the family level; the changes in selected genera are depicted in the supplemental Figure 4-2. Dietary intervention with 7.5 g/d fructans did not induce significant changes in fecal or mucosal microbiota (Figure 4-3). A high dose of β-fructans predominantly altered fecal microbiota; the abundance of *Coriobacteriaceae, Erysipelotrichaceae* and *Lachnospiraceae* and *Dialister* spp. from *Veillonellaceae* increased significantly, suggesting the luminal microbiota as primarily involved in the prebiotic fermentation. In mucosal microbiota, the relative abundance of *Anaerococcus* spp. and *Bradyrhizobiaceae* family changed significantly.

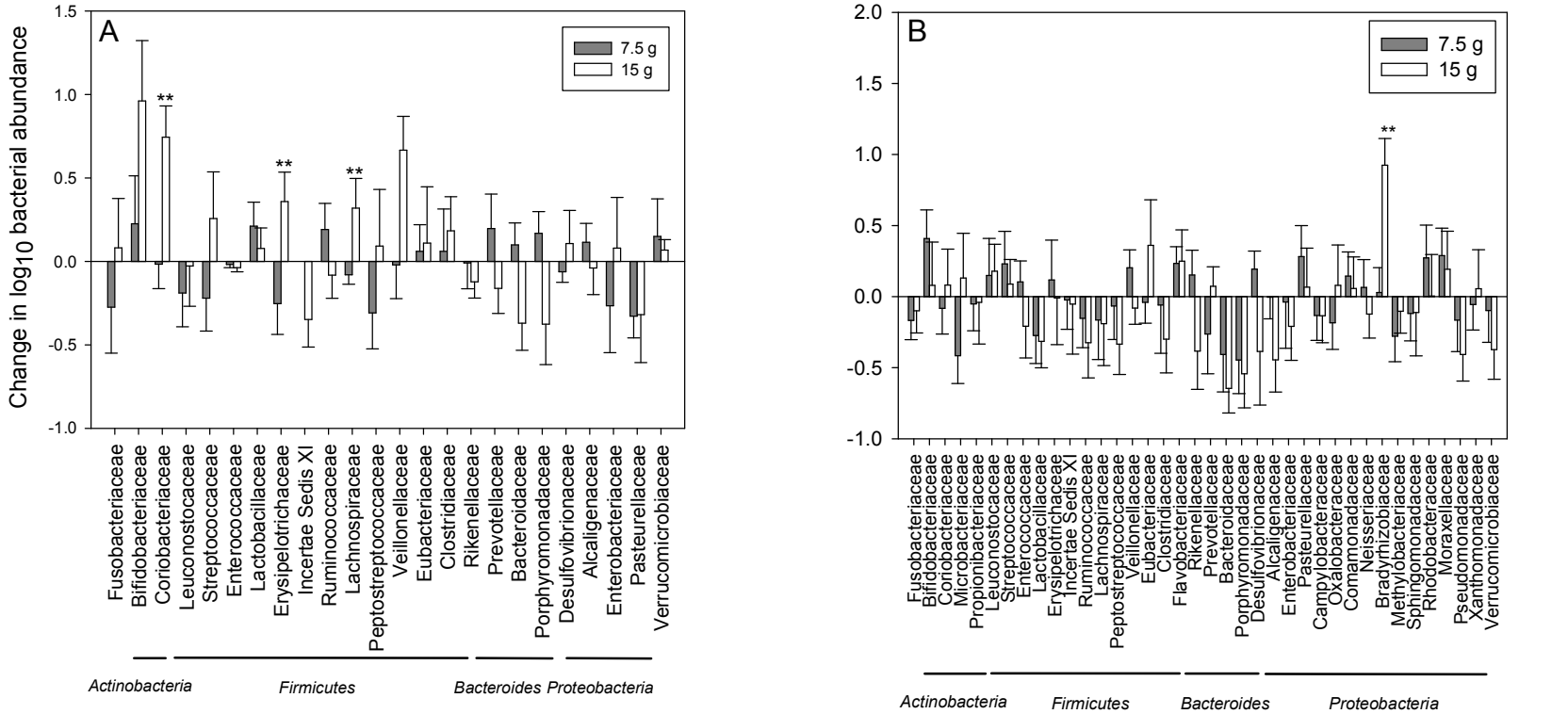


**Figure 4-3.** Comparison of the luminal (A) and mucosal (B) microbiota identified at Family level after 16S rRNA tags sequencing of DNA from fecal and biopsy samples of UC patients treated with 7.5 g or 15 g FOS/inulin for 9 weeks. Results are expressed as $\log_{10}$.

### 4.3.4. Inflammation is associated with specific microbiota

To determine whether the inflammation modulated the composition of gut microbiota, a multiple correlation analysis employing principle component analysis (PCA, Figure 4-4), Mann-Whitney two-sided test (Table 4-2) and Spearman's correlation analysis (Suppl. Table 4-4) was performed. The diversity of mucosal microbiota increased as measured by Shannon's and Simpson's indices increased with decreasing inflammation (Suppl. Tab. 4-4); the same trend was also seen in fecal samples.

PCA analysis showed that patients with reduced inflammation clustered separately from non-responding patients (Figure 4-4 A and B). All measured inflammation markers (Mayo score, endoscopy score, and fecal calprotectin) grouped in the same quadrants of the PCA plot (Figure 4-4 C and D). In fecal samples, inflammation was associated with *Lachnospiraceae, Eubacteriaceae, Rikenellaceae, Bacteroidaceae* and *Porphyromonadaceae* (Figure 4-4C). Mann-Whitney test further confirmed that stool samples from non-responding were characterized by a significantly higher proportion of *Blautia* ssp. from *Lachnospiraceae* (Table 4-2).

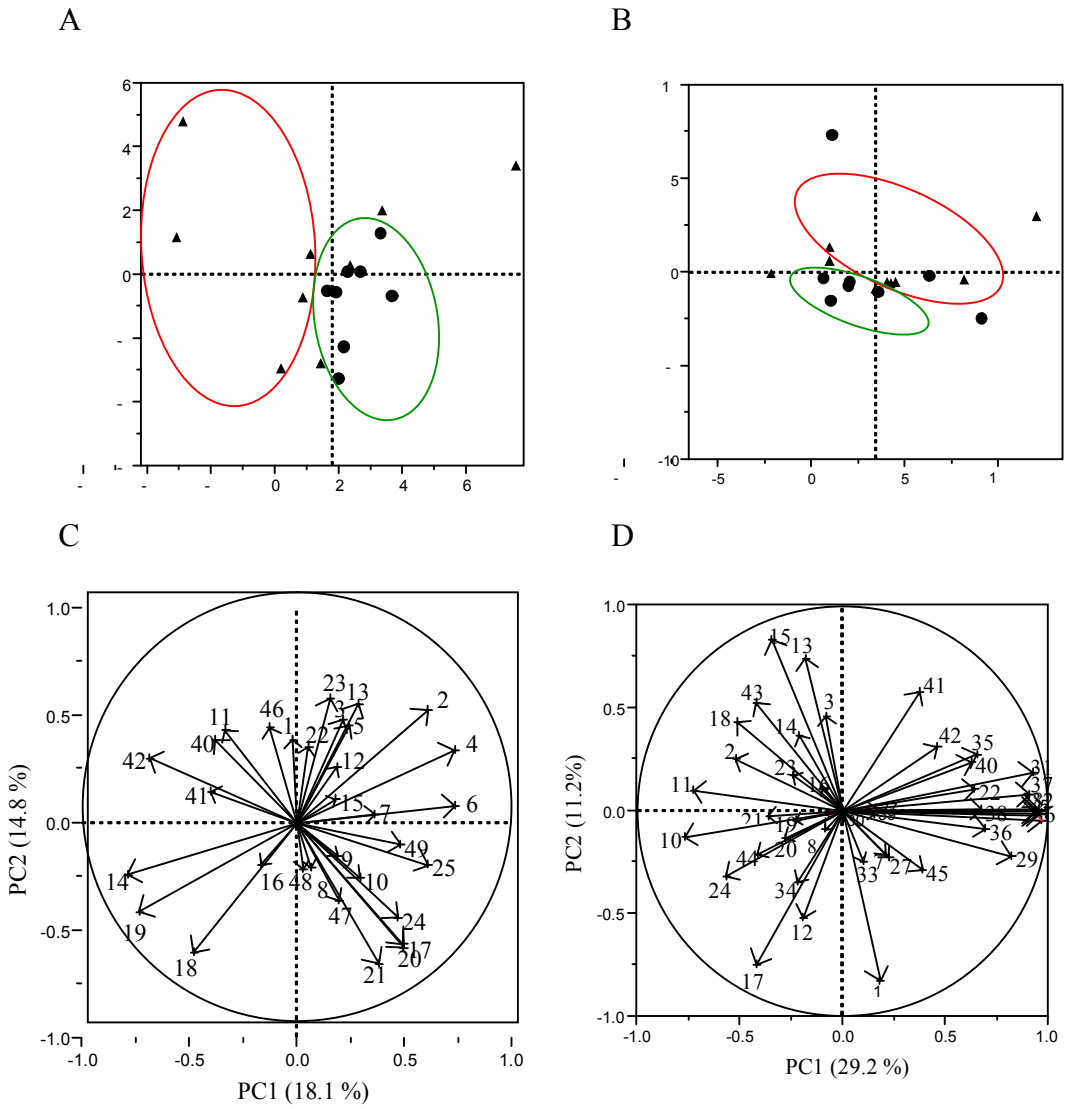


**Figure 4-4.** Principle component analysis of fecal (A, C) and biopsy (B, D) samples, based on inflammation markers and abundance of bacterial groups determined by pyrosequencing of 16S rRNA tags.

1 – *Fusobacteriaceae*; 2 – *Bifidobacteriaceae*; 3 – *Coriobacteriaceae*; 4 – *Leuconostocaceae*; 5 – *Streptococcaceae*; 6 – *Enterococcaceae*; 7 – *Lactobacillaceae*; 8 – *Erysipelotrichaceae*; 9 - *Incertae Sedis* XI; 10 –

*Ruminococcaceae*; 11 – *Lachnospiraceae*; 12 – *Peptostreptococcaceae*; 13 – *Veillonellaceae*; 14 – *Eubacteriaceae*; 15 – *Clostridiaceae*; 16 – *Rikenellaceae*; 17 – *Prevotellaceae*; 18 – *Bacteroidaceae*; 19 – *Porphyromonadaceae*; 20 – *Desulfovibrionaceae*; 21 – *Alcaligenaceae*; 22 – *Enterobacteriaceae*; 23 – *Pasteurellaceae*; 24 – *Verrucomicrobiaceae*; 25 - *Incertae Sedis* XIII; 26 – *Microbacteriaceae*; 27 – *Propionibacteriaceae*; 28 – *Flavobacteriaceae*; 29 – *Campylobacteraceae*; 30 – *Oxalobacteraceae*; 31 – *Comamonadaceae*; 32 – *Neisseriaceae*; 33 – *Bradyrhizobiaceae*; 34 – *Methylobacteriaceae*; 35 – *Sphingomonadaceae*; 36 – *Rhodobacteraceae*; 37 – *Moraxellaceae*; 38 – *Pseudomonadaceae*; 39 – *Xanthomonadaceae*; 40 – Mayo Clinical Score; 41 – endoscopy activity score; 42 – fecal calprotectin; 43 – butytyl-CoA-transferase gene; 44 – MCT1 relative expression; 45 – MUC2 relative expression; 46 – acetate; 47 – propionate; 48 – butyrate; 49 – total SCFA.

PCA of mucosal parameters (Figure 4-4D) placed inflammation markers in one quadrant with *Enterobacteriaceae, Comamonadaceae, Neisseriaceae, Sphingomonadaceae, Moraxellaceae,* and *Flavobacteriaceae.* The role of *Neisseriaceae* and more specifically *Microvirgula* ssp. in inflamed mucosa was validated by Mann-Whitney test (Table 4-2). Non-responders had significantly increased abundance of this taxon at the end of the study. In contrast, *Clostridiaceae* and *Dialister* ssp. from *Veillonellaceae* family were significantly reduced in non-responders (Table 4-2).

**Table 4-2**. Bacterial taxa identified by pyrosequencing of 16S rDNA sequence tags from fecal and biopsy samples of UC patients. Only bacterial taxa for which significant differences between responders and non-responders were observed are shown. Responders are defined as patients with decreased fecal calprotectin levels with 500 μg / g stool or more at the end of study.

| Phylum or Family (Genus) | Responders | | Non-Responders | | *P* value |
|---|---|---|---|---|---|
| | 0 week | 9 week | 0 week | 9 week | |
| **Stool** | | | | | |
| ***Firmicutes*** | | | | | |
| *Lachnospiraceae* ***(Blautia)*** | 2.15±0.94 | 0.78±0.15 | 1.11±0.38 | 2.46±0.81 | 0.049 |
| **Biopsies** | | | | | |
| ***Firmicutes*** | | | | | |
| *Clostridiaceae* | 0.12±0.06 | 1.68±1.23 | 0.72±0.42 | 0.065±0.029 | 0.019 |
| *Veillonellaceae* ***(Dialister)*** | 0.075±0.08 | 0.11±0.05 | 0.11±0.06 | 0.015±0.015 | 0.039 |
| ***Proteobacteria*** | | | | | |
| *Neisseriaceae* ***(Microvirgula)*** | 0.26±0.09 | 0.19±0.08 | 0.13±0.05 | 0.33±0.09 | 0.043 |

The associations between variables were further assessed by Spearman's correlation analysis. In fecal microbiota, only few bacterial taxa were correlated to inflammation. *Lactobacillus* and *Veillonella* were positively associated with Mayo score; *Prevotella*, *Odoribacter, Roseburia, Sutterella* and *Akkermansia* were negatively correlated either to Mayo score or to fecal calprotectin (Supplementary Table 4-4).

In contrast to fecal microbiota, mucosal microbiota were strongly associated with inflammation (Figure 4-5 and Supplementary Table 4-4). Inflammation was characterized by very distinct consortium of facultative anaerobic taxa mainly from *Proteobacteria* as well as *Enterococcaceae.* Among them *Microvirgula* showed the strongest correlation to fecal calprotectin.

Reduction of inflammation was correlated to increased abundance of the strict anaerobes *Dialister* ssp, *Faecalibacterium* ssp., *Roseburia* ssp. and unclassified *Clostridiaceae* in the mucosa. Sulfate-reducing *Desulfovibrio* ssp. and oxygen tolerant *Sutterella* ssp. were also negatively associated with inflammation.

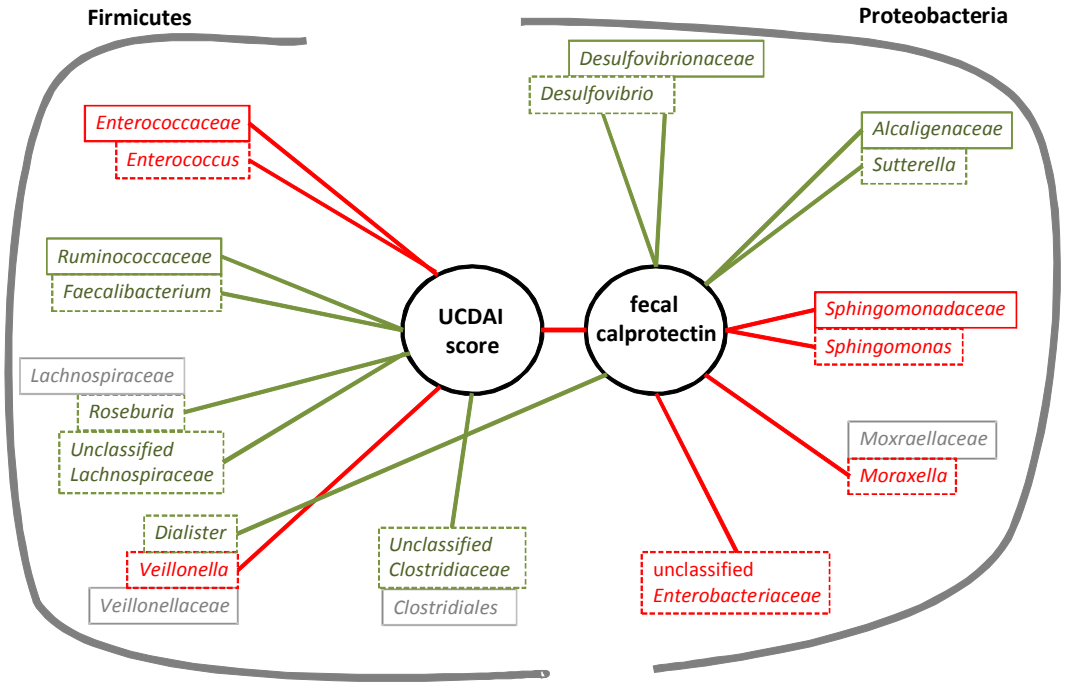


**Figure 4-5.** Network based on Spearman correlation analysis between disease activity measured by fecal calprotectin and Mayo score with bacterial groups from mucosa-associated microbiota. Only statistically significant ($P<0.05$) correlations are used in the network. Red colors indicate positive correlations, green colors indicate negative correlations. Family names are printed in grey if a genus within the family but not the family itself correlated to inflammation markers.

