THE EFFECT OF β-FRUCTANS ON INTESTINAL PERMEABILITY IN PATIENTS WITH ULCERATIVE COLITIS IN CLINICAL REMISSION

by

Reem Abdelaziz Rashed

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

Master of Science

Department of Medicine
University of Alberta

ABSTRACT

Introduction: β-fructans are non-digestible fermentable carbohydrates that are metabolized by commensal gut bacteria such as *Faecalibacterium prausnitzii* and *Roseburia spp.* By-products of β-fructans fermentation include short-chain fatty acids, shown to improve intestinal epithelial integrity and the mucosal barrier in pre-clinical studies via pH reduction, and homeostasis of energy metabolism and inflammation. Patients with inflammatory bowel diseases exhibit increased paracellular intestinal permeability and tight junction dysfunction ("leaky gut"), which may exacerbate disease progression. In a recent placebo-controlled intervention β-fructans reduced the severity of subclinical relapse in patients with ulcerative colitis (UC). However, it is unknown if this protective effect was mediated by improved intestinal permeability.

Hypothesis: We hypothesized that 6-month supplementation of β-fructans may improve barrier integrity in UC patients with inactive disease.

Methods: Serum samples and colonic biopsies were collected during a randomized placebo-controlled 6-month clinical study with 73 UC patients in remission treated with inulin-type β-fructans (Synergy 1, Beneo GmbH) or maltodextrin (placebo). mRNA expression of tight junction proteins including, *claudin-2* and *occludin*, were quantified using real-time quantitative polymerase chain reaction from colonic biopsies. In addition, serum concentrations of markers for circulating Gram-negative bacteria [lipopolysaccharides (LPS) and LPS-binding protein (LBP)] and Gram-positive bacteria [lipoteichoic acid (LTA)] were assessed at baseline and endpoint by enzyme-linked immunosorbent assay.

Results: *Claudin-2* and *occludin* mRNA gene-expression showed a positive correlation to each other (Placebo: R^2=0.604, p=0.001; β-fructans: R^2=0.0528, p=0.007). Intergroup analysis showed that tight-junction proteins were down-regulated in patients treated with β-fructans. This was particularly valid for those patients in the β-fructans group who remained in clinical and biochemical remission (one-way ANOVA *claudin-2* p=0.0332; *occludin* p=0.0640). Assessment of the serum LBP, LPS, and LTA showed no difference over the course of the study within treatment and between treatment groups (Placebo: LBP p=0.4939, LPS p=0.6437, LTA p=0.2738; β-fructans: LBP p=0.3092, LPS p= 0.397 LTA p=0.1207; between group differences: LBP p=0.2149, LPS p=0.3176, LTA p=0.0633). Further multivariate analysis including FCP and fecal SCFAs, and markers for intestinal permeability identified that fecal valerate was positively correlated to *claudin-2* gene expression (R^2=0.413, p=0004).

Conclusion: The efficacy of inulin-type β-fructans was partially mediated by down-regulation in tight-junction proteins *claudin-2* and *occludin* mRNA gene expression in patients with UC who are in clinical remission.

PREFACE

This thesis is an original work by Reem Abdelaziz Rashed. The research project is a sub-analysis to the original study, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, under the project name "Relapse prevention of Ulcerative colitis by prebiotic efficacy and protective mechanism" Study ID Pro00041938, July 14, 2015. The study is publicly accessible at the U.S. National Institute of Health database (clinicaltrials.gov identification number NCT02865707).

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Leo Dieleman for his invaluable guidance, unfaltering support, and exceptional mentorship during my master's degree. His profound knowledge, dedication to academic excellence, and commitment to my professional development have been inspiring. Being his final graduate student has truly been an honour.

I am deeply grateful to Simona Veniamin for playing a pivotal role in shaping my academic journey and accomplishments. She created a positive and enriching experience during my degree. Her exceptional patience, brilliant teaching abilities, and calm demeanor fueled my curiosity and greatly contributed to my learning. I am immensely grateful for her contributions to my academic journey and for the lasting impact she has had on my personal and professional life.

I would like to extend my heartfelt gratitude and appreciation to Dr. Rosica Valcheva. She selflessly dedicated countless hours of her personal time to support, guide, impart her valuable experience, and provide encouragement throughout my journey. Despite the challenges posed by the time difference and late-night meetings, Dr. Valcheva always showed a willingness to help in every possible way. Her unwavering dedication and passion to my work and success is truly priceless and highly valued.