### 4.3.5. Butyrate metabolism is associated with colitis reduction

Short chain fatty acids in fecal samples were quantified to determine the effect of β-fructans on colonic fermentation. A trend toward a dose-dependent increase in the SCFA production was observed, with an average of 2 fold and 3.2 fold increase of SCFA in the low and high fructans group, respectively. However, changes in the concentration of SCFA over 9 weeks of treatment, or the changes in the proportion of individual SCFA among the total SCFA were not significant (Suppl. Figure 4-3). Only the fecal butyrate concentrations correlated to the Mayo score (Table 4-3). SCFA quantification was complemented by quantification of butyryl-CoA transferase, representing a key gene in bacterial butyrate formation, and by quantification of the expression of MCT1, a transport protein mediating butyrate transport in colonic epithelial cells (Cuff *et al*., 2002; Ritzhaupt *et al*., 1998). The gene copy numbers for butyryl-CoA transferase varied widely among patients and gene copy numbers did not correlate to Mayo score or fecal calprotectin. However, the expression of MCT1 was inversely correlated to fecal calprotectin (Table 4-3). Based on these findings the relationship of bacterial taxa and butyrate formation was further analyzed (Figure 4-6). Positive correlations with the copy numbers of butyryl-CoA-transacetylase, butyrate, or MCT1 expression were observed with bacterial taxa in the phyla *Firmucutes* and *Bacteroidetes* (Figure 4-6). Virtually all taxa in the phylum *Proteobacteria* negatively correlated to MCT1 expression, indicating impaired butyrate transport or tissue damage.

***Table 4-3.** Correlations (r) between inflammation (measured by Mayo score and fecal calprotectin) and butyrate metabolism (butyrate in feces, Butyryl-CoA-Transferase gene copy numbers, and MCT1 mRNA relative expression).

| Inflammation | Fecal calprotectin | Butyrate | Butyryl-CoA-transferase | MCT1 |
|---|---|---|---|---|
| **Mayo Clinical Score** | **0.53 (0.016)** | -0.46 (0.073) | NS | NS |
| **Fecal calprotectin** | NS | NS | NS | **-0.45 (0.048)** |

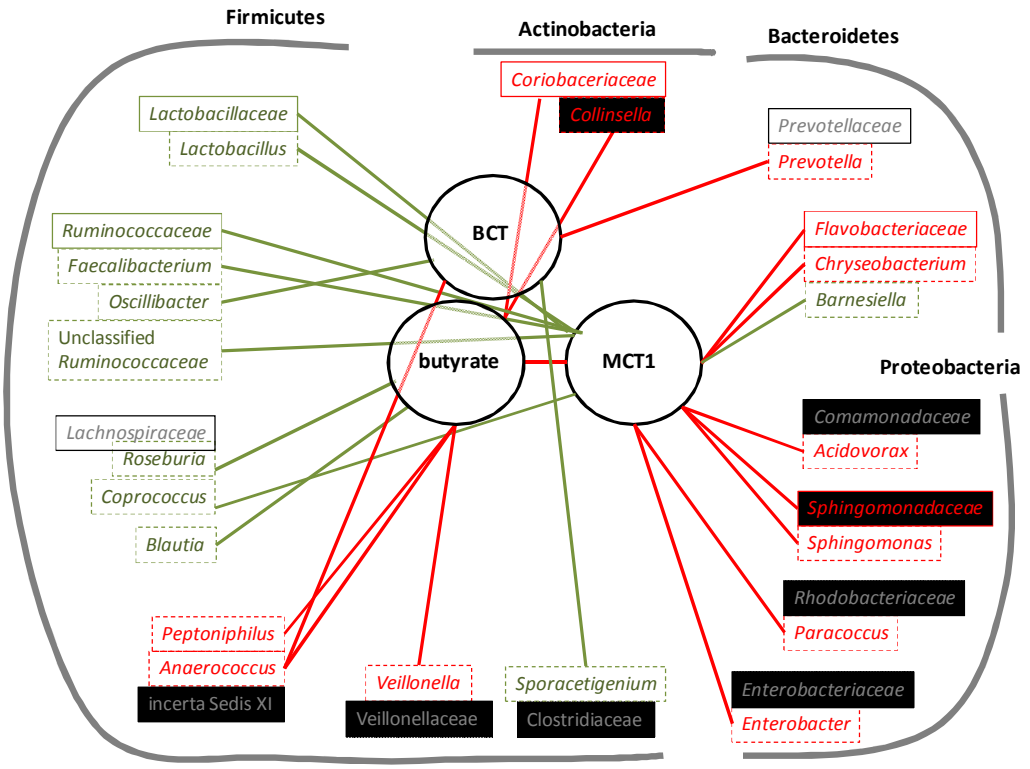


**Figure 4-6.** Network based on Spearman correlation analysis between butyrate metabolism as measured by butyrate production, fecal copy numbers of butyryl-CoA-transacetylase, MCT1 expression with bacterial taxa in mucosal samples. Only statistically significant ($p<0.05$) correlations are used in the network. Red colors indicate negative correlations, green colors indicate positive correlations. Family names are printed in grey if a genus within the family but not the family itself correlated to inflammation markers.

## 4.4. Discussion

IBD is associated with dysfunction of the intestinal microbiota (Morgan *et al.,* 2012; Erickson *et al*., 2012; Sokol *et al*., 2008) and dietary intervention to restore intestinal microbiota may be a successful approach for prevention or treatment of IBD. β-Fructans reduced colitis in transgenic rodent models, (Koleva *et al*., 2012; Hoentjen *et al*., 2005) but the assessment of their clinical efficacy is incomplete. Clinical studies using β-fructans applied a treatment duration that was too short to assess clinical as well as endoscopic efficacy, and neglected to assess potential protective mechanism (Casellas *et al*., 2007; Furrie *et al*., 2005). Therefore, the present study identified the effect of dietary fructans on the gut microbiome of UC patients and correlated bacterial taxa and metabolites to reduced disease activity.

Microbiome analysis performed in this study employed dynamic sampling within the same patient before and after treatment. This design complements previous studies that were based on a comparative approach of IBD patients, often including active and inactive disease, to healthy subjects (Willing *et al*., 2009; Joossens *et al*., 2011; Willing *et al*., 2010). Our analysis and others detected great inter-individual differences of mucosal and fecal microbiota (Willing *et al*., 2009; Momozawa *et al*., 2011; Hong *et al*., 2011). The disparity between fecal and mucosal microbiome within the individual was confirmed (Momozawa *et al*., 2011; Bajaj *et al*., 2012; Gorkiewicz *et al*., 2013). In keeping with the presence of oxygen gradient at the interface of aerobic host tissue and the anaerobic gut lumen, fecal microbiota were dominated by strict anaerobic *Bacteriodetes* and *Firmicutes,* whereas facultative anaerobic proteobacteria and *Leuconostocaceae*

were the most abundant taxa in UC mucosal microbiota. However, even strict anaerobes as *F. prausnitzii* may grow at oxic-anoxic interphase when exogenous antioxidants, as riboflavine and glutathione, are present (Khan *et al*., 2012). Depletion of antioxidants in the inflamed colonic tissue (Isman *et al*., 2003) may then result in decreased abundance of oxygen-sensitive bacteria in the UC mucosa.

Besides its specific composition, mucosal microbiota showed distinct function with respect to inflammation, supporting the hypothesis that mucosal microbes have more effect on gut immunity compared to fecal microbiome (Bajaj *et al*., 2012; Lepage *et al*., 2005). Colonic inflammation was characterized by a decreased richness of phylotypes in addition to increased abundance of mucosal *Proteobacteria*, validating previous observations (Lepage *et al*., 2011). Earlier *Escherichia / Shigella* was detected more often in the intestinal epithelium of UC patients versus that of non-IBD controls (Lepage *et al*., 2011; Kotlowski *et al.,* 2007). Here we showed that other *Proteobacteria,* notably *Microvirgula* ssp., were an ever stronger indicator of mucosal inflammation. This genus with one cultured representative *M. aerodenitrificans* uses nitrate, nitrite and nitrous oxide as alternative electron acceptors in anoxic conditions (Patureau *et al*., 1998). Nitric oxide production is increased in experimental colitis and colonic biopsy samples from UC patients (Abdelouhab *et al*., 2012; Dijkstra *et al*., 1998; Southey *et al*., 1997), thus possibly serving as a source of reducing equivalents for *Microvirgula*. The relative increase of oxygen-tolerant mucosal *Proteobacteria*

may then be regarded as a result of the host-mediated inflammation rather than triggering the inflammation perse (Lin *et al*., 2012; Lupp *et al*., 2007).

Decreased inflammation was associated by increased abundance of strict anaerobe *Firmicutes* as *Dialister, Faecalibacterium*, *Roseburia* and unclassified *Clostridiaceae. Roseburia* was previously reported to be under-represented in UC (Morgan *et al*., 2012; Vermeiren *et al*., 2012) however little is known about their function in colon. *In vitro* study showed that butyrate formation by *Roseburia/Eubacterium rectale* group is pH dependent (Walker *et al*., 2005). An increase of the pH from 5.5 to 6.5 resulted in a fourfold drop in the butyrate concentration, coupled with virtual elimination of *Roseburia* spp, despite the continued supply of polysaccharides, suggesting that the colonic environment rather the diet alone affects the growth and activity of *Roseburia.* In the frame of the UC pathogenesis we may then speculate that resolution of mucosal inflammation favors *Roseburia* (and other butyrate-producing bacteria) growth. Likewise, under-representation of *F. prausnitzii* and *Dialister* was documented in CD patients (Willing *et al*., 2009; Joossens *et al*., 2011) but not in UC. Our results demonstrated that these taxa may be protective in UC. In addition, *Dialister* were virtually absent in biopsy samples from all inflamed patients at the end of the study. *Dialister* also correlated to reduced plasma IL-6 concentrations in healthy subjects consuming whole grains (Martinez *et al*., 2013). We propose that *Dialister* is a suitable indicator microorganism for mucosal health.

Dietary intervention with β-fructans induced significant dose-dependent changes in the fecal microbiota as seen for other fibers (Martinez *et al*., 2013; Davis *et al*.,

2010). The high dose of β-fructans specifically stimulated fecal *Lachnospiraceae, Erysipelotrichaceae* and *Dialister* ssp. within *Firmicutes* and *Coriobacteriaceae* from *Actinobacteria.* Some *Lachnospiraceae* as *Roseburia inulinivorans* are able to directly ferment β-fructans and their enzyme system for inulin break down is well described (Scott *et al*., 2011). In addition, *Dialister* together with *Faecalibacterium* and *Phascolarctobacterium* were significantly increased by dietary fibers (polydextrose or soluble corn fiber) (Hooda *et al*., 2012). *Coriobacteriaceae* and *Erysipelotrichaceae* were previously related to cholesterol metabolism and high fat diet (Fleissner *et al*., 2010) or colorectal cancer (Chen *et al*., 2012) which directly linked them to energy production or adiposity but also implicated these taxa as major contributors to the obesity and colonic cancer. Many of the currently classified bacteria in *Erysipelotrichaceae* were formal *Lachnospiraceae* or *Clostridiaceae* with saccharolytic activity and SCFA production (Kageyama *et al*., 2000). Some *Coriobacteriaceae* as *Eggerthella* sp. were also shown to be involved in the metabolism of polyphenols (Queipo-Ortuno *et al*., 2012), lignan conversion (Mabrok *et al.,* 2012), and production of equol, a daidzein metabolite with estrogenic and antioxidative activities (Yokoyama *et al*., 2008; Wikoff *et al*., 2009). Therefore, it can be proposed that microbial fermentation of β-fructans in UC involved a number of bacterial groups, which are linked to increased energy production in an energy-deprived inflamed colon (Tims *et al*., 2013).

The colonic fermentation of β-fructans leads to the production of SCFA (Cherbut *et al*., 2003). Butyrate is a major energy source for colonocytes (Donohoe *et al*.,

2011) and reduces inflammation by preventing bacterial translocation (Lewis *et al*., 2010) and by modulation of signal transduction in the nuclear factor kappa B (NFκB) pathway (Segain *et al*., 2000). Microbiota of Crohn's patients are depleted of major butyrate-producing *Firmicutes*, including *Roseburia* spp. and *Faecalibacterium* spp. as well as butyrate-producing metabolic pathways (Morgan *et al*., 2012; Erickson *et al*., 2012). Fecal SCFA levels represent a balance between microbial production and host absorption (Regmi *et al*., 2011). We therefore additionally assessed microbial expression of butyryl-CoA transferase, and host expression of the monocarboxylate transporter 1 (MCT1). Butyryl-CoA transferase harboring microorganisms display lower abundance in both the luminal and mucosal fractions of UC patients compared to healthy controls (Vermeiren *et al*., 2012). However, our results show no direct association between inflammation and the quantity of this enzyme encoding gene. PCA analysis indicated that MCT1 expression and fecal butyrate concentrations are highly related and inversely associated with disease activity. Moreover, MCT1 expression and fecal butyrate concentrations correlated to fecal calprotectin and Mayo score, respectively. Phylotypes that correlated with increased butyrate synthesis, notably in the fecal *Erysipelotrichaceae* and *Lachnospiraceae,* also correlated to lowered disease activity and were stimulated by the high-dose β-fructan intervention. These data all support the hypothesis that β-fructans reduce intestinal inflammation through stimulation of butyrate-producing bacteria. However, lack of a placebo group to serve as a control for monitoring the microbiome changes in absence of β-fructans limit conclusions on their potential

clinical efficacy in active UC. Therefore, host / inflammation-mediated changes of the intestinal microbiota composition and function, including butyrate formation, should not be ruled out.

In conclusion, this small open label study indicates that a dose of 15 g/day β-fructans induced significant changes in the intestinal microbiota and identified butyrate-producing *Firmicutes* from *Lachnospiraceae, Erysipelotrichaceae* as well as *Dialister* as the most relevant microorganisms involved in the colonic fermentation of β-fructan in active UC. The presence of colonic inflammation was not correlated to the composition of luminal microbiota but reflected in a different composition of mucosal microbiota. Improvements in inflammation were associated with increased bacterial diversity and enhanced butyrate production. The high dose β-fructan treatment is suitable for further studies in active UC.

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## 4.6. Supplementary material

***Supplementary Table 4-1.** Patient demographic and baseline characteristics.

| Characteristic | 7.5 g FOS/inulin group | 15 g FOS/inulin group |
|---|---|---|
| **Number of patients (n)** | 17 | 12 |
| **Gender** | | |
| **Male** | 8 (47%) | 3 (25%) |
| **Female** | 9 (53%) | 9 (75%) |
| **Mean age at entry (yr)** | 35 (range, 18 – 58) | 43 (range, 20 – 65) |
| **Disease location** | | |
| **Proctosigmoiditis** | 1 (6%) | 4 (33%) |
| **Left-sided colitis** | 11 (65%) | 6 (50%) |
| **Pancolitis** | 5 (29%) | 2 (17%) |
| **Concomitant medications** | | |
| **No medication** | 4 (24%) | 3 (25%) |
| **5-aminosalicylic acid (5-ASA)** | 13 (76%) | 9 (75%) |

**Supplementary Table 4-2.** Abundance (%) of bacterial groups in fecal microbiota and colonic biopsies of patients with mild to moderate ulcerative colitis at 0 week (before inulin plus FOS treatment) and 9 week (after the treatment), determined by pyrosequencing of 16S rRNA tags.

| Phylum | Family | Genus | Fecal samples | | Biopsy samples | |
|---|---|---|---|---|---|---|
| | | | 0 week | 9 week | 0 week | 9 week |
| *Fusobacteria* | | | 1.57±1.56 | 0.02±0.02 | 0.33±0.29 | 0.02±0.01 |
| | *Fusobacteriaceae* | | 1.57±1.56 | 0.03±0.02 | 0.33±0.29 | 0.01±0.01 |
| | | *Fusobacterium* | 1.54±1.54 | 0.02±0.02 | 0.29±0.26 | 0.01±0.01 |
| *Actinobacteria* | | | 3.14±1.33 | 5.13±2.15 | 5.04±1.59 | 6.03±0.50 |
| | *Microbacteriaceae* | | - | - | 2.21±1.44 | 1.94±1.21 |
| | | *Microbacterium* | - | - | 2.19±1.43 | 1.93±1.20 |
| | *Propionibacteriaceae* | | - | - | 0.06±0.02 | 0.10±0.03 |
| | *Bifidobacteriaceae* | | 2.37±1.16 | 4.47±2.12 | 2.08±0.63 | 3.28±1.11 |
| | | *Bifidobacterium* | 2.28±1.12 | 4.39±2.10 | 2.05±0.62 | 3.23±1.09 |
| | *Coriobacteriaceae* | | 0.74±0.25 | 0.63±0.16 | 0.58±0.24 | 0.53±0.19 |
| | | *Collinsella* | 0.58±0.24 | 0.32±0.14 | 0.46±0.22 | 0.44±0.18 |
| | | *Eggerthella* | 0.06±0.03 | 0.15±0.08 | - | - |
| *Firmicutes* | | | 50.84±5.77 | 56.36±6.42 | 48.31±2.97 | 52.46±3.44 |
| | *Leuconostocaceae* | | 0.057±0.03 | 0.07±0.05 | 14.39±2.68 | 21.36±3.27 |
| | | *Leuconostoc* | - | | 10.33±1.89 | 14.94±2.19 |
| | | *Weissella* | - | | 3.85±0.76 | 6.12±1.09 |
| | *Streptococcaceae* | | 0.86±0.46 | 0.18±0.07 | 3.19±0.62 | 4.49±0.76 |

| Phylum | Family | Genus | Fecal samples | | Biopsy samples | |
|---|---|---|---|---|---|---|
| | | *Streptococcus* | 0.82±0.46 | 0.18±0.07 | 0.48±0.09 | 0.66±0.13 |
| | | *Lactococcus* | - | - | 2.70±0.53 | 3.82±0.66 |
| | *Enterococcaceae* | | 0.09±0.09 | 0.06±0.05 | 0.49±0.11 | 0.71±0.13 |
| | | *Enterococcus* | 0.09±0.08 | 0.05±0.04 | 0.47±0.11 | 0.67±0.13 |
| | *Lactobacillaceae* | | 0.09±0.07 | 1.95±1.46 | 0.02±0.01 | 0.10±0.05 |
| | | *Pediococcus* | 0.03±0.02 | 0.43±0.40 | - | - |
| | | *Lactobacillus* | 0.06±0.05 | 1.52±1.42 | 0.01±0.01 | 0.10±0.04 |
| | *Erysipelotrichaceae* | | 0.57±0.17 | 0.97±0.46 | 1.25±0.47 | 3.16±2.05 |
| | | *Coprobacillus* | 0.28±0.10 | 0.27±0.12 | 0.96±0.47 | 2.68±2.04 |
| | | *Holdemania* | 0.10±0.05 | 0.12±0.05 | - | - |
| | | *Catenibacterium* | 0.05±0.05 | 0.48±0.47 | 0.11±0.11 | 0.34±0.31 |
| | *Incertae Sedis XIII* | | 0.09±0.07 | 0.02±0.01 | - | - |
| | *Incertae Sedis XI* | | 0.39±0.20 | 0.04±0.02 | 0.26±0.19 | 0.34±0.30 |
| | | *Parvimonas* | 0.31±0.19 | 0.03±0.02 | 0.01±0.01 | 0.25±0.24 |
| | | *Peptoniphilus* | - | - | 0.14±0.09 | 0.04±0.04 |
| | | *Anaerococcus* | - | - | 0.05±0.05 | 0.03±0.02 |
| | *Ruminococcaceae* | | 17.48±4.32 | 18.69±4.55 | 5.83±1.05 | 5.11±1.82 |
| | | *Faecalibacterium* | 5.48±2.43 | 8.64±3.47 | 3.22±0.70 | 3.24±1.25 |
| | | *Ruminococcus* | 0.11±0.05 | 0.32±0.20 | 0.18±0.07 | 0.19±0.10 |
| | | *Anaerotruncus* | 0.16±0.08 | 0.12±0.04 | 0.07±0.02 | 0.05±0.04 |
| | | *Subdoligranulum* | 8.18±4.22 | 4.97±3.43 | 0.58±0.24 | 0.54±0.28 |
| | *Oscillospiraceae* | | 0.48±0.12 | 0.79±0.25 | 0.35±0.08 | 0.08±0.04 |
| | | *Oscillibacter* | 0.48±0.12 | 0.79±0.25 | 0.35±0.08 | 0.08±0.04 |
| | *Lachnospiraceae* | | 14.90±3.11 | 15.97±2.37 | 15.97±2.66 | 11.21±2.54 |
| | | *Blautia* | 1.56±0.46 | 1.67±0.47 | 3.89±0.70 | 2.68±0.49 |

| Phylum | Family | Genus | Fecal samples | | Biopsy samples | |
|---|---|---|---|---|---|---|
| | | *Coprococcus* | 0.79±0.30 | 0.68±0.22 | 0.83±0.19 | 0.56±0.19 |
| | | *Dorea* | 0.93±0.32 | 1.29±0.41 | 1.28±0.41 | 0.80±0.27 |
| | | *Roseburia* | 1.21±0.29 | 1.85±0.63 | 0.80±0.16 | 0.438±0.13 |
| | *Peptostreptococcaceae* | | 0.54±0.19 | 1.76±1.44 | 1.57±0.57 | 0.75±0.28 |
| | | *Sporacetigenium* | 0.33±0.14 | 1.28±1.17 | 0.33±0.10 | 0.19±0.08 |
| | *Veillonellaceae* | | 7.27±2.51 | 8.01±2.49 | 0.57±0.19 | 0.98±0.52 |
| | | *Megamonas* | - | - | 0.05±0.05 | 0.52±0.51 |
| | | *Dialister* | 5.27±2.18 | 6.59±2.41 | 0.09±0.04 | 0.06±0.03 |
| | | *Veillonella* | 0.64±0.33 | 0.45±0.28 | 0.31±0.15 | 0.33±0.08 |
| | | *Phascolarctobacterium* | 0.59±0.32 | 0.47±0.29 | 0.10±0.07 | 0.05±0.03 |
| | | *Megasphaera* | 0.24±0.24 | 0.01±0.01 | - | - |
| | *Eubacteriaceae* | | 0.25±0.09 | 0.23±0.10 | 0.17±0.07 | 0.05±0.02 |
| | | *Eubacterium* | 0.25±0.09 | 0.22±0.10 | 0.15±0.06 | 0.05±0.02 |
| | *Clostridiaceae* | | 0.78±0.65 | 0.39±0.15 | 0.42±0.22 | 0.78±0.56 |
| | | *Butyricicoccus* | 0.36±0.24 | 0.55±0.31 | 0.14±0.04 | 0.21±0.07 |
| | | *Clostridium* | 0.65±0.56 | 0.26±0.11 | 0.34±0.16 | 0.63±0.45 |
| *Bacteroidetes* | | | 36.27±6.24 | 33.26±6.03 | 23.20±2.71 | 11.96±2.29 |
| | *Rikenellaceae* | | 0.73±0.39 | 0.84±0.53 | 0.41±0.20 | 0.13±0.10 |
| | | *Alistipes* | 0.73±0.39 | 0.83±0.53 | 0.41±0.20 | 0.13±0.10 |
| | *Prevotellaceae* | | 1.06±0.97 | 0.27±0.15 | 1.14±0.71 | 0.52±0.39 |
| | | *Prevotella* | 0.79±0.76 | 0.09±0.07 | 0.43±0.35 | 0.10±0.05 |
| | *Bacteroidaceae* | | 29.43±5.26 | 27.93±5.73 | 18.03±2.27 | 9.14±2.06 |
| | | *Bacteroides* | 29.43±5.26 | 27.93±5.73 | 18.03±2.27 | 9.14±2.06 |
| | *Porphyromonadaceae* | | 3.84±1.67 | 3.26±1.53 | 2.57±0.90 | 1.02±0.52 |

| Phylum | Family | Genus | Fecal samples | | Biopsy samples | |
|---|---|---|---|---|---|---|
| | | *Butyricimonas* | - | - | 0.08±0.04 | 0.01±0.01 |
| | | *Barnesiella* | 0.46±0.30 | 1.22±0.87 | 0.18±0.09 | 0.16±0.14 |
| | | *Parabacteroides* | 3.17±1.60 | 1.39±0.79 | 2.24±0.91 | 0.78±0.49 |
| | | *Odoribacter* | 0.11±0.07 | 0.15±0.12 | - | - |
| | *Flavobacteriaceae* | | - | - | 0.69±0.15 | 0.93±0.16 |
| | | *Flavobacterium* | - | - | 0.04±0.02 | 0.06±0.02 |
| | | *Chryseobacterium* | - | - | 0.62±0.13 | 0.80±0.14 |
| *Proteobacteria* | | | 4.36±2.06 | 1.25±0.27 | 20.25±4.18 | 25.08±4.55 |
| | *Campylobacteraceae* | | - | - | 0.23±0.06 | 0.23±0.05 |
| | | *Sulfurospirillum* | - | - | 0.05±0.02 | 0.03±0.01 |
| | | *Arcobacter* | - | - | 0.18±0.04 | 0.20±0.05 |
| | *Desulfovibrionaceae* | | 0.07±0.05 | 0.03±0.01 | 0.12±0.07 | 0.01±0.01 |
| | | *Desulfovibrio* | - | - | 0.10±0.07 | 0.01±0.01 |
| | *Sutterellaceae* | | 0.41±0.14 | 0.24±0.11 | 0.91±0.30 | 0.63±0.25 |
| | | *Sutterella* | 0.17±0.08 | 0.08±0.05 | 0.68±0.31 | 0.35±0.21 |
| | | *Parasutterella* | 0.25±0.13 | 0.16±0.11 | 0.23±0.08 | 0.28±0.18 |
| | *Oxalobacteraceae* | | - | - | 0.07±0.04 | 0.13±0.10 |
| | | *Massilia* | - | - | 0.05±0.04 | 0.11±0.10 |
| | *Comamonadaceae* | | - | - | 0.35±0.08 | 0.48±0.10 |
| | | *Comamonas* | - | - | 0.08±0.02 | 0.10±0.03 |
| | | *Acidovorax* | - | - | 0.23±0.05 | 0.33±0.07 |
| | *Neisseriaceae* | | - | - | 0.36±0.10 | 0.49±0.13 |
| | | *Microvirgula* | - | - | 0.20±0.05 | 0.27±0.06 |
| | | *Vitreoscilla* | - | - | 0.11±0.03 | 0.12±0.04 |
| | *Bradyrhizobiaceae* | | - | - | 9.40±4.14 | 13.29±4.37 |

| Phylum | Family | Genus | Fecal samples | | Biopsy samples | |
|---|---|---|---|---|---|---|
| | | *Bradyrhizobium* | - | - | 1.11±0.55 | 1.54±0.60 |
| | | *Afipia* | - | - | 0.04±0.02 | 0.05±0.02 |
| | *Methylobacteriaceae* | | - | - | 0.15±0.09 | 0.28±0.18 |
| | | *Methylobacterium* | - | - | 0.15±0.09 | 0.28±0.18 |
| | *Sphingomonadaceae* | | - | - | 0.04±0.01 | 0.05±0.02 |
| | | *Sphingomonas* | - | - | 0.04±0.01 | 0.04±0.02 |
| | *Rhodobacteraceae* | | - | - | 0.08±0.02 | 0.13±0.03 |
| | | *Paracoccus* | - | - | 0.04±0.02 | 0.04±0.02 |
| | *Moraxellaceae* | | - | - | 3.44±0.72 | 4.61±0.87 |
| | | *Acinetobacter* | - | - | 3.11±0.66 | 4.29±0.82 |
| | | *Enhydrobacter* | - | - | 0.29±0.06 | 0.26±0.06 |
| | *Pseudomonadaceae* | | - | - | 0.06±0.02 | 0.08±0.02 |
| | | *Pseudomonas* | - | - | 0.06±0.02 | 0.08±0.02 |
| | *Enterobacteriaceae* | | 3.51±2.06 | 0.64±0.29 | 4.12±0.71 | 3.63±0.65 |
| | | *Escherichia/Shigella* | 2.18±1.67 | 0.51±0.27 | 1.12±0.66 | 0.11±0.04 |
| | | *Enterobacter* | - | - | 0.23±0.04 | 0.34±0.08 |
| | | *Klebsiella* | - | - | 0.09±0.02 | 0.09±0.03 |
| | | *Citrobacter* | 1.00±1.00 | 0.01±0.01 | 1.33±0.27 | 1.66±0.33 |
| | *Pasteurellaceae* | | 0.35±0.12 | 0.19±0.06 | 0.11±0.05 | 0.13±0.08 |
| | | *Haemophilus* | 0.25±0.09 | 0.16±0.06 | 0.07±0.04 | 0.11±0.07 |
| | *Xanthomonadaceae* | | - | - | 0.38±0.25 | 0.29±0.19 |
| | | *Stenotrophomonas* | - | - | 0.37±0.25 | 0.27±0.18 |
| *Verrucomicrobia* | | | 1.13±0.92 | 1.66±0.74 | 0.95±0.84 | 0.04±0.02 |
| | *Verrucomicrobiaceae* | | 1.13±0.93 | 1.66±0.74 | 0.95±0.84 | 0.04±0.02 |
| | | *Akkermansia* | 1.13±1.66 | 1.66±0.74 | 0.95±0.84 | 0.04±0.02 |

| Phylum | Family | Genus | Fecal samples | | Biopsy samples | |
|---|---|---|---|---|---|---|
| *Cyanobacteria* | | | 0.01±0.01 | 0.29±0.28 | 0.04±0.01 | 0.08±0.02 |
| | *Chloroplast* | | 0.01±0.01 | 0.29±0.28 | 0.04±0.01 | 0.08±0.02 |
| | | *Streptophyta* | 0.01±0.01 | 0.29±0.28 | 0.04±0.01 | 0.08±0.02 |

**Supplementary Table 4-3.** Bacterial taxa identified by pyrosequencing of 16S rDNA sequence tags from fecal and biopsy samples of UC patients. Only bacterial taxa for which significant differences were observed in comparison of samples taken before or after treatment with β-fructans for 9 weeks are shown.

| Bacterial Taxon | | | Fecal microbiota | | Mucosal microbiota | |
|---|---|---|---|---|---|---|
| **Phylum** | **Family** | **Genus** | **0 week** | **9 week** | **0 week** | **9 week** |
| ***Bacteroidetes*** | | | 36.27±6.24 | 33.26±6.03 | **23.20±2.71[A]** | **11.96±2.29** |
| | *Bacteroidaceae* | | 29.43 ± 5.26 | 27.93 ± 5.73 | **18.03±2.27[A]** | **9.14 ± 2.06** |
| | | *Bacteroides* | 29.43±5.26 | 27.93±5.73 | **18.03±2.27[A]** | **9.14±2.06** |
| ***Firmicutes*** | | | 50.84±5.77 | 56.36±6.42 | 48.31±2.97 | 52.46±3.44 |
| | *Leuconostocaceae* | | 0.06±0.03 | 0.07±0.05 | **14.39±2.68[B]** | **21.36±3.27** |
| | *Lactobacillaceae* | *Lactobacillus* | 0.06±0.05 | 1.52±1.42 | **0.01±0.01[A]** | **0.10±0.04** |
| | *Oscillospiraceae* | *Oscillibacter* | 0.48±0.12 | 0.79±0.25 | **0.35±0.08[A]** | **0.08±0.04** |

Results are presented as means and standard error of the mean. [A] Means for the relative abundance of the same taxon that shows statistically significant difference ($P<0.05$). [B] Means for the relative abundance of the same taxon approaching statistically significant differences ($p<0.1$) at 0 week versus 9 week.

**Supplementary Table 4-4.** Correlations between inflammation (measured by Mayo score and fecal calprotectin) and butyrate metabolism (butyrate in feces, Butyryl-CoA-Transferase gene copy numbers, and MCT1 mRNA relative expression), as well as between the abundance in mucosa-associated bacterial taxa and fecal calprotectin, Mayo score, butyrate, Butyryl-CoA-Transferase gene copy numbers, and MCT1 expression in colonic biopsy samples.

| | | **Shannon's index** | | | | **Simpson's index** | |
|---|---|---|---|---|---|---|---|
| **Fecal calprotectin** | | **-0.64 (0.001)** | | | | **-0.55 (0.008)** | |
| **Phylum or Family** | **Genus** | **Abund. (%)** | **Mayo score** | **Fecal calprotect.** | **Butyrate** | **Butyryl-CoA-Transfe.** | **MCT1** |
| ***Actinobacteria*** | | | | | | | |
| | Unc. *Bifido-bacteriaceae* | 0.0 – 0.6 | | -0.49 (0.043) | | | |
| ***Firmicutes*** | | | | | | | |
| ***Lactobacillaceae*** | | 0.0 – 24.4 | | | | | |
| | *Lactobacillus* | 0.0 – 24.3 | 0.54 (0.025) | | -0.58 (0.049) | | |
| ***Erys-ipelotrichaceae*** | | 0.05 – 8.05 | | | 0.58 (0.049) | | |
| | *Holdemania* | 0.0 – 0.9 | | | | -0.59 (0.011) | |
| | *Catenibacterium* | 0.0 – 0.9 | | | 0.59 (0.043) | | |
| ***Lachnospiraceae*** | | 0.06 – 40.2 | | | 0.75 (0.0051) | | |
| | *Roseburia* | 0.0 – 9.3 | | -0.51 (0.037) | | | |
| | Unc. *Lachnospiraceae* | 0.6 – 33.2 | | | 0.64 (0.024) | | |

| Phylum or Family | Genus | Abund. (%) | Mayo score | Fecal calprotect. | Butyrate | Butyryl-CoA-Transfe. | MCT1 |
|---|---|---|---|---|---|---|---|
| ***Eubacteriaceae*** | | 0.0 – 1.5 | | | | 0.63 (0.0062) | |
| | *Eubacterium* | 0.0 – 1.5 | | | | 0.61 (0.0091) | |
| ***Pepto-streptococcaceae*** | | 0.0 – 24.6 | | | 0.62 (0.030) | | |
| ***Veillonellaceae*** | | 0.3 – 42.7 | | | | | |
| | *Veillonella* | 0.0 – 4.5 | 0.52 (0.034) | | -0.64 (0.025) | | |
| | *Phasco-larctobacterium* | 0.0 – 4.7 | | | | | |
| ***Clostridiaceae*** | | 0.0 – 10.2 | | | | | |
| | *Sporacetigenium* | 0.0 – 1.85 | | | 0.65 (0.021) | | |
| ***Bacteroidetes*** | | | | | | | |
| ***Prevotellaceae*** | | 0.0 – 17.6 | -0.67 (0.0032) | | | | |
| | *Prevotella* | 0.0 – 13.7 | -0.69 (0.001) | | | | |
| | Unc. *Prevotellaceae* | 0.0 – 3.75 | -0.49 (0.047) | | | | |
| ***Porphyromonadaceae*** | | 0.0 – 22.6 | | | | | |
| | *Parabacteroides* | 0.0 – 22.5 | | | | | 0.59 (0.012) |
| | *Odoribacter* | 0.0 – 2.0 | -0.55 (0.023) | | | | 0.54 (0.024) |
| ***Proteobacteria*** | | | | | | | |
| ***Alcaligenaceae*** | | 0.0 – 2.05 | | | | | |
| | *Sutterella* | 0.0 – 1.2 | | | | | 0.48 (0.049) |
| ***Pasteurellaceae*** | | 0.0 – 1.55 | | | | | -0.60 (0.011) |