I would also like to express my thanks to my supervisory committee members, Dr. Karen Madsen, and Dr. Eytan Wine, for their valuable guidance throughout my research. Additionally, I extend my gratitude to my arm's length examiner, Dr. Marie-Claire Arrieta, for her valuable contributions. Special thanks go to Dale Archer and Christopher Cheng for their assistance in qPCR, which greatly contributed to the success of my research.

I would like to express my sincere gratitude to Janssen, Canada for their generous educational grant, as well as The Centre of Excellence for Gastrointestinal Inflammation and Immunity Research (CEGIIR), The University of Alberta and Faculty of Medicine and Dentistry. Thank you for recognizing the importance of investing in students' learning experiences.

Finally, I am deeply grateful to my family and husband for their unwavering love, encouragement, and support throughout my journey in pursuing my master’s degree. Their constant belief in me and their understanding during the challenging moments have been a

source of strength and inspiration. Their sacrifices, patience, and understanding have made it possible for me to pursue my academic goals with confidence. I am truly blessed to have such a loving and supportive family, and I am forever grateful for their presence in my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-ASA: 5-aminosalicylic acid

AJC: Apical junctional complex

AMP: Antimicrobial proteins

BAs: Bile acids

Caco-2 cells: immortalized cell line of human colorectal adenocarcinoma cells

CARD15: Caspase activating recruitment domain 15

CD: Crohn's disease

cDNA: complementary deoxyribonucleic acid

CXCL: chemokine ligand

DSS: dextran sulfate sodium

EIM: Extraintestinal manifestations

ELISA: enzyme-linked immunosorbent assay

FDR: first-degree relatives

FOS: Fructo-oligosaccharides

H2S: Hydrogen sulfide

HFD: High-fat diet

HLA: human leukocyte antigen

IBD: Inflammatory bowel disease

IEC: intestinal epithelial cell

IgA: Immunoglobulin A

IL: Interleukin

INF-γ: Interferon gamma

JAK: Janus kinase

JAM: junctional adhesion molecules

LAB: lactic acid bacteria

LBP: Lipopolysaccharide binding protein

LMR: Lactulose: mannitol ratio

LOMD: Loss of microbial diversity

LP: Lamina propria

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

MDP: muramyl dipeptide

MIP: macrophage inflammatory protein

MPO: myeloperoxidase

MUC: mucin

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NOD: nucleotide-binding oligomerization domain-containing protein

NSAID: non-steroidal anti-inflammatory drug

PAMPs: Pathogen-associated molecular patterns

PAR: Perijunctional-actinomyosin ring

PCR: polymerase chain reaction

RT-qPCR: real-time quantitative polymerase chain reaction

SCFA: short chain fatty acid

SD: standard deviation

TCA: Taurocholic acid

TJ: Tight junction

TJP: tight junction proteins

TLR: Toll-like receptor

TNBS: trinitrobenzene sulfonic acid

TNF-α: Tumor necrosis factor alpha

UC: Ulcerative colitis

UCAI: Ulcerative Colitis Disease Activity Index

UPR: Unfolded protein response

XOS: xylo-oligosaccharide

ZO-1: Zonula-occluden-1

Chapter I: Introduction

1.1 Overview of Ulcerative Colitis

Ulcerative colitis (UC) is a one of the major phenotypes of Inflammatory Bowel Disease (IBD). First identified in 1859, Sir Samuel Wilks described UC in a case report titled "Morbid appearances in the intestine of Miss Bankes."[1] In 1955, the natural disease course in patients with moderate-to-severe UC had a devastating mortality rate of over 50% which greatly decreased after the introduction of corticosteroids in UC therapy.[2] As with all IBD phenotypes, UC acts in a relapsing and remitting fashion, and many patients may live with a high symptom burden as well as risk of disability. Apart from colectomy,[3] surgical removal of the affected colon,[3] there is currently no cure for UC. Even then, complications with colectomy include anastomotic leak,[4] infection, ileus,[5] and deep vein thrombosis.[6] Despite advancement in medical therapy, patients may suffer from repeated flare-ups or even complete intestinal failure.[7,8] Furthermore, patients with longstanding or extensive UC are at a two-fold increased risk of colorectal cancer as compared to the general population.[9] Many questions relating to the etiology of this idiopathic disease remains at large. However, it is understood that it is caused by a complex interplay between genetics, environmental factors (e.g., nutrition, smoking, stress, hygiene), gut microbiome dysbiosis, and a dysregulated immune system.[10]