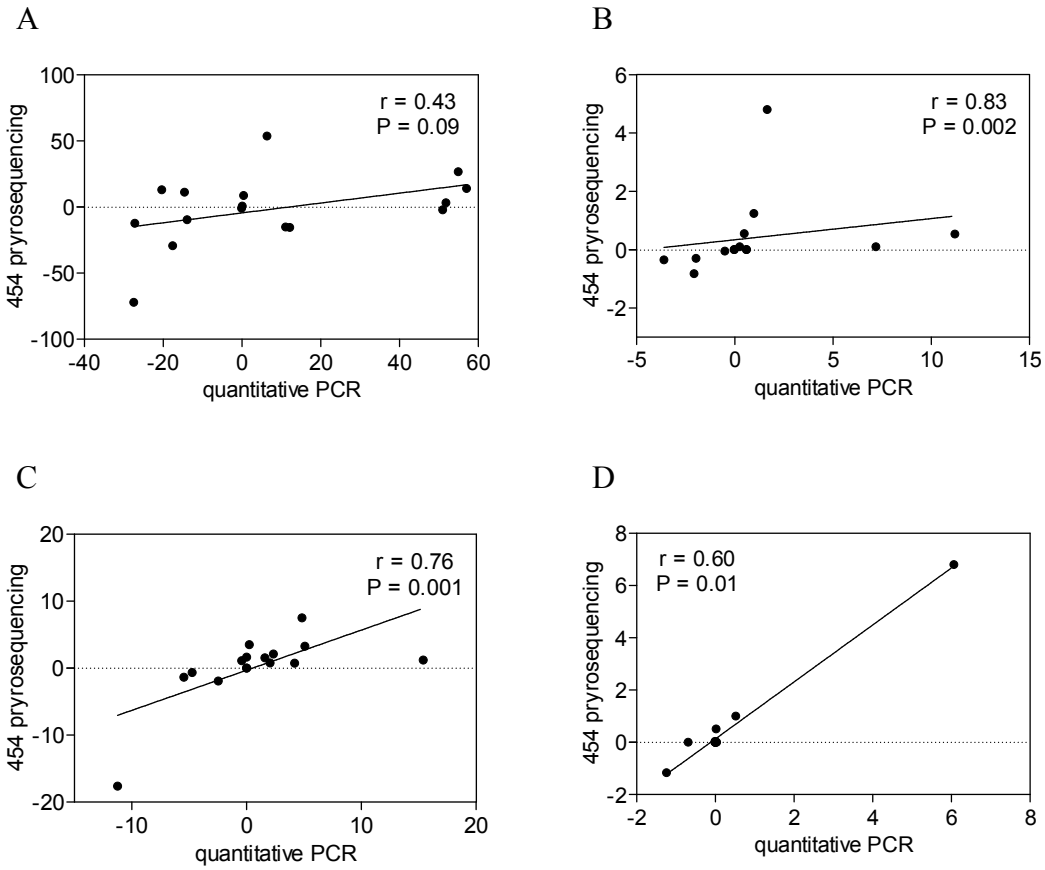
| Phylum or Family | Genus | Abund. (%) | Mayo score | Fecal calprotect. | Butyrate | Butyryl-CoA-Transfe. | MCT1 |
|---|---|---|---|---|---|---|---|
| | *Haemophilus* | 0.0 – 1.3 | | | | | -0.59 (0.011) |
| ***Verrucomicrobia*** | | | | -0.49 (0.047) | | | 0.70 (0.015) |
| ***Verrucomicrobiaceae*** | | 0.0 – 16.7 | | -0.49 (0.047) | | | 0.71 (0.001) |
| | *Akkermansia* | 0.0 – 16.7 | | -0.49 (0.047) | | | 0.71 (0.001) |
| ***Actinobacteria*** | | | | | | | |
| ***Coriobacteriaceae*** | | 0.0 – 4.6 | | | -0.73 (0.0047) | | |
| | *Collinsella* | 0.0 – 4.1 | | | -0.76 (0.0026) | | |
| ***Firmicutes*** | | | | | 0.67 (0.012) | | |
| ***Leuconostocaceae*** | | 0.1 – 51.5 | | | | | |
| | Unc. *Leuconostocaceae* | 0.0 – 0.95 | 0.53 (0.025) | | | -0.50 (0.045) | |
| ***Enterococcaceae*** | | 0.0 – 1.65 | 0.51 (0.029) | | | | |
| | *Enterococcus* | 0.0 – 1.65 | 0.48 (0.043) | | | | |
| ***Lactobacillaceae*** | | 0.0 – 0.65 | | -0.51 (0.038) | | | 0.57 (0.014) |
| | *Lactobacillus* | 0.0 – 0.65 | | -0.51 (0.038) | | | 0.57 (0.014) |
| ***Erysipelotrichaceae*** | | 0.0 – 37.3 | | | | | |
| | *Coprobacillus* | 0.0 – 9.05 | | | | | |
| ***Ruminococcaceae*** | | 0.05 – 26.95 | -0.52 (0.027) | | | | 0.54 (0.019) |
| | *Faecalibacterium* | 0.0 – 19.1 | -0.61 | | | | 0.47 (0.048) |

| Phylum or Family | Genus | Abund. (%) | Mayo score | Fecal calprotect. | Butyrate | Butyryl-CoA-Transfe. | MCT1 |
|---|---|---|---|---|---|---|---|
| | | | (0.007) | | | | |
| | *Oscillibacter* | 0.0 – 1.2 | | | | 0.65 (0.0064) | |
| | *Subdoligranulum* | 0.0 – 4.9 | | | | | |
| | Unc. *Ruminococcaceae* | 0.0 – 8.9 | | | | | 0.48 (0.046) |
| ***Lachnospiraceae*** | | 0.05 – 31.5 | | | | | |
| | *Blautia* | 0.0 – 11.05 | | | 0.71 (0.0061) | | |
| | *Roseburia* | 0.0 – 2.4 | -0.52 (0.027) | | 0.69 (0.0087) | | |
| | *Coprococcus* | 0.0 – 3.1 | | | | | 0.58 (0.012) |
| | Unc. *Lachnospiracea* | 0.05 – 24.7 | -0.47 (0.047) | | | | |
| ***Incertae Sedis* XI** | | 0.0 – 5.5 | | | | | |
| | *Peptoniphilus* | 0.0 – 1.7 | | | -0.66 (0.013) | | |
| | *Anaerococcus* | 0.0 – 1.1 | | | -0.65 (0.017) | -0.62 (0.010) | |
| ***Veillonellaceae*** | | 0.05 – 9.75 | | | | | |
| | *Dialister* | 0.0 – 0.65 | | -0.76 (0.0004) | | | |
| | *Veillonella* | 0.0 – 3.2 | 0.52 (0.028) | | -0.65 (0.017) | | |
| ***Clostridiaceae*** | | 0.0 – 10.2 | | | | | |
| | *Sporacetigenium* | 0.0 – 1.9 | | | | 0.51 (0.045) | |
| | Unc. *Clostridiales* | 0.0 – 1.9 | -0.50 (0.037) | | | | |
| ***Bacteroidetes*** | | | | | | | |
| ***Rikenellaceae*** | | 0.0 – 3.5 | | | | | |

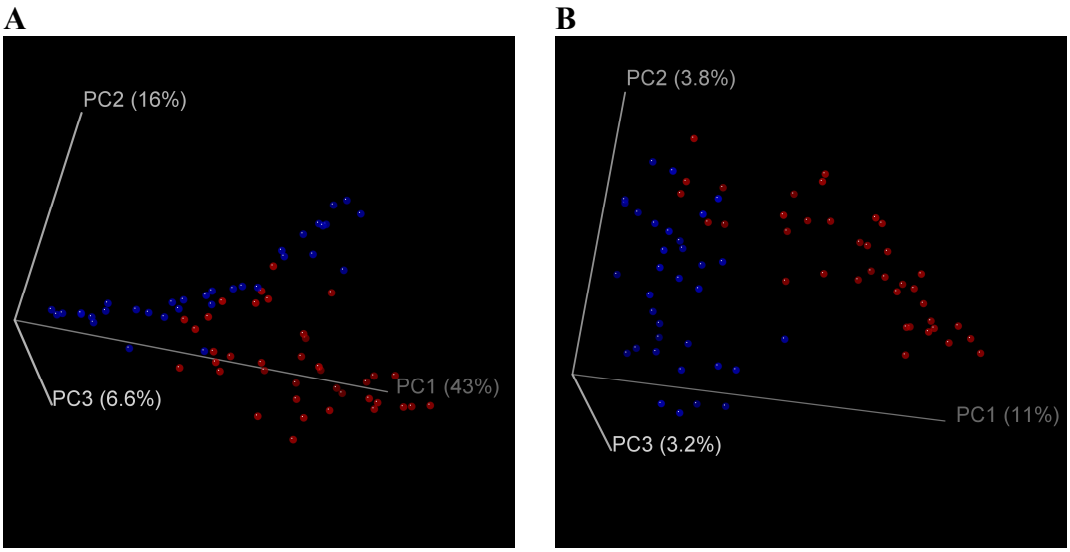
| Phylum or Family | Genus | Abund. (%) | Mayo score | Fecal calprotect. | Butyrate | Butyryl-CoA-Transfe. | MCT1 |
|---|---|---|---|---|---|---|---|
| | *Alistipes* | 0.0 – 3.5 | | | | | |
| ***Prevotellaceae*** | | 0.0 – 12.1 | | | | | |
| | *Butyricimonas* | 0.0 – 0.55 | | | | | |
| | Unc. *Prevotellaceae* | 0.0 – 12.1 | | | | -0.55 (0.029) | |
| ***Flavobacteriaceae*** | | 0.0 – 2.2 | | | | | -0.53 (0.022) |
| | *Chryseobacterium* | 0.0 – 1.9 | | | | | -0.58 (0.011) |
| | *Barnesiella* | 0.0 – 2.5 | | | | | 0.57 (0.014) |
| | Unc. *Flavobacteriaceae* | 0.0 – 0.2 | | | | | |
| ***Proteobacteria*** | | | | | | | |
| ***Desulfovibrionaceae*** | | 0.0 – 1.25 | | -0.50 (0.039) | | | |
| | *Desulfovibrio* | 0.0 – 1.25 | | -0.50 (0.043) | | | |
| ***Alcaligenaceae*** | | 0.0 – 5.3 | | -0.55 (0.023) | | | |
| | *Sutterella* | 0.0 – 5.3 | | -0.60 (0.011) | | | |
| ***Comamonadaceae*** | | 0.0 – 1.4 | | | | | |
| | *Acidovorax* | 0.0 – 1.2 | | | | | -0.58 (0.011) |
| ***Neisseriaceae*** | | 0.0 – 1.9 | | | | | |
| | *Microvirgula* | 0.0 – 1.0 | | 0.63 (0.0071) | | | |
| | *Vitreoscilla* | 0.0 – 0.6 | | | | -0.51 (0.043) | |
| ***Sphingomonadaceae*** | | 0.0 – 0.3 | | 0.56 (0.019) | | | -0.58 (0.011) |

| Phylum or Family | Genus | Abund. (%) | Mayo score | Fecal calprotect. | Butyrate | Butyryl-CoA-Transfe. | MCT1 |
|---|---|---|---|---|---|---|---|
| | *Sphingomonas* | 0.0 – 0.3 | | 0.65 (0.0051) | | | -0.57 (0.015) |
| ***Rhodobacteriaceae*** | | 0.0 – 0.45 | | | | | |
| | *Paracoccus* | 0.0 – 0.45 | | | | | -0.51 (0.029) |
| ***Moraxellaceae*** | | 0.0 – 12.15 | | | | | |
| | *Enhydrobacter* | 0.0 – 0.7 | | | | | -0.49 (0.041) |
| | Unc. *Moraxellaceae* | 0.0 – 0.3 | | | | 0.50 (0.048) | |
| ***Enterobacteriaceae*** | | 0.0 – 12.6 | | | | | |
| | *Enterobacter* | 0.0 – 1.0 | | | | | -0.48 (0.044) |
| | Unc. *Enterobacteriaceae* | 0.0 – 4.5 | | 0.53 (0.029) | | | |

[a]Results are expressed as correlation coefficients (r) and P value indicated in brackets. Correlations are shown only for r ≥0.4 and P <0.05; Unc., unclassified




**Supplementary Figure 4-1.** Spearman correlation analysis of data generated by 16S rRNA pyrosequencing and qPCR: A) *Bacteroides* group; B) *Enterobacteriaceae* family; C) *Bifidobacterium* spp.; D) *Lactobacillus* ssp.




**Supplementary Figure 4-2.** Communities clustered using principle coordinate analysis of (A) weighted and (B) unweighted UniFrac distance matrix. Blue points correspond to samples with luminal origin and red points denotes for samples with colon biopsy origin.

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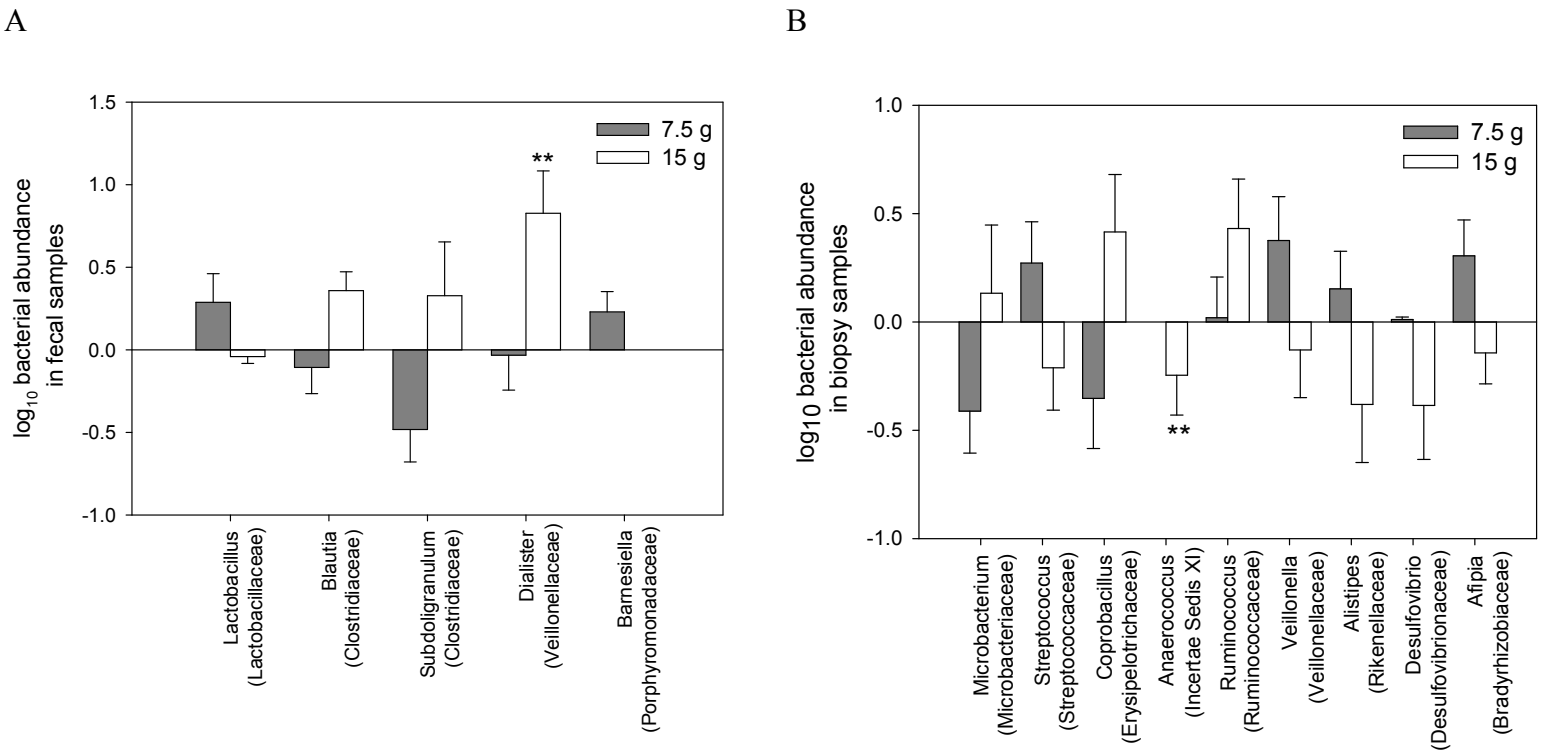
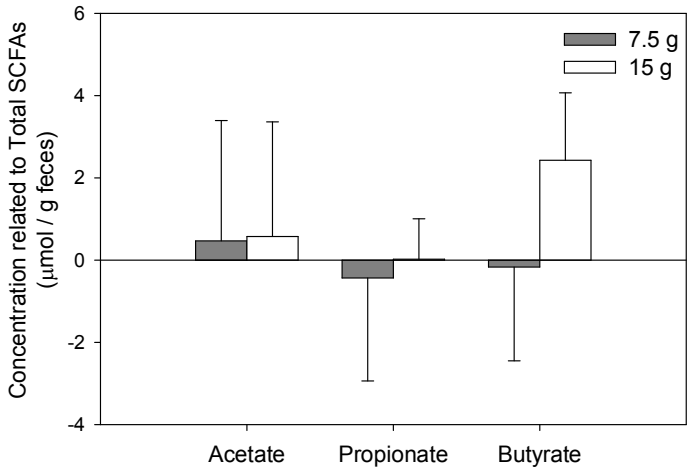
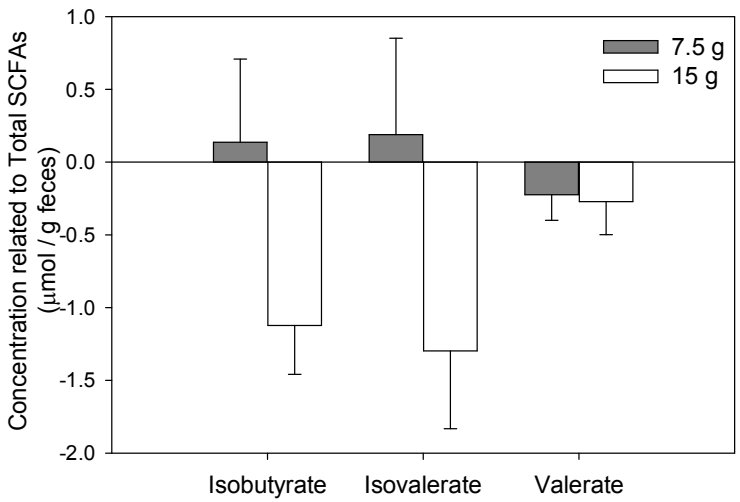



**Supplementary Figure 4-3.** Comparison of the luminal (A) and mucosal (B) microbiota identified at Genus level from fecal and biopsy samples of UC patients treated with 7.5 g or 15 g FOS/inulin for 9 weeks. Results are expressed as $\log_{10}$ of changes of 9 week minus week 0 and represent mean ± SEM. Significant differences ($P<0.05$) between the two treatment groups are indicated with '**'.

A




B




**Supplementary Figure 4-4.** Concentration of major (A) and minor (B) short-chain fatty acids in fecal samples of UC patients collected at the end point of treatment with 7.5 g or 15 g FOS/inulin.

# *Escherichia coli* Virulence Factors and their Association with Intestinal Barrier Dysfunction in Inflammatory Bowel Disease

A version of this chapter is in preparation for submission to Journal of Infection and Immunity. Dr. Wine's laboratory from the Department of Pediatrics at the University of Alberta, contributed to this study with the isolation of *E. coli* strains from biopsy samples obtained from non-IBD subjects and CD patients.

## 5.1. Introduction

*Escherichia coli* is the most abundant facultative anaerobe of the human intestinal microbiota and normal inhabitant of the gastrointestinal tract of other warm-blooded animals and reptiles (Kaper *et al*., 2004; Lukjancenko *et al*., 2010). *E. coli* strains are capable of adapting to many different ecological conditions and have been isolated from soil, water, sediment and food. Commensal *E. coli* normally do not cause illness, however when the host is immunocompromised or if the intestinal epithelial barrier does not function properly they can lead to infections. Some strains of *E. coli* have evolved pathogenic behavior and can induce different diseases in humans and animals (Kaper *et al.,* 2004). Pathogenic *E. coli* can be classified as intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC). IPEC strains are further divided into six groups depending on virulence factors involved and clinical manifestation of the disease. The six pathotypes of *E. coli* include: enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC; closely related to *Shigella*) and diffusely adherent *E. coli* (DAEC) (Kaper *et al*., 2004). Recently, two more pathogenic subsets of *E. coli* have been discovered, such as shiga toxin producing enteroaggregative *E. coli* (STEAEC) and adherent invasive *E. coli* (AIEC) (Clements *et al*., 2012).

Multiple studies have proposed that AIEC play a role in the pathogenesis of inflammatory bowel disease (IBD). IBD comprises of two major phenotypes, ulcerative colitis (UC) and Crohn's disease (CD), and are characterized as chronic

and relapsing disorders with uncontrolled inflammation within the gastrointestinal system (Kaser *et al*., 2010). It is generally accepted that intestinal microbiota play a crucial role in the pathogenesis of IBD (Farrel and La Mont, 2002). AIEC have been reported to be highly associated with mucosal microbiota of ileal specimens in CD patients and strains of this *E. coli* pathotype have been isolated from ileal biopsies from up to 36 % of patients with CD (Darfeuille-Michaud *et al*., 2004). Less frequently, these pathogenic bacteria have been also found in the mucosa-associated microbiota of UC patients (Elliott *et al*., 2013), however little is known regarding the role of AIEC in UC pathogenesis. Translocation of this *E. coli* subtype through the intestinal mucosa and epithelial barrier could be important for the worsening of intestinal inflammation and may be involved in IBD pathogenesis.

AIEC are different from the other pathotypes; they do not cause diarrhogenic infections and no specific virulence factors have been associated with this group of *E. coli*. However, AIEC have the ability to adhere to and invade intestinal epithelial cells and which function is facilitated by the binding of type I fimbriae encoded by the *fim* cluster to the epithelial surface receptor carcinembryogenic antigen cell adhesion molecule 6 (CEACAM6) (Barnich *et al*., 2007). Binding of AIEC to epithelial cells can also be mediated through the interaction of long polar fimbriae, encoded by the *lpf* operon, with a glycoprotein (GP2) on the surface of some specialized erythrocytes such as M cells (Hase *et al*., 2009). Once they reach the lamina propria, AIEC can survive and replicate in macrophages and

induce the synthesis of tumor necrosis factor alpha (TNF-α) (Glasser *et al*., 2001; Darfeuille-Michaud *et al*., 2004).

Intestinal barrier dysfunction as defect of innate immune responses has been also associated with IBD (Laukoetter *et al*., 2008; McGuckin *et al*., 2009). It is characterized with increased epithelial permeability, breached mucus layer, uncontrolled immune responses due to increased bacterial translocation, Paneth cells dysfunction and altered secretion of antimicrobial factors (McGuckin *et al*., 2009). Mucosa-associated *E coli* are more abundant in IBD patients compared to healthy controls (Swidsinski et al., 2002; Baumgart *et al*., 2007; Rehman *et al*., 2010) and this observation cannot be explained alone by the pathogenesis of this bacterial species. Due to the dysregulated function of the intestinal barrier in IBD patients it is likely that commensal *E. coli* strains can gain easy access through the intestinal mucosa. Our current study aimed to investigate the hypothesis that the altered function of the intestinal epithelial barrier in IBD patients can promote the survival of commensal *E. coli* with similar virulence characteristics as *E. coli* from non-IBD subjects, and thus enhancing and perpetuating the chronic intestinal inflammation.

## 5.2. Materials and Methods

### 5.2.1. Study subjects and sample collection

All the patients recruited in the study gave their informed consent before participation. Analysis of gene expression and quantification of *E. coli* virulence factors and screening of virulence genes in *E. coli* isolates was performed using

biopsy samples obtained from three different studies. Ethics approval of those studies was provided by Health Research Ethics Board at the University of Alberta.

Specimens from 24 UC patients used in this study were described previously (Valcheva *et al*., 2012). Twenty four patients, diagnosed with mild to moderately active UC based on total Mayo Clinical Score (with clinical score from 3 to 8 on the 12 point scale) were recruited into the study and treated with two different doses (7.5 and 15 g) of the fiber mixture inulin plus fructo-oligosaccharides (FOS) (ratio 1:1) for 9 weeks (detailed information for the patients, study design, exclusion and inclusion criteria are provided in Chapter 4). At the end of the fiber intervention, patients with Mayo score <2 were defined as patients in remission and those subjects with Mayo score ≥3 were considered in relapse.

Biopsy specimens from CD patients were obtained from the neo-terminal ileum after ileocecal resection of 38 subjects. Post-operative recurrence of CD patients was defined by the scoring system developed by Rutgeerts *et al.* (1990) with a scale from 0 to 4. Patients with score 0-2 were considered in remission or in mild relapse (*n*=20), whereas those with score 3 and 4 - in significant relapse (*n*=18). Additionally, ileal (n=6) and colonic (n=7) biopsy samples obtained from subjects with non-inflammatory conditions (non-IBD) were used as control samples.

In addition, *E. coli* strains were also isolated from fresh biopsy samples obtained from UC (n=12) and CD (n=5) patients with active disease, and from individuals with non-inflammatory conditions (non-IBD; n=6).

### 5.2.2. Culture-dependent analysis

Biopsy tissues from 6 UC patients with active disease were thoroughly disrupted with tissue grinder pestle in 1 mL of peptone water, followed by vigorous vortexing. Then, the suspension was used to generate 10-fold serial dilutions, where 100 µL of dilutions $10^{-3}$ to $10^{-6}$ were spread plated in duplicates. Modified MRS agar (mMRS; MRS supplemented with additional carbone sources: fructose (5 g/L), lactose (1 g/L), maltose (10 g/L), L-cystein (0.5 g/L) and vitamins) and Endo agar, a selective medium for Gram-negative bacteria were used as growth media. The bacterial cultures were incubated under aerobic conditions at 37°C overnight. Single bacterial colonies with different morphology were picked up, subcultured twice on the respective agar media followed by a subculture in mMRS or Luria-Bertani (LB) broth. The purified bacterial strains were stored in glycerol at -80°C until further analysis.

### 5.2.3. Identification of *E. coli* isolates

Unknown isolates were identified as *E. coli* with species-specific PCR targeting the gene encoding the universal stress protein (*uspA*) and sequencing of a partial region of 16S rRNA gene (Table 5-1; Chen and Griffiths, 1998). Characterization of the *E. coli* isolates was performed via random amplified polymorphic DNA (RAPD). For the RAPD assay the universal M13 primer (Table 5-1) was used with the following amplification conditions: 1 cycle at 94°C for 5 min; 2 cycles at 94°C for 5 min, 40°C for 5 min, and 72°C for 5 min; 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 5 min (Rossetti and Giraffa, 2005). The RAPD profiles were compared by BioNumeric software

(version 6.01 Applied Maths) and isolates from the same sample that shared identical RAPD patterns were considered identical and were not further analyzed. Representative strains of each RAPD pattern were further identified by 16S rRNA sequencing (Table 5-1; Weisburg *et al*., 1991). The RAPD outcomes with M13 primer were also confirmed with the universal primer 1283, which was previously estimated for RAPD fingerprinting of *E. coli* by Wang and co-workers (Wang *et al*., 1993). Additionally, *E. coli* isolates were cultured on tryptone bile agar (TBA) to check for lactose fermenting strains and to differentiate *E. coli* from *Shigella* species based on the color change of the media and production of gas.

Seventeen *E. coli* strains identified as EHEC were kindly provided by Health Canada and used as a reference group in this study. Unknown strains were isolated from gut washes of 6 UC patients, 6 CD patients and 5 non-IBD individuals, and were provided by Dr. Wine's laboratory from the Department of Pediatrics at the University of Alberta. Those isolates were screened for *E. coli* strains using species-specific PCR (Table 5-1). Detailed information of all stains used in the current study is provided in the Supplementary Table 5-1.

### 5.2.4. Total DNA extraction from pure cultures, feces and biopsy samples

Genomic DNA extraction from pure bacterial cultures is briefly explained below. Overnight *E. coli* inoculates were washed with T100E buffer (25 mM Tris-HCl, pH=8.5; 10 mM EDTA, pH=7.5) and resuspended in 500 µl of the same buffer. 10 mg/mL lysozyme was added to the suspensions and samples were incubated for 3 h at 37ºC. Subsequently, centrifugation for 5 min at 10 000 rpm was applied and pellets were homogenized in 500 µl of T100E buffer. 20 % (w/v) SDS was

added in order to disrupt the bacterial cell membranes, followed by the addition of 300 µl potassium acetate (3 M, pH=5.2) and incubation for 20 min at 4ºC. Another centrifugation for 10 min at 13 000 rpm was carried out and the supernatant was mixed with the same volume of cold isopropanol. The precipitated DNA was centrifuged for 20 min at 13 000 rpm at 4ºC and washed with 70 % (w/v) ethanol. The last step of the extraction was to dry the DNA at room temperature and to dilute it in water.

Bacterial total DNA from fecal and biopsy samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Mississauga, ON, Canada). Isolation of DNA from feces was performed following the manufacturer's protocol. Obtaining bacterial DNA from biopsy samples started with incubation of the biopsies in 1 ml of TN150 buffer (10 mM Tris-HCl; 150 mM NaCl, pH=8.0) together with 10 % w/v SDS and 20 mg/mL proteinase K at 55ºC for 2 h. Then, bead-beating step was applied in order to physically disrupt the cells. Zirconium beads with 0.1 mm diameter (500 mg) were used and the bead-beating step was performed in a FastPrep-24 (MP Biomedical, Solon, OH) in 3 cycles of 30 s at 4 m/s speed cooling on ice for 5 min after each cycle. Samples were then incubated at 95ºC for 15 min and further processed along with the QIAamp DNA Stool Mini Kit protocol.

### 5.2.5. Phylotyping and detection of *E. coli* virulence factors

Modified multiplex PCR, as described previously (Clermont *et al*., 2000; Doumith *et al*., 2012), was performed to determine the phylotyping group (A, B1, B2 and D) of each *E. coli* isolate.

The presence of 11 virulence genes was detected in the *E. coli* isolates and the primers specific for those genes are listed in Table 5-1. The targeting genes included: adhesins, such as *fimH* – type I fimbriae; *papC* - P fimbriae chaperone; *sfa/focDE* – S fimbriae; *lpfA* – long polar fimbriae; and toxins typical for enterotoxigenic *E. coli* (ETEC), enterohemoragic *E. coli* (EHEC) and/or extraintestinal *E. coli* (ExPEC), such as STa and STb – heat stable enterotoxins; LT – heat labile enterotoxin; *astA* – enteroaggregative heat stable enterotoxin; *vt1* – shiga-like toxin 1; *vt2* – shiga-like toxin 2; *clbB* and *clbN* – markers for the colibactin polyketide synthesis system. Additionally, the gene *aggR* encoding transcriptional activator found in enteroaggregative *E. coli* was also targeted. PCR amplifications were carried out on a Gene Amp PCR System 9700 (Applied Biosystems) and the reactions were run in final volume of 50 µL containing 50 ng of genomic DNA, 10 µL of GoTaq Reaction Buffer, 3 µL of $MgCl_2$ (50 mM), 2 µL of dNTPs, 1 µL of each primer (20 pmol/µL), 2 unites of GoTaq Polymerase (Promega), and water. PCR cycling conditions were the following: initial denaturation of 94ºC for 5 min; 35 cycles of denaturation for 30 s at 94ºC, annealing for 30 s at the optimal temperature for the respective primer pair (Table 5-1), and extension at 72ºC for 30s, followed by a final extension at 72ºC for 5 min.

**Table 5-1.** Primer pairs used in the study for, RAPD-PCR analysis, ribosomal sequencing of 16S rRNA gene, identification of *E. coli*, phylotyping primers and primers targeting genes encoding *E. coli* virulence factors.

| Target gene | Oligonucleotide sequence (5'→3') | Tm (°C)* | Reference |
|---|---|---|---|
| | ***E. coli* identification** | | |
| 16S (544 bp)** | ECP79F: GAAGCTTGCTTCTTTGCT<br>ECP620R: GAGCCCGGGGATTTCACAT | 54 | Sabat *et al.,* 2000 |
| *uspA* (884 ) | F: CCGATACGCTGCCAATCAG<br>R: ACGCAGACCGTAGGCCAGAT | 70 | Chen and Griffiths, 1998 |
| | **RAPD analysis** | | |
| | M13: TTATGTAAAACGACGGCCAGT | 40/60 | Rossetti and Giraffa, 2005 |
| | 1283: GCGATCCCCA | 36 | Wang *et al*., 1993 |
| | **Ribosomal sequencing** | | |
| 16S (1500 bp) | rP1: ACGGTTACCTTGTTACGACTT<br>630V: AAGGAGGTGGATCCARCC | 52 | Weisburg *et al*., 1991 |
| | **Phylotyping** | | |
| *chuA* (281 bp) | F: ATGATCATCGCGGCGTGCT<br>R: AAACGCGCTCGCGCCTAAT | 55 | Doumith *et al*., 2012 |
| *yjaA* (216 bp) | F: TGTTCGCGATCTTGAAAGCAAACGT<br>R: ACCTGTGACAAACCGCCCTCA | 55 | Doumith *et al*., 2012 |
| *Tsp* (152 bp) | F: GAGTAATGTCGGGGCATTCA<br>R: CGCGCCAACAAAGTATTACG | 55 | Clermont *et al*., 2000 |
| | **Toxins** | | |
| STa (193 bp) | F: ATGAAAAAGCTAATGTTGGC<br>R: TACAACAAAGTTCACAGCAG | 56 | Han *et al*., 2007 |
| STb (204 bp) | F: AATATCGCATTTCTTCTTGC<br>R: GCATCCTTTTGCTGCAAC | 56 | Han *et al*., 2007 |
| LT (291 bp) | F: CTATTACAGAACTATGTTCGG<br>R: TACTGATTGCCGCAATTG | 56 | Han *et al*., 2007 |
| *astA* (109 bp) | F: TGCCATCAACACAGTATATCC<br>R: GCGAGTGACGGCTTTGT | 56 | Han *et al*., 2007 |
| *clbB* (579 bp) | F: GATTTGGATACTGGCGATAACCG<br>R: CCATTTCCCGTTTGAGCACAC | 57 | Johnson *et al*., 2008 |
| *clbN* (733 bp) | F: GTTTTGCTCGCCAGATAGTCATTC<br>R: CAGTTCGGGTATGTGTGGAAGG | 58 | Johnson *et al*., 2008 |
| *stx1* (614 bp) | F: ACACTGGATGATCTCAGTGG<br>R: CTGAATCCCCCTCCATTATG | 55 | Yilmaz *et al*., 2006 |
| *stx2* (779 bp) | F: CCATGACAACGGACAGCAGTT<br>R: CCTGTCAACTGAGCACTTTG | 55 | Yilmaz *et al*., 2006 |
| | **Transcription factor** | | |
| *aggR* (457 bp) | F: CTAATTGTACAATCGATGTA<br>R: AGAGTCCATCTCTTTGATAAG | 50 | Cerna *et al*., 2003 |
| | **Adhesins** | | |
| *fimH* (400 bp) | F: CGTGCTTATTTTGCGACAGA<br>R: CTCCGGTACGTGCGTAATTT | 60 | Yang *et al*., 2004 |
| *papC* (200 bp) | F: GTGGCAGTATGAGTAATGACCGTTA<br>R: ATATCCTTTCTGCAGGGATGCAATA | 63 | Le Bouguenec *et al*., 1992 |
| *sfa/focDE* (410 bp) | F: CTCCGGAGAACTGGGTGCATCTTAC<br>R: CGGAGGAGTAATTACAAACCTGGCA | 63 | Le Bouguenec *et al*., 1992 |
| *lpfA* (273 bp) | F: CCTTGCGTACTGTCCGTTGA<br>R: AGCGACCAGGGTATTGCTGT | 62 | Vidal *et al*., 2006 |

*Tm – annealing temperature; F – forward primer; R – reverse primer; RAPD – random amplified polymorphic DNA;

**Product size (bp)

*fimH* – type I fimbriae; *papC* - P fimbriae chaperone; *sfa/focDE* - fimbriae; *lpfA* – long polar fimbriae; STa and STb – heat stable enterotoxins; LT – heat labile enterotoxin; *astA* – enteroaggregative heat stable enterotoxin; *aggR* – transcriptional activator typical for enteroaggregative *E. coli*; *vt1* – shiga-like toxin 1; *vt2* – shiga-like toxin 2; *clbB* and *clbN* – markers for the 5' and 3' ends of the *pks* island, encoding the machinery required for the synthesis of colibactin.