UC is distinguished by its trademark superficial mucosal inflammation beginning at the rectum and extending to the proximal colon in a continuous manner.[11,12] This is opposed to Crohn's disease (CD), which occurs in non-continuous lesions anywhere along the gastrointestinal tract but primarily in the terminal ileum of the small intestine with transmural inflammation which may lead to fibrosis, strictures, and fistulas.[11,13] Common clinical features of UC include abdominal pain, rectal bleeding, and diarrhea.[14] Endoscopic features include diffuse continuous inflammation proximal to the rectum, a so-called "sandpaper" appearance of mucosa, friable mucosa, small superficial ulcers.[15–17] Histologic features include crypt abscesses, crypt architectural distortion, inflammation limited to the mucosa, peri appendiceal inflammation alone with distal colitis.[18]

Patients with UC also present with extraintestinal manifestations (EIM). The two types of EIMs include immune-related manifestations of IBD which are consequential manifestations due

to intestinal inflammation such as anemia,[19] arthritis,[20] erythema nodosum, pyoderma gangrenosum, aphthous stomatitis, episcleritis, and iritis/uveitis.[21,22] The second type of EIMs are autoimmune disorders related to IBD which are independent of intestinal activity such as primary sclerosing cholangitis, alopecia areata, and thyroid autoimmune disease. Anemia and other EIMs include metabolic bone disease such as osteoporosis[23] and hepatobiliary disease such as primary sclerosing cholangitis.[21,24] Bacterial translocation across a "leaky" intestinal barrier is implicated in the role of activating the adaptive immune system which cannot distinguish between epitopes of bacteria and skin/joints; therefore, causing EIMs. Autoimmune response triggers are associated with human leukocyte antigen (HLA) with UC patients having complications in the HLA-DR103 genotype.[25]

Several disease classification indices have been validated to assess the extent of UC. Notably, the Montreal classification is used in adults,[26,27] and the Paris classification is useful in assessing disease severity in pediatric patients.[28] As for severity scoring systems, the Ulcerative colitis overall disease (UCAI) severity index are most utilized by clinicians in adult UC patients and the Pediatric UCAI in children.[28] Furthermore, the Mayo Score [29] and partial Mayo score [30] are widely used in clinical trials and can be applied to clinical practice. Disease severity aids in distinguishing types of UC with increasing severity, from proctitis, left-sided colitis, extensive colitis, pancolitis, and toxic colitis.

Unfortunately, Canada belongs to the group of regions that have the one of the highest incidence and prevalence of IBD which is only projected to rise.[31] In 2023, the national prevalence of IBD in Canada is estimated to be 0.83%.[31] By 2030, about 0.98 % or 403,000 Canadians are predicted to be living with IBD.[31] These statistics are in comparison to the global incidence of IBD plateauing at about 3-15 per 100,000 persons in Western countries.[32] UC is most commonly diagnosed in adolescents between 20-30 years, seniors over 65, and afflicts females and males at the same rate.[31,33]

Optimal treatment is critical in preventing complications that lead to surgery, disability, and morbidity. 5-aminosalicylic acid (5-ASA) is indicated as first-line therapy for mild-to-moderate UC.[27] In those who do not optimally respond to 5-ASA, immunosuppressive or biologic therapies are administered.[27] Surgical options for UC are important to discuss with patients and include restorative options such as an ileoanal pouch or, rarely, an ileorectal anastomosis or a permanent ileostomy.[27] The current treatment guidelines for moderate-to-severe

UC is the use of biologic therapy to achieve endoscopic and clinical remission which decreases the need of corticosteroids, invasive surgeries and hospitalization.[27] The decision to use a particular agent is a collaborative process between the patient and clinician based on IBD phenotype and behaviour, previous treatment exposure and response, potential side-effects, and co-morbidities. Commonly used biologic agents include anti-tumor necrosis factor-alpha (TNF-α) agents (infliximab, adalimumab, certolizumab, and golimumab); anti-integrin agents (vedolizumab, natalizumab); and an anti-interleukin (IL) 12-23 agent (ustekinumab).[27] Finally, tofacitinib, a non-biologic small molecule and janus kinase inhibitor (JAK), is approved for the treatment of moderate-severe UC.[27] Upcoming novel and emerging therapies for IBD include other selective JAK inhibitors, anti-interleukin 23 and leukocyte trafficking/migrating inhibitors (such as sphingosine-1-phosphate receptor modulator).[34] Despite the number of available medications, there are considerable rates of primary non-response, loss of response, or adverse reactions thereby necessitating additional medical and adjuvant therapy options.