### 5.2.6. Total RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from ileal and colonic biopsy samples was extracted in TRIzol reagent (Invitrogen, Burlington, ON, Canada) using the RNeasy kit (Qiagen). The quantity and quality of RNA for each sample were checked on Nano-Drop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) and 300 ng of the isolated RNA was reverse transcribed employing QuantiTect Reverse Transcription kit (Qiagen).

### 5.2.7. Quantification of *E. coli* virulence factors and host gene expression

Quantitative PCR (qPCR) was performed using QuantiFast SYBR Green PCR master mix (Qiagen) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Samples were quantified in duplicates and the following cycling programme was used: initial denaturation at 95ºC for 5 min; forty cycles of 95ºC for 15 s, primer annealing at the optimal temperatures (Tables 5-1 and 5-2) for 30 s, and extension at 72ºC for 30 s, where the fluorescent emission was measured at the extention step. Melting curves were obtained by a stepwise increase of the

temperature from 60 to 95ºC (at 10 s/0.5ºC) and used to verify the amplification of the correct targeted PCR products. For generation of standard curves, 10-fold serial dilutions of purified and quantified PCR products were used. The standard curves of the individual qPCR assays were obtained by PCR using primers listed in Table 5-1 and genomic DNA isolated from pure *E. coli* cultures positive for the respective gene.

The relative quantification of the genes involved in the intestinal barrier function was calculated based on the efficiency corrected calculation model in comparison to a reference gene and control samples. The equation used to calculate the relative expression ratio was based on the primers' efficiency of the target gene and the $\Delta C_P$ deviation of control minus unknown sample of a target gene, and divided by the efficiency and the $\Delta C_P$ deviation of a reference gene. The housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a reference gene for the normalization of the cDNA leves, and was run on the same PCR plate for each quantified gene. Ileal and colonic biopsy specimens from non-IBD subjects were used as control samples. The negative PCR controls contained water or DNase treated RNA instead of template DNA.

**Table 5-2.** Primers targeting genes involved in the intestinal barrier function.

| Target gene* | Oligonucleotide sequence (5'→3') | Tm (ºC) | Reference |
|---|---|---|---|
| MUC1 | F: AGAGAAGTTCAGTGCCCAGC | 63 | Dharmaraj *et* |
| (112 bp) | R: TGACATCCTGTCCCTGAGTG | | *al*., 2009 |
| MUC2 | F: CTTCGACGGACTCTACTACAGC | 58 | Lopez-Ferrer *et* |
| (387 bp) | R: CTTTGGTGTTGTTGCCAAAC | | *al*., 2001 |
| MUC4 | F: CTTACTCTGGCCAACTCTGTAGTG | 55 | Lopez-Ferrer *et* |
| (468 bp) | R: GAGAAGTTGGGCTTGACTGTC | | *al*., 2001 |
| TFF3 | F: GTGCCAGCCAAGGACAG | 57 | Wiede *et al*., |
| (303 bp) | R: CGTTAAGACATCAGGCTCCAG | | 1999 |
| HD-5 | F: CCCAGCCATGAGGACCATC | 60 | Fahlgren *et al*., |
| (306 bp) | R: TCTATCTAGGAAGCTCAGCGACAG | | 2003 |
| HD-6 | F: AGCGACCCTAGCCATGAGAACC | 63 | Fahlgren *et al*., |
| (406 bp) | R: ACAAAGTTGATGGCAATGTATGGGA | | 2003 |
| Lysozyme | F: GAACTCTGAAAAGATTGGGAATGGA | 63 | Fahlgren *et al*., |
| (356 bp) | R: ACAACCTTGAACATACTGACGGACA | | 2003 |
| GAPDH | F: GAAGGTGAAGGTCGGAGTCAA | 60 | Thiele *et al*., |
| (258 bp) | R: GGTGAAGACGCCAGTGGACT | | 1999 |
| TNF-α | F: CCCAGGGACCTCTCTCTAATCA | 60 | Zilbauer *et al*., |
| (104 bp) | R: GCTACAGGCTTGTCACTCGG | | 2010 |
| CEACAM6 | F: CGCATACAGTGGTCGAGAGA | 60 | Kim *et al*., |
| (286 bp) | R: GTCATGTTGCCATTGGACAG | | 2013 |

*Produc size (bp)

**Tm – annealing temperature; F – forward primer; R – reverse primer

GAPDH - glyceraldehyde-3-phosphate dehydrogenase; TNFα – tumor necrosis factor α; CEACAM6 – carcinembryogenic antigen cell adhesion molecule 6

### 5.2.8. Statistical analysis

Data were analyzed statistically using the Statistical Analysis Systems (SAS Institute, Inc., Cary, NC). Differences between remission or mild relapse versus severe relapse of virulence genes and host factors for CD patients were evaluated using Mann-Whitney test. The Kruskal-Wallis test followed by Wilcoxon signed-rank test was performed to check for significant differences between start of treatment, relapse and remission within the same UC patient. The categorical data for *E. coli* phylotypes and virulence factors was analyzed using the catmod

procedure (PROC CATMOD) in SAS. Further examination of differences between divergent groups of *E. coli* isolates was performed using contrast statements. Correlations between disease activity and host factors were assessed by Spearman's correlation test using GraphPad Prism version 5.00 (GraphPad Software).

## 5.3. Results

### 5.3.1. Culture-dependent analysis of mucosa-associated microbiota of UC and CD patients

A total of 93 strains were cultured on mMRS and ENDO from biopsy samples obtained from 26 individuals (8 non-IBD; 12 UC, and 6 CD patients). Fifty eight of those isolates were further identified as *E. coli* on the basis of 16S rRNA gene sequencing or species-specific PCR. *E. coli* isolates were all lactose-fermenting strains, which excluded the presence of *Shigella* strains. The non-*E. coli* isolates were dominated by other Gram-negative bacteria including, *Klebsiella* spp., *Providencia* spp., and *Serratia* spp., belonging to γ-*Proteobacteria*, and *Neisseria* spp., a member of β-*Proteobacteria*. *Enterococcus* spp., *Streptococcus* spp., and *Staphylococcus*, Gram-positive cocci part of the *Firmicutes* phylum, were aslo identified. In addition, few bacterial species belonging to *Actinobacteria* phylum, including *Bifidobacterium bifidum*, *Rothia mucilaginosa* and *Corynebacterium* spp., were also isolated from fresh biopsy samples.

### 5.3.2. Phylotyping of *E. coli* isolates and characterization of virulence factors

To investigate the phylotype grouping of each *E. coli* isolate, multiplex PCR was performed. Phylotypes B1 and D were the most abundant in the foodborne pathogens group, whereas phylotype B2 was not detected in any of those isolates (Table 5-3). In contrast, B2 phylogroup together with D were the only phylogenetic types observed in *E. coli* strains isolated from non-IBD subjects and CD patients (Table 5-3). B2 and D phylotypes were also highly abundant in UC patients, however A and B1 phylogroups were detected in 24 % of UC isolates. Phylotypes of *E. coli* strains were not significantly different between isolates from non-IBD individuals and IBD patients.

To characterize and determine virulence factors, *E. coli* isolates were screened for several virulence genes. The genes STa, STb, LT and *aggR* were not detected in any of the isolates. The majority of strains carried the gene encoding for *fimH* (Table 5-3). The adhesin *papC* was more abundant in non-IBD subjects and IBD patients and was present in only 6 % of the foodborne *E. coli* pathogens. The *lpfA* gene was detected in 18 % of the pathogenic *E. coli*, but not in any of the IBD strains. The shiga-like toxins 1 and 2 were identified only in foodborne pathogens, whereas *clbB* and *clbN* genes, markers for the genotoxin colibactin, were present in non-IBD, UC and CD isolates only, however, no relationship with IBD was observed.

**Table 5-3.** Summary of phylotypes and virulence factors detected in *E. coli* foodborne pathogens, as well as *E. coli* strains isolated from non-IBD and IBD subjects.

| Characteristics | Virulence factor/ Phylotype | Foodborne pathogens (n=17) | non-IBD (n=10) | UC (n=34) | CD (n=11) |
|---|---|---|---|---|---|
| **Phylotyping** | A | 6 %$^{A}$ | 0 %$^{B}$ | 12 %$^{A}$ | 0 %$^{B}$ |
| | B1 | 47 %$^{A}$ | 0 %$^{B}$ | 12 %$^{C}$ | 0 %$^{B}$ |
| | B2 | 0 %$^{A}$ | 30 %$^{B}$ | 32 %$^{B}$ | 36 %$^{B}$ |
| | D | 47 %$^{A}$ | 70 %$^{A}$ | 44 %$^{A}$ | 64 %$^{A}$ |
| **Adhesins** | *fimH* | 94 %$^{A}$ | 100 %$^{A}$ | 100 %$^{A}$ | 100 %$^{A}$ |
| | *papC* | 6 %$^{A}$ | 50 %$^{B}$ | 32 %$^{B}$ | 45 %$^{B}$ |
| | *sfa/focDE* | 0 %$^{A}$ | 0 %$^{A}$ | 12 %$^{B}$ | 0 %$^{A}$ |
| | *lpfA* | 18 %$^{A}$ | 0 %$^{A}$ | 0 %$^{B}$ | 0 %$^{B}$ |
| **Toxins** | *astA* | 29 %$^{A}$ | 20 %$^{AB}$ | 6 %$^{B}$ | 9 %$^{AB}$ |
| | *vt1* | 41 %$^{A}$ | 0 %$^{B}$ | 0 %$^{B}$ | 0 %$^{B}$ |
| | *vt2* | 29 %$^{A}$ | 0 %$^{B}$ | 0 %$^{B}$ | 0 %$^{B}$ |
| | *clbB* | 0 %$^{A}$ | 30 %$^{B}$ | 29 %$^{B}$ | 27 %$^{B}$ |

*STa, STb, LT and *aggR* were not detected in any group of isolates. Results of *clbN* were the same as results for clbB.

**Frequency of the phylotypes and virulence factors for the different diseases are presented as percentage. Data for *E. coli* phylotypes and virulence factors was analyzed using the catmod procedure (PROC CATMOD) in SAS, followed by contrast statements to check for differences between divergent groups of *E. coli* isolates. $^{A,B,C}$Percentages without common letters for each phylotype and virulence factor are significantly different ($P<0.05$).

UC – ulcerative colitis; CD – Chron's disease; non-IBD - individuals with no inflammatory conditions *fimH* – type I fimbriae; *papC* - P fimbriae chaperone; *sfa/focDE* - fimbriae; *lpfA* – long polar fimbriae; STa and STb – heat stable enterotoxins; LT – heat labile enterotoxin; *astA* – enteroaggregative heat-stable enterotoxin; *aggR* – transcriptional activator typical for enteroaggregative *E. coli*; *vt1* – shiga-like toxin 1; *vt2* – shiga-like toxin 2; *clbB* and *clbN* – markers for the 5' and 3' ends of the *pks* island, encoding the machinery required for the synthesis of colibactin.

### 5.3.3. Quantification of *E. coli* and virulence-associated genes in intestinal samples from UC and CD patients either in remission or relapse

*E. coli* virulence-associated genes were quantified in luminal and biopsy samples from UC and CD patients, respectively, to obtain a second method to verify *E. coli* virulence deterimants in IBD (Figure 5-1). Percentage of *E. coli* related to total bacteria for patients in relapse and remission were 0.78±0.50 and 0.55±0.51, respectively for UC; and 0.23±0.09 and 0.12±0.07, respectively for CD. Similar to the results of the *E. coli* isolates, virulence genes encoding for STa, STb, LT, *aggR*, *vt1* and *vt2* were not detected in any sample obtained from the IBD patients. Gene copies of *fimH, papC*, *sfa/focDE, lpfA* and *clbB* were quantified in luminal and biopsy samples of UC and CD patients, correspondingly, but they did not differ significantly for patients between relapse and remission (Figure 5-1).

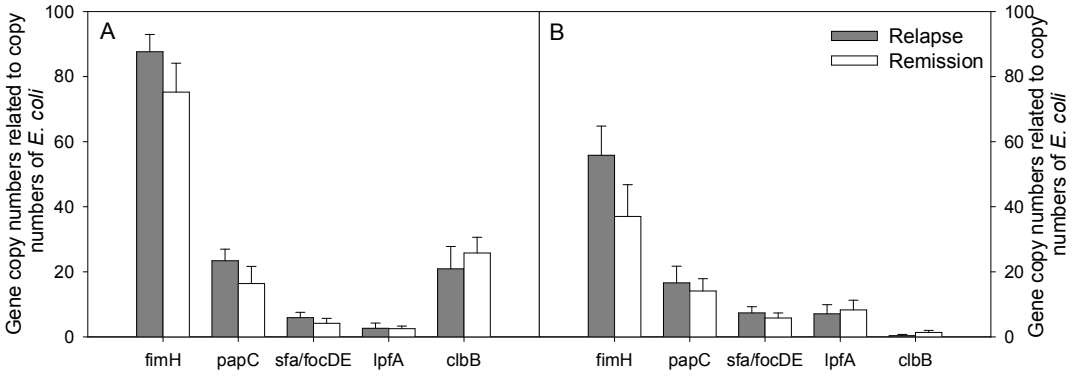



**Figure 5-1.** Quantification of *E. coli* virulence factors in luminal and mucosa-associated microbiota in ulcerative colitis (A) and Crohn's disease (B) patients, respectively. In UC patients, remission was defined by the Mayo clinical score; for CD patients, remission was defined by the Rutger's score. Results are expressed as means ± SEM. Differences between remission and relapse of virulence genes for CD and UC patients were evaluated using Mann-Whitney test. *fimH* – type I fimbriae; *papC* - P fimbriae chaperone; *sfa/focDE* - the central region of the *sfa/foc* operon, encoding S fimbriae and F1C fimbriae; *lpfA* – long polar fimbriae; *clbB* and *clbN* – markers for the 5' and 3' ends of the *pks* island, encoding the machinery required for the synthesis of colibactin.

### 5.3.4. Relative expression of genes involved in the intestinal barrier function

UC and CD are characterized with dysregulated intestinal barrier (Laukoetter *et al*., 2008; McGuckin *et al*., 2009). The mRNA expression levels of genes involved in the epithelial barrier function were measured in order to investigate if the disease status had affected their expression, as well as to establish the

relationship of those genes with *E. coli* virulence determinants. Four genes involved in the formation of the mucus layer were studied in colonic and ileal biopsies of UC and CD patients (Figure 5-2). No significant differences were observed for MUC1, MUC2, MUC4 and TFF3 between patients in relapse and in remission for UC (Figure 5-2 A). Mucin genes and the gene encoding for the trefoil factor 3 (TFF3) were less expressed in CD patients in comparison with UC, which confirmed previous observations (Sheng *et al*., 2011). Expression levels of MUC1 and MUC4 were higher in patients in remission versus patients in relapse, however only values for MUC1 gene reached statistical significance (Figure 5-2 B). In addition, the expression of two α-defensins, HD-5 and HD-6, two β-defensins, hBD-1 and hBD-2, and lysozyme were studied in biopsy samples from UC and CD patients (Figure 5-3). The three defensin molecules showed higher expression in patients in remission compared to patients in relapse for UC (Figure 5-3 A). Adjunct treatment of UC patients with β-fructans significantly decreased level of expression of lysozyme after 9 weeks. HD-5 and HD-6 genes were predominantly expressed in ileal samples of CD patients in comparison with UC, however no significant differences were identified between the CD patients with severe relapse versus the group in remission or with mild recurrent ileitis (Figure 5-3 B). In contrast, hBD-2 relative expression was more pronounced in UC patients in comparison to patients with CD. As shown in Fig. 5-3A, hBD-2 was less expressed in UC patients in remission versus those in relapse or 0 week, however due to the small sample size and the big individual variability those differences did not reach statistical significance. Further, Spearman's correlation

analysis confirmed the results above and revealed no association between levels of expression of the studied host genes and chronic intestinal inflammation (data not shown). Moreover, the correlation test showed that there was no relationship between level of expression of mucin genes, anti-microbial peptides, and gene copy numbers of *E. coli* and their virulence factors.

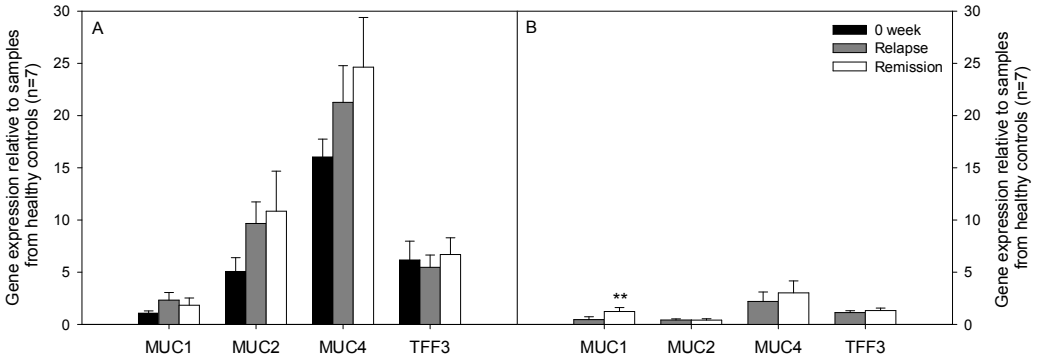



**Figure 5-2.** Relative expression of genes involved in the formation of the mucus layer in (A) – UC patients and (B) – patients with CD. A house-keeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH), was used for normalization and the expression was determined relative to a control group of non-IBD individuals (n=7). Differences between remission and relapse of mucin genes for CD patients were evaluated using Mann-Whitney test, whereas comparison of 0 week, relapse and remission for UC patients was performed by Kruskal-Wallis test. Vertical bars indicate mean±SEM. Statistical significant differences are shown with '**'; $P<0.05$.

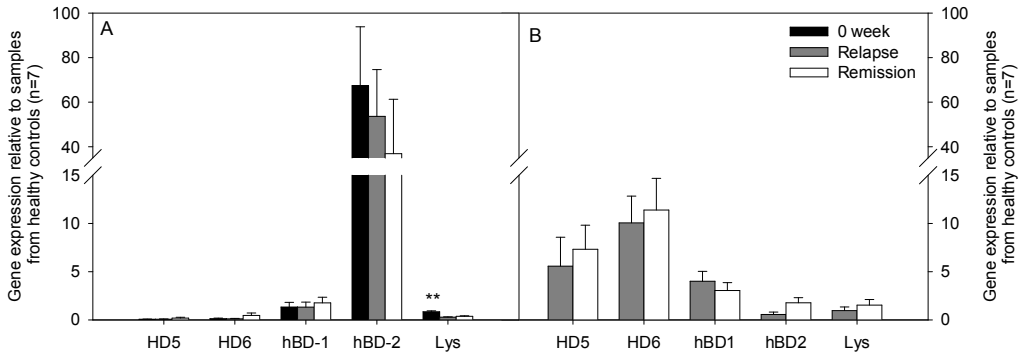



**Figure 5-3.** Levels of expression of genes encoding antimicrobial peptides for (A) – UC and (B) – patients with CD. A house-keeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH), was used for normalization and the expression was determined relative to a control group of non-IBD individuals (n=7). Comparison of antimicrobial genes between relapse and remission for CD patients was performed by Mann-Whitney test. Kruskal-Wallis test was used to assess differences between 0 week, relapse and remission for UC samples. The results are expressed as mean±SEM. Statistical significant differences are indicated with '**'; $P<0.05$.

### 5.3.5. Comparison of expression levels of CEACAM6 and TNF-α in biopsy samples from UC and CD patients in relapse and remission

AIEC are able to induce the expression of the epithelial receptor CEACAM6, either directly by adhering to the epithelial cells or indirectly by the increased secretion of TNF-α from infected macrophages with AIEC (Carvalho *et al.*, 2009). Therefore, we measured levels of expression of the epithelial receptor CEACAM6 and TNF-α. Expression of those host factors did not differ between patients in remission versus relapse both for UC and CD (Figure 5-4). β-Fructans

treatment of UC patients with active disease did not modify the expression of the targeted gene. Interestingly, CEACAM6 was expressed 6 fold higher in colonic biopsies from UC patients in comparison with the ileal samples from CD subjects (Table 5-4).

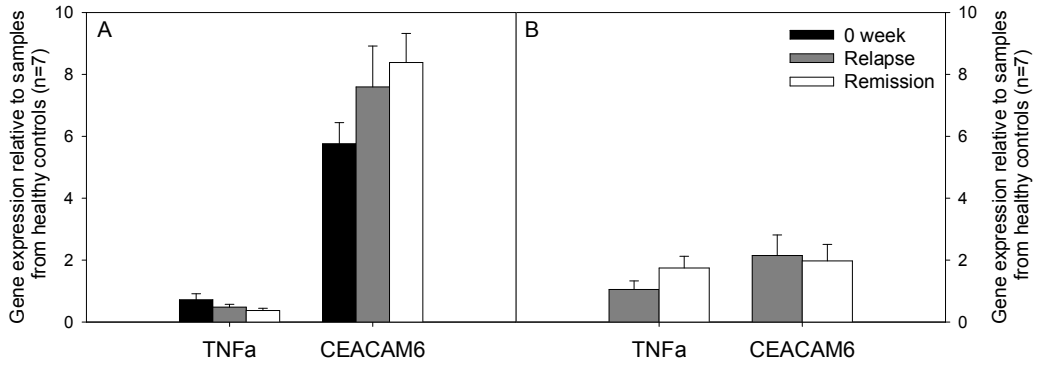



**Figure 5-4.** TNF-α and CEACAM6 mRNA gene expression in (A) – ulcerative colitis patients and (B) – patients with Crohn's disease. The expression of TNF-α and CEACAM6 was normilized with the expression of a house-keeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH), and determined relative to a control group of non-IBD individuals (n=7). The results are expressed as mean±SEM. Comparison of host factors between relapse and remission for CD patients was performed by Mann-Whitney test., whereas differences between 0 week, relapse and remission for UC samples were assessed by Kruskal-Wallis test. TNF-α – tumor necrosis factor α; CEACAM6 – carcinembryogenic antigen cell adhesion molecule 6.

### 5.4. Discussion

It is still unclear if IBD-associated *E. coli* strains are directly involved in the pathogenesis of this chronic inflammatory disorder. AIEC, which were recently

related to IBD, and particularly with CD, cannot be clearly distinguished from other commensal *E. coli* strains on the basis of genomic content (Nash *et al*., 2010). It is debated whether their high abundance is a result of the inflammatory response, the altered microbial environment and/or the abnormal intestinal barrier in IBD. Therefore, this study investigated the virulence determinants of *E. coli* isolated from IBD patients and non-IBD subjects. Quantification of those virulence genes in luminal and biopsy samples from IBD patients was performed as a second method to obtain insight into their connection with IBD. Expression levels of genes involved in the intestinal barrier function was examined in colonic and ileal biopsies from UC and CD patients to assess the integrity intestinal epithelial barrier.

Similar to previous observations, in this study mainly *E. coli* strains were isolated from fresh biopsy specimens obtained from UC and CD patients with active disease (Kotlowski *et al*., 2007; Sepehri *et al*., 2011). The non-*E. coli* isolates found in the biopsy samples, were members of γ-*Proteobacteria*, particularly *Enterobacteriaceae*, as well as *Enterococcus*, *Streptococcus* and *Staphylococcus* spp. *E. coli* strains cluster into four major groups including A, B1, B2 and D (Herzer *et al*., 1990). In the current study, *E. coli* isolates from IBD patients were characterized to belong to the phylogenetic groups B2 and D (Table 5-3), which is in agreement with previous studies (Sepehri *et al*., 2011; Elliott *et al*., 2013). AIEC strains, found to be highly related to ileal mucosa in CD patients, also cluster in phylogroup B2 (Leimbach *et al*., 2013). Interestingly, *E. coli* isolates from non-IBD subjects were also characterized as phylotypes B2 and D (Table 5-

3). Similar results were observed in a recent study, which showed that healthy mucosal *E. coli* strains were grouped as B2 and D phylotypes (Elliott *et al*., 2013). The outcomes from this study and our findings suggested that no specific phylotype of *E. coli* was associated with IBD disease status. Elliott *et al.* (2013) proposed that phylogenetic groups B2 and D consist of diverse evolutionary lineages that facilitate the adaptation of the mucosa-associated *E. coli* to the harsh ecological environment of the submucous shield.

*E. coli* possesses a multitude of virulence factors that could contribute resisting host defense mechanisms and facilitating their colonization and invasion of the host (Clements *et al*., 2012). *E. coli* contributors to virulence include diverse adhesins, toxins, siderophores, polysaccharide coating and invasins (Clements *et al*., 2012; Leimbach *et al*., 2013). The divergent combinations of those genetic elements result in different *E. coli* pathothypes (Kaper *et al*., 2004). However, *E. coli* uses a number of mechanisms to exchange genetic material, which may lead to the emergence of novel strains representing combinations of different pathotypes with improved ability to adhere to and infect the host (Muniesa *et al.*, 2012). Several of those virulence elements such as different adhesins and toxins were screened in the current study. The phage encoded *vt1* and *vt2* proteins are the major toxins synthesized by EHEC strains (Johannes and Römer, 2010), and together with the enteroaggregative heat-stable enterotoxin (*astA*) were detected only in the foodborne pathogen *E. coli*, which were included as a reference group in this study. Not surprisingly, the heat-stable (STa and STb) and heat-labile (LT) enterotoxins, produced by ETEC strains as molecular mechanisms of virulence

(Nagy and Fekete, 2005), were not found in any of the *E. coli* isolates, and were below the detection limit in specimens from IBD patients. Additionally, the 5' and 3' regions of the *pks* genomic island (respectively *clbB* and *clbN* genes) were amplified in order to screen for the genotoxin colibactin. This toxin has been recently associated with chronic inflammation and demonstrated to potentiate the development of colon cancer (Arthur *et al*., 2012). Colibactin was detected only in intestinal *E. coli* isolates however we did not find any significant association with IBD disease status. Interestingly, certain commensal strains isolated from healthy individuals also carry the *pks* cluster in their genome (Le Gall *et al*., 2007).

Similar to some EHEC strains, certain AIEC use long polar fimbriae (*lpfA*) and type I fimbriae (*fimH*) for their adherence to the epithelial cells (Fitzhenry *et al*., 2006; Barnich *et al*., 2007; Hase *et al*., 2009). Interestingly, *lpfA* was detected in 18 % of the foodborne pathogens, but not in any of the isolates from IBD patients, whereas *fimH* was carried by the majority of the intestinal *E. coli* isolates, as well as the reference EHEC strains. This finding is consistent with previous reports that type I fimbriae are the most common adhesins produced by *E. coli* strains (Mossman *et al*., 2008). However, gene copies of *fimH* did not correlate with expression levels of the epithelial receptor CEACAM6 in which overexpression has been previously associated with IBD (Carvalho *et al*., 2009). Additionally, two other adhesins, *papC* and *sfa*/*focDE*, ExPEC-related virulence determinants, were also studied, but they did not show any relationship with IBD. In the current work, no *E. coli*-associated virulence determinants were found unique to IBD and foodborne pathogenic strains clustered separately.

The common set of genetic elements of *E. coli* isolates from IBD and non-IBD individuals suggests that any *E. coli* is able to invade the epithelial barrier in a host with a compromised immune system. Patients with IBD have been characterized with several defects related to their intestinal barrier function (Laukoetter *et al*., 2008; McGuckin *et al*., 2009). Changes in expression levels of mucin genes have been previously associated with IBD (Tytgat *et al*., 1996; Buisine *et al*., 2001; Longman *et al*., 2006). We investigated the impact of disease activity on the expression of the mucin genes, encoding for MUC1, MUC2 and MUC4 and treifoil factor 3 (TFF3), and the correlation between their level of expression and *E. coli* virulence factors. Not surprisingly, expression of the targeted mucin genes in the colon exceeded expression levels of the same genes in the ileum by a factor up to 20 (Figure 5-2). MUC2 is a major secretory protein and decreased secretion of this mucin was previously observed in UC (Tytgat *et al.,* 1996; Longman *et al*., 2006), however expression of MUC2 did not differ between remission and relapse in CD and UC. The surface mucins, MUC1 and MUC4, were expressed in higher amounts in CD patients who were in remission versus CD patients in relapse, which was in agreement with previous reports (Buisine et al., 2001; Longman *et al*., 2006). In UC, the gene encoding for MUC1 was reported to be overexpressed in severe disease (Longman *et al*., 2006), however we did not observe any changes in the expression levels of this mucin in remission versus in relapse. Alterations in mucin expression in IBD may lead to a thinner mucus layer and disruption of the intestinal wall, and particularly UC has been characterized with a diminished colonic mucus layer and mucosal

inflammation (Leiper *et al*., 2001). The disturbed extracellular mucus shield can expose the epithelial cells to direct contact with luminal bacteria, and thus trigger an inappropriate immune response.

Impaired expression of antimicrobial peptides in IBD may lead to a decrease of antimicrobial activity, making the susceptible IBD patients more vulnerable to attachment and invation of intestinal bacteria, such as *E. coli* - a normal gut inhabitant. Epithelial cells, and especially Paneth cells, secrete a wide variety of molecules with antimicrobial activity, such as defensins, lysozymes, cathelicidins, secretory phospholipase A2, and C-type lectins (Clevers *et al*., 2013). HD-5 and HD-6 are human α-defensins produced primarily by the Paneth cells in the small intestines and in lesser amounts by colonic erythrocytes (Clevers *et al*., 2013). A decreased expression of these α-defensins was reported in tissue specimens in CD patients, and their reduction was even more distinct in patients with NOD-2 mutations (Wehkamp *et al*., 2004). Surprisingly, we did not show any changes in the expression levels of HD-5 and HD-6 in CD, although those antimicrobial peptides were expressed in higher amounts in biopsy specimens from CD patients in comparison with UC patients. The human β-defensins hBD-1 and hBD-2 are expressed in the gut (Pazgier *et al.,* 2006), where hBD-1 is produced constitutively, and hBD-2 expression is induced with inflammation in IBD, particularly UC (Wehkamp *et al*., 2003). Mucosal hBD-1 and hBD-2 expression did not differ between remission and relapse in both CD and UC. In agreement with previous observations hBD-2 expression was increased extensively in samples from UC patients but not in CD (Wehkamp *et al*., 2003). Expression of

lysozyme was significantly reduced by dietary intervention with β-fructan in UC patients. Previously, increased colonic expression of lysozyme has been associated with active UC, whereas no changes were observed in CD (Fahlgren *et al.*, 2003). Impaired expression of antimicrobial peptides in IBD may lead to a decrease of antimicrobial activity, making the susceptible IBD patients more vulnerable to attachment and invasion of intestinal bacteria, such as *E. coli* - a normal gut inhabitant.

Taken together, the present study demonstrates that *E. coli* isolates obtained from IBD patients harbour common genetic elements found in *E. coli* strains isolated from non-IBD subjects. Furthermore, copy numbers of virulence genes in specimens from IBD patients in remission were not distinguishable from those in relapse. Interestingly, we demonstrated that changes in levels of expressions of mucins and antimicrobial peptides were associated with IBD disease activity, however their expression levels did not correlate with *E. coli* virulence determinants. These findings confirm and extend previous work that epithelial barrier dysfunction may contribute to the increased numbers of mucosa-associated *E. coli* strains in patients with IBD.