1.2 The Gut Microbiota

The gut microbiota plays a crucial role in various aspects of human health, including nutrition, energy metabolism,[35–37] host defense, and immune system development.[38] With its vast number of genes, surpassing the human genome by a hundredfold, the microbiota influences not only gastrointestinal diseases but also systemic conditions such as obesity[39,40] and metabolic syndrome.[41] Consequently, the term "mucosal barrier" refers to the protective system in the intestines that prevents harmful substances, such as pathogens and toxins, from entering the body while allowing essential nutrients to be absorbed.[42,43] It is comprised of several elements such as commensal microbes of the gut microbiota, the epithelial layer including tight junctions, the mucus layer, antimicrobial peptides, and immune cells.[42,43] Maintaining a healthy mucosal barrier is essential for overall gut health, as any disruption in its function can lead to increased intestinal permeability, bacterial translocation, and inflammation, potentially contributing to various gastrointestinal disorders and extraintestinal diseases.[42,43]

The mucosal barrier accurately emphasizes the dynamic interaction between the gut and gut microbiota, highlighting that it is not a static shield but an active, dynamic system with specialized responses.[44] The concept of "permeability" defines the functional aspect of this barrier, which enables the coexistence of beneficial microbial symbionts and absorption of

nutrients while preventing the passage of macromolecules and pathogens.[44] "The last human body organ" is a term used to describe the combination of the mucus barrier and the gut microbiome.[45] This dynamic duo consists of approximately 100 trillion symbiotic microbial cells as well as over 9000 carbohydrate-degrading enzymes.[45] Together, they form a complex and intricate system within the human body. The mucus barrier acts as a protective layer, while the gut microbiome, with its vast array of microbial cells and enzymes, plays a vital role in various physiological processes, including digestion, nutrient absorption, immune function, and maintaining overall health.[45] The symbiotic relationship between the mucus barrier and the gut microbiota highlights the profound influence this dynamic organ has on host well-being.[45] Intestinal bacteria have developed various mechanisms to adhere to the protective mucus barrier in the gut. These strategies include the utilization of adhesins, flagella, and fimbriae, which enable them to attach firmly.[46–48] Additionally, intestinal bacteria engage in cross-feeding through the degradation of mucin, allowing them to derive nutrients and establish cooperative interactions within the gut microbiota.[49,50] Furthermore, microbes employ a commensal type VI secretion system to maintain colonization resistance, which serves as a defense mechanism against potential pathogens.[51] These evolutionary adaptations of intestinal bacteria highlight their ability to establish symbiotic relationships with the host and contribute to the intricate dynamics of the gut ecosystem.

There is mounting evidence pointing to gut dysbiosis as a fundamental cause in the etiology of UC. An abnormal mucosal immune response to the dysbiosis that consists of abnormal microbiota composition, function, and by-products. Healthy microbiota communities are involved in managing the fluctuating balance of production, secretion, expansion, and proteolysis of mucus components.[52] Furthermore, commensal bacteria and their fermentation products such as short-chain fatty acids (SCFA) aid in regulating the production and secretion of mucin 2 (MUC2), the major component of mucus, in sentinel goblet cells at crypt openings. β-oxidation of SCFA in colonocytes generate carbon dioxide, which is converted into bicarbonate by carbonic anhydrase ($HCO3^{-}$).[52] $HCO3^{-}$ can precipitate calcium, which in turns raises the pH at the epithelial surface.[48] This rise in pH promotes the ideal stratification of the mucus layer and the proper unfolding of mucin.[48]