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## 5.6. Supplementary material

**Supplementary Table 5-1.** *Escherichia coli* strains used in the study.

| Subject No. | Strain ID | Origin | Phylotype | Serotype; virulence genes |
|---|---|---|---|---|
| 1 | 1935[c] | Health Canada | D | O157:H7; *astA, vt1, vt2, fimH, lpfA* |
| 2 | 7236[c] | Health Canada | D | O157:H7; *astA, fimH, lpfA* |
| 3 | C0283[c] | Health Canada | D | O157:H7; *vt1, vt2, fimH* |
| 3 | E0122[c] | Health Canada | D | O157:H7; *astA, vt2, fimH* |
| 4 | 03-6430[c] | Health Canada | D | O145:NM; *vt1,fimH* |
| 5 | 05-6544[c] | Health Canada | B1 | O26:H11; *vt1,fimH* |
| 5 | 09-0525[c] | Health Canada | A | O133:H4; *astA, vt1, fimH* |
| 5 | 09-0523[c] | Health Canada | B1 | O76:H19; *vt1,fimH* |
| 5 | 05-6545[c] | Health Canada | D | O45:H2; *vt1,fimH* |
| 5 | 03-2832[c] | Health Canada | B1 | O121:H19; *vt2,fimH* |
| 5 | 03-4064[c] | Health Canada | B1 | O121:NM; *vt2,fimH* |
| 6 | PARC 444[c] | Health Canada | B1 | O103:H2; *fimH* |
| 6 | PARC 445[c] | Health Canada | B1 | O103:H2; *fimH* |
| 7 | PARC 447[c] | Health Canada | B1 | O111:NM; *fimH* |
| 7 | PARC 448[c] | Health Canada | B1 | O26:H11; *astA, fimH* |
| 7 | PACR 449[c] | Health Canada | D | O145:NM; *fimH* |
| 7 | PARC 450[c] | Health Canada | D | O44:H18; *lpfA, papC* |
| 8 | 16LA2[a] | non-IBD | D | *fimH, papC* |
| 8 | 16MC1[a] | non-IBD | B2 | *fimH, clbB, papC* |
| 9 | 17LA2[a] | non-IBD | B2 | *astA, fimH, clbB, papC* |
| 10 | 19MC1[a] | non-IBD | D | *astA, fimH, papC* |
| 10 | 19MRS1[a] | non-IBD | D | *fimH* |
| 11 | 20LAN2[a] | non-IBD | D | *fimH* |
| 11 | 20MC1[a] | non-IBD | B2 | *fimH, clbB* |
| 11 | 20MRS3[a] | non-IBD | D | *fimH, papC* |
| 12 | 27LA1[a] | non-IBD | D | *fimH* |
| 12 | 27MC2[a] | non-IBD | D | *fimH* |
| 13 | In8[ab] | UC | D | *fimH* |
| 13 | In25[ab] | UC | D | *fimH* |
| 13 | Non10[ab] | UC | D | *fimH* |
| 13 | Non13[ab] | UC | D | *fimH* |
| 14 | I1[ab] | UC | B2 | *fimH, clbB* |
| 14 | N1[ab] | UC | A | *fimH* |
| 14 | N3[ab] | UC | A | *fimH* |
| 15 | Bm4[ab] | UC | D | *fimH* |
| 15 | Be2[ab] | UC | D | *fimH* |
| 15 | Cm19[ab] | UC | D | *fimH* |
| 15 | Cmac1[ab] | UC | D | *fimH* |
| 16 | Ie4[ab] | UC | B2 | *fimH, clbB, papC,* |

| | | | | |
|---|---|---|---|---|
| | | | | *sfa/focDE* |
| 16 | Ib4[ab] | UC | B2 | *fimH, clbB, papC, sfa/focDE* |
| 16 | Nm1[ab] | UC | B2 | *fimH, clbB, papC, sfa/focDE* |
| 16 | Nc3[ab] | UC | B2 | *fimH, clbB, papC, sfa/focDE* |
| 17 | Am6[ab] | UC | B2 | *fimH, clbB, papC* |
| 17 | Ae5[ab] | UC | A | *fimH* |
| 17 | Ac5[ab] | UC | B2 | *fimH, clbB, papC* |
| 17 | Im9[ab] | UC | A | *fimH* |
| 17 | Ib6[ab] | UC | B2 | *fimH, clbB, papC* |
| 18 | Ine2[ab] | UC | B1 | *fimH* |
| 18 | Inc12[ab] | UC | B1 | *fimH* |
| 19 | 12LA1[a] | UC | B1 | *astA, fimH* |
| 20 | 14MC2[a] | UC | D | *fimH* |
| 20 | 14MRSN1[a] | UC | B2 | *fimH, papC* |
| 20 | 14MRSN3[a] | UC | D | *fimH, papC* |
| 21 | 15LAN1[a] | UC | D | *fimH* |
| 21 | 15MRSN3[a] | UC | D | *fimH* |
| 22 | 18LA1[a] | UC | D | *fimH* |
| 22 | 18MC3[a] | UC | B1 | *fimH* |
| 23 | 25LA1[a] | UC | B2 | *fimH, clbB* |
| 23 | 25MRS1[a] | UC | D | *fimH, papC* |
| 24 | 28LA2[a] | UC | D | *fimH* |
| 24 | 28MC2[a] | UC | B2 | *astA, fimH, clbB, papC* |
| 25 | 11LA1[a] | CD | D | *fimH* |
| 25 | 11MC1[a] | CD | B2 | *fimH, papC* |
| 26 | 13LA1[a] | CD | D | *astA, fimH* |
| 26 | 13MCN3[a] | CD | D | *fimH* |
| 27 | 21MC2a | CD | B2 | *fimH, clbB, papC* |
| 27 | 21MRS2a | CD | D | *fimH, papC* |
| 28 | 22LA1[a] | CD | D | *fimH* |
| 28 | 22MC2[a] | CD | B2 | *fimH, clbB, papC* |
| 29 | 23LA2[a] | CD | B2 | *fimH, clbB, papC* |
| 29 | 23MC3[a] | CD | D | *fimH* |
| 30 | 30MC3[a] | CD | D | *fimH* |

[a]Strains were identified by species-specific PCR targeting uspA and 16S rRNA genes.

[b]Strains were sequenced and approximately 1000 bp sequences were identified to type strain *E. coli*.

[c]Isolates provided from Health Canada and characterized as EHEC strains.

STa, STb, LT and *aggR* were not detected in any group of isolates. Results of *clbN* were the same as results for clbB.

EHEC - enterohaemorrhagic *E. coli;* non-IBD - individuals with non-inflammatory conditions; UC – ulcerative colitis; CD – Chron's disease; *fimH* – type I fimbriae; *papC* - P fimbriae chaperone; *sfa/focDE* - fimbriae; *lpfA* – long polar fimbriae; STa and STb – heat stable enterotoxins; LT – heat labile enterotoxin; *astA* – enteroaggregative heat-stable enterotoxin; *aggR* – transcriptional activator typical for enteroaggregative *E. coli*; *vt1* – shiga-like toxin 1; *vt2* – shiga-like toxin 2; *clbB* and *clbN* – markers for the 5' and 3' ends of the *pks* island, encoding the machinery required for the synthesis of colibactin.

# General Discussion and Conclusions

## 6.1. General Discussion

Inflammatory bowel disease (IBD) is a group of debilitating disorders that reduce the quality of life in patients, mainly due to chronic relapses and its complications. Non-digestible fermentable carbohydrates that manipulate intestinal microbiota and their metabolic activities represent a novel and potentially promising treatment for IBD. This thesis compared the influence of two β-fructans with different degree of polymerisation in a preventive study of IBD, and examined the effects of different diets on the protective ani-inflammatory properties of two oligosaccharides with different structures on colitis development in HLA-B27 transgenic rats, a validated animal model of IBD. The clinical outcomes from the experimental studies were confirmed in an open label pilot study using patients with active ulcerative colitis (UC). The particular human study investigated if the dietary mixture of fructo-oligosaccharides (FOS) and inulin could reduce intestinal inflammation and induce compositional and functional shifts in gut microbiota.

Additionally, virulence determinants of *Escherichia coli* strains isolated from IBD and non-IBD subjects, and their association with dysregulated intestinal barrier function in IBD, were also investigated in an *in vitro* study. This research was conducted to assess if the high abundance of *E. coli* strains is related to IBD as causal infection or as a consequence of the chronic intestinal inflammation and disrupted epithelial barrier function.

### 6.1.1. Dysbiosis of intestinal microbiota – cause or consequence of chronic intestinal inflammation?

The human gastrointestinal (GI) tract is inhabited by a vast number and diverse collection of bacteria that are collectively termed 'intestinal microbiota'. The intestinal microbiota play an important role in maintaining the host health status and are necessary for the appropriate gut development and function. An alteration in intestinal microbial composition and function, or abnormal response of the host towards the gut bacteria, may lead to the development of chronic intestinal inflammation. A wide variety of clinical observations and animal experiments have suggested that shifts in the gut microbiota (also called dysbiosis) is associated with a number of GI and metabolic disorders including IBD (Frank *et al*., 2007), colon cancer (Azcárate-Peril *et al.,* 2011), irritable bowel syndrome (IBS) (Malinen *et al*., 2005), diabetes and obesity (Holmes *et al*., 2011). The pathogenesis of those diseases is multifactorial, with both environmental and genetic components (van Heel *et al.,* 2004; Schmidt *et al.,* 2008; Lindgren *et al*., 2009). The role of intestinal microbiota is currently considered as an environmental factor contributing to the triggering and perpetuation of the disease (Bäckhed et al., 2004). The role of gut microbiota in the pathogenesis of IBD has been studied widely in the last decade and dysbiosis in IBD has been linked to reduced microbial diversity (Andoh *et al*., 2007; Sokol *et al.,* 2008), depletion of bacteria belonging to phyla *Bacteroidetes* and *Firmicutes* (Frank *et al*., 2007), where the loss of *Firmicutes* is primarly associated with decrease abundance of members from clostridial clusters IV and XIVa (Frank *et al*., 2007; Sokol *et al*.,

2008); and elevated relative abundance of bacteria from *Enterobacteriaceae* family (Sokol *et al*., 2006). Although a number of studies have suggested that intestinal bacteria play an important role in IBD pathogenesis, it is still not clear if such changes of the compositional structure of gut microbiota are a cause or a consequence of IBD.

IBD can occur in genetically susceptible individuals with impaired epithelial mucosal integrity. Linkage analysis and genome-wide association studies have shown that mutations in multiple genes can be linked with either UC or CD, or both (Lees *et al*., 2011). The host genotype has an important role in shaping and controlling the intestinal bacterial community (Zoentendal *et al*., 2001; Benson *et al*., 2010), and the multiple genes associated with IBD are involved in different pathways maintaining host intestinal homeostasis (van Limbergen *et al.,* 2009; Lees *et al*., 2011). For instance, a genotype-phenotype correlation and gene-environment interaction was investigated in a study of IBD patients and the results revealed that the genetic loci NOD2 (Leu1007fs, R702W, G908R alleles) and ATG16L1 (T300A allele) implicated in IBD could influence the composition of the intestinal microbiota (Frank *et al*., 2011).

The mucosal microbiota is considered to be more significant compared to the luminal microbiota in prompting the mucosal immune response (Swidsinski *et al*., 2005). In the UC clinical trial conducted in this thesis, the presence of colonic inflammation was not correlated to the composition of luminal microbiota but was associated with shifts of the mucosal microbiota composition (Chapter 4). In IBD, the intestinal mucosal barrier is characterized with abnormalities in both its

structural integrity and mucus barrier function (Laukoetter *et al*., 2008; McGuckin *et al*., 2009). Microbial dysbiosis in IBD could be a consequence of the effect of the mucosal inflammation. Increased mucosal permeability (McGuckin *et al*., 2009) and altered bacterial clearance due to changes in antibacterial peptide expression and genetic predisposition of the host (Nuding *et al*., 2007) have been observed in IBD patients. In the current thesis, we found that the expression of genes involved in intestinal epithelial barrier function was different between patients in relapse and those in CD and UC remission (Chapter 5). Disrupted epithelial barrier function leads to excessive stimulation of Toll-like receptors (TLR), production of proinflammatory cytokines and activation of innate and adaptive immune responses, and loss of immune tolerance to commensal bacterial antigens (Sartor and Muehlbauer, 2007). We therefore hypothesize that this dysregulated mechanism of host tolerance contributes to microbial dysbiosis.

The influence of diet on the composition of the microbiota has been shown in a number of experimental and human studies (Turnbaugh *et al*., 2009; De Filippo *et al*., 2010; Fallani *et al*., 2010). Dietary effects have also been associated with increased incidence of some chronic diseases of the GI tract, including IBD, IBS, celiac disease, as well as some metabolic disorders such as obesity, and type 1 and type 2 diabetes (Brown *et al*., 2012). Recent evidence suggests that a Western diet could induce dysbiosis of the intestinal microbiota, which could lead to altered immune functions and higher risk of disease. For instant, Western diet is high in refined sugars and processed fats, and low in fibers, fruits and vegetables, and it has been shown to cause microbial dysbiosis, that could subsequently impact the

GI tract host metabolism and immune homeostasis (Sekirov *et al*., 2010). In a study with CEABAC10 mice, a Western diet induced dysbiosis in gut microbial composition and altered host homeostasis (Martinez-Medina *et al.,* 2013). These findings were seen in conjunction with increased colonisation of adherent-invasive *E. coli* (AIEC) in genetically susceptible mice. The current thesis revealed that diet, supplementation with oligosaccharides, as well as the chronic intestinal inflammation itself all affected the composition of intestinal microbiota (Chapter 1, Chapter 2). Comparison of IBD with other gut disorders that lead dysbiosis may help to elucidate the contribution of diet and inflammation on intestinal microbiota. For example, celiac disease is an inflammatory disorder of the small intestines associated with immune-mediated food intolerance to wheat gluten and similar proteins of barley and rye in genetically susceptible individuals. Celiac disease also induces dysbiosis of the gut microbiota (Collado *et al.,* 2008; Sanchez *et al*., 2010). However the intestinal dysbiosis in celiac patients is not a cause, but a consequence of the disease. Similar to IBD, abundance of the *Enterobacteriaceae* family is higher in fecal and biopsy samples obtained from celiac patients compared to control subjects (Collado *et al.,* 2008). *E. coli* strains isolated from subjects with this GI disorder carry a higher number of virulence factors compared to healthy individuals (Sanchez *et al*., 2010).

Adherent-invasive *E. coli* (AIEC) have been previously associated with IBD, and particularly CD, and their role in the pathogenesis in IBD as a cause or a consequence of the chronic intestinal inflammation has been in debate for decades. The high abundance of AIEC in ileal mucosa of CD patients could be

associated with the fact that the dysregulated epithelial barrier and immune deficiency in CD patients favors the selection and expansion of this pathotype of *E. coli*. It was shown that pro-inflammatory cytokines can induce the expression levels of (CEACAM6) (Carvalho *et al*., 2009), an epithelial surface binding site receptor for *E. coli* type I fimbriae (Barnich *et al*., 2007). The increased numbers of such epithelial binding sites can lead to a selective proliferation of bacteria with AIEC phenotype that are already present in the intestine in small numbers. Similarly, loss of antibacterial function of Paneth cells in IBD patients could also contribute to the relatively high emergence of *E. coli* (Wehkamp *et al*., 2004). The study described in Chapter 5 supported the hypothesis that the elevated relative abundance of *E. coli* in IBD patients is a consequence rather that the cause of the chronic intestinal inflammation.

### 6.1.2. Future directions

The intestinal microbiota have been extensively studied due to its importance to human health and disease. Abbarent microbiota patterns have been associated with the pathogenesis of IBD, and modulation of the intestinal dysbiosis can be used as a therapeutic option for the treatment of this GI disorder. It has been demonstrated in the current thesis that gut microbiota can be manipulated by different dietary fibers in experimental and human IBD. Assessment of the microbiota changes in response to dietary treatments in the current thesis was achieved by the use of quantitative PCR (qPCR) targeting dominant intestinal bacterial groups. Nonetheless, qPCR is limited to the measurement of a few targeted bacterial groups and thus cannot provide a global assessment of the

bacterial community. The development of gene-sequencing technologies and powerful bioinformatic tools have enabled novel insights into the microbial composition in experimental and human IBD. Herein, pyrosequencing analysis was also performed to determine changes in luminal and mucosal microbial composion and their association with reduction of inflammation in UC patients with active disease. The high throughput sequencing provided more comprehensive information about the gut microbiota taxonomy and composition in comparison with the qPCR method. However, culture-independent methods provided information about intestinal bacterial populations and composition, albeit not microbiota metabolic functions. To fully understand the revalence of a particular bacterial group to host health and particularly IBD, the characterization of the physiological functions of intestinal isolates is still needed based on the traditional culture-dependent techniques in combination with metagenomic approaches.

### 6.1.3. Anticipated significance of the research

The design of the current prevention studies in experimental IBD and the small clinical trial provided an important mechanistic understanding of the complex interactions between the gut microbiota and IBD disease. The studies identified potential protective mechanisms of three fibers, including inulin, FOS and isomalto-oligosaccharides (IMO), with different structure and degree of polymerization in a well-characterized colitis model. Structural differences of non-digestible fermentable dietary fibers play an important role in stimulation and growth of different groups of bacteria in the colon and might have various health

effects on the host. An understanding of their protective mechanisms of action is required in order to determine which fibers or which mixture of fibers will be ideal for the prevention or treatment of IBD.

## 6.2. General Conclusions

In this thesis, I was able to demonstrate that dietary interventions with non-digestible fermentable oligosaccharides differing in their structure and composition have divergent effects on colitis development and intestinal microbiota composition in experimental IBD. Those observations were associated with positive correlation of *Clostrdium* cluster XI with tissue inflammation and negative correlation of *Bifidobacterium* spp. and butyrate with colitis. The beneficial anti-inflammatory properties of dietary fibers depended on the diet of the host, however the protective effects of the oligosaccharides were unrelated to any specific bacterial group or genera of the targeted ones. The results from the experimental colitis studies were supported in a clinical trial with UC patients, and it was demonstrated that β-fructans could be suitable as an adjunct therapeutic option for UC patients with mild to moderately active disease. Overall, the promising results obtained from this thesis contribute to our understanding of the role of intestinal bacteria in the pathogenesis of IBD. Future studies that provide an improved functional characterization of gut microbiota in health and disease through metagenomic approaches, and account for the interaction of dietary fiber with other dietary components are necessary to establish dietary fiber as novel

therapeutic options to improve the quality of life of patients with this chronic gastrointestinal disorder.

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