Colonic epithelium is blanketed by a mucus layer composed of a loose outer layer that is colonized with commensal microbes, and a firm inner layer which is relatively sterile.[53,54] At

homeostasis, the gut microbiota at the outer mucus layer modulates mucin production and secretion and mucus stratification mediated by HCO_3^- to maintain mucus barrier integrity. A healthy mucus layer is well attached, stratified, and hydrated.[52] Not only does it provide protection and lubrication over the epithelium, but it also a conducive environment for microbial colonization and nourishment to commensal microbiota, thereby stabilizing microbial communities and promoting symbiotic interactions and resulting in microbial commensalism.[52,55] In active UC, several mucus barrier abnormalities indicate that a faulty mucus barrier and microbiome may precede the onset of chronic inflammation involved in UC.[56,57]

1.2.1 Structure and Function of a "Healthy" Gut Microbiota

There is yet to be a clearly defined "gold standard" for what a healthy gut microbiota is. Relative abundance and distribution of gut bacteria is unique, especially at the strain level,[58] changes in growth rates, and structural variants within microbial genes [59,60] mostly owing to environmental exposure,[61] host-genetics, age, ethnicity, sex, and health status.[61] Mode of birth and access to breastfeeding [62] play important roles in shaping the infant microbiota which is molded further by environmental exposures[63] during childhood such as sanitation, exposure to animals, antibiotics, pollutions, and stress. [63] The microbiota then remains relatively stable until a natural decline in diversity occurs related to aging and a weakening immune system.[62] Generally, high taxonomic diversity, high microbial gene richness and stable microbiome functional cores make up healthy microbial communities. Generally, high microbial diversity is observed in healthy people and is associated with healthy levels of SCFA production, an intact mucosal barrier, and no overt inflammation.[64] The concept of Loss of Microbiota Diversity (LOMD) refers to the consistent observation of reduced diversity in the gut microbiota of individuals with intestinal dysbiosis, especially in the context of modern Western lifestyles.[62] The hypothesis proposes that this reduction in diversity is linked to the loss of commensals, which could be associated with changes in dietary habits, hygiene practices, and other lifestyle factors in the modern world.[62] A loss in diversity has consistently been observed in inflammatory and metabolic disorder states as IBD,[65] and metabolic syndrome.[66] Although high gut bacterial diversity and richness are important, on their own, they do not indicate a healthy microbiota since intestinal transit time can effect microbial richness.[67] In conditions with prolonged transit

time, an increase in microbial richness may be observed, however, this does not necessarily mean that the individual is host to a "healthy" gut microbiome.[67]

With the high rate of taxonomic variation, defining a "healthy" microbiome as being composed of an ideal set of specific microbes is no longer sought after. However, searching for a healthy "functional core" looks to define metabolic and molecular functions that are performed by the microbiota but may not necessarily be carried out by the same organisms between people.[60] Resistance to external and internal stressors along with resilience to recover back to a healthy functional profile are traits sought after for a "healthy" microbiota.[60] Commensal bacteria such as *Bifidobacterium* can synthesize vitamins such as vitamin K and water-soluble B vitamins.[60] Symbionts such as adult-like *Bacteroides, Parabacteroides, Clostridium, Lactobacillus, Bifidobacterium*, and *Faecalibacterium prausnitzii* provides several determinants of a healthy microbiome when introduced to the infant microbiome.[68] These species are the main producers of SCFAs, an important source of energy from nondigestible carbohydrates.[68] SCFAs are immunomodulatory,[69] inhibit common pathogens, and are hypothesized to possess tumor-suppressive properties.[70,71] The gut microbiota is an inextricable requirement for immune system education and the establishment of these beneficial genera early in life promotes immune tolerance and can consequently attenuate or prevent autoimmune diseases.[72–76]

The gut microbiota additionally contributes to host defense though colonization resistance. Colonization resistance is the process of competition where commensal bacteria occupy the physical and nutritional niches of the gastrointestinal system to prevent colonization of pathogenic bacteria.[60] For example, *Bacteroides* (*B.*) *thetaiotaomicron*, an anaerobe residing in the colon, utilizes carbohydrate compounds also used by *Citrobacter rodentium*, which cause direct exclusion of the pathogen in the lumen.[77] In terms of indirect competition, commensal microbes can activate immune responses for the exclusion of pathogens. For example, lipopolysaccharide (LPS) and flagellin produced by the gut microbiota enhance the expression of antimicrobial peptide and RegIIIγ cells by stimulating Toll-like receptor (TLR) 4^+ stromal cells and $TLR5^+$ $CD103^+$ dendritic cells.[78,79] Segmented filamentous bacteria, such *as Candidatus Arthromitus*, promote the maturation of the mucosal immune system and can promote the secretion of IgA from B cells, antimicrobial peptides, and the development of Th_{17} cells in the intestinal mucosa.[80,81]

Many population-scale projects have undertaken the great feat of characterizing the human gut microbiome. In 2010, the Metagenomes of the Human Intestinal Tract (MetaHIT) reported gut metagenomes from a predominantly healthy cohort made up of 124 European adults.[82] In 2012, the Human Microbiome Project (HMP) used 16S rRNA profiling on 242 healthy adults in the United States along with metagenomic sequencing.[83] Finally, a study with 145 gut metagenomes from a Chinese study have been added to the roster. [84]

Microbiome-directed interventions have gained traction as sustainable options with minor side effects. Untargeted interventions include individualized nutrition, fecal microbiota transplantation, prebiotics, probiotics, synbiotics, and postbiotics.[85] These untargeted interventions are applied for the general improvement in microbial community composition and function.[85] Targeted interventions include bio-engineered commensals, drugs targeting selected microbial metabolism, bacteriophage therapy, and CRISPR-Cas9 editing which result in highly specific modifications in metabolism-related gut microbiota.[85]

1.2.2 Alterations in Gut Microbiota in Ulcerative Colitis

Microbial dysbiosis has not only been implicated in IBD, but a plethora of metabolic disorders such as malnutrition, obesity, type 2 diabetes, and cardio-metabolic disease.[84,86,87] Numerous microbiome studies have identified associations with UC, highlighting microbial dysbiosis and temporal shifts related to UC.[88] Furthermore, the gut microbiota in the large intestine is denser in comparison to the small intestine, made up of mostly Gram-negative Bacteroidetes and Gram-positive Firmicutes. This phenomenon is conserved in humans and mice alike.[89] Interestingly, Scaldaferri et al. reported that UC patients demonstrate the highest abundance of bacteria at sites of most severe inflammation.[69] It has been suggested that changes in innate characteristics of the gut microbiota could be used as a diagnostic and prognostic tool in UC.

Adherent invasive *Escherichia coli* adheres to intestinal epithelial cells (IECs) through the process of microtubular polymerization to induce inflammatory factors.[90] Zhang XJ et al. reported that the abundance of Bacteroidetes, Proteobacteria and Firmicutes which are involved in tryptophan catabolism may aggravate intestinal injury in DSS-induced colitis in mice.[91] Furthermore, alteration of the diversity and composition of microbiota involved in T cell balance (Th_1/Th_2, Th_{17}, T_{reg}) may be a therapeutic target for UC.[92]

In a recent Chinese study,[93] they found a difference in α- and β-diversity between active/remission UC patients and healthy controls. Furthermore, the study supported the finding of previous studies showing a gut microbiota profile dominated by Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria, in varying proportions between the three groups. Firmicutes and Bacteroidetes are involved in lipid and bile acid metabolism regulation and homeostasis of energy in the host. Proteobacteria levels were high in active UC patients, alluding to its roll in inflammation. *Blautia* and *Lachnoclostridium* were shown to be abundant in UC remission.[94] *Blautia* is an anaerobic microbe with probiotic traits that promotes the production of SCFA, prevents inflammation, and maintains homeostasis. Furthermore, *Blautia* spp. has been shown to be reduced in colorectal cancer and more abundant in UC remisson.[94,95] In active UC, *Rothia, Actinomyces, Pediococcus,* and *Saccharibacteria* (TM7) were significantly abundant.[94]

It is well-documented that patients with active UC display lower levels of *Bifidobacterium* spp, *Faecalibacterium prausnitzii*, and organic acids.[74,96] Sulfate-reducing bacteria, whose metabolites (e.g., hydrogen sulfide) can block the use of butyrate by colonocytes[97,98] have also been found to be increased in abundance in patients with IBD [99]. Lloyd-Price et al. [100] demonstrated lower relative abundances of *Desulfobacterota* and *Verrucomicrobiota* in active UC patients compared to healthy controls. Higher levels of pathogenic bacteria and lower levels of butyrate-producing bacteria were also detected during active UC.[100] In general, an increase in facultative anaerobes at the expense of obligate anaerobes in the gut microbiota of UC patients has been demonstrated.[93]

The gut microbiota plays a pivotal role in the education of the host immune system. The immune system in turn plays a role in further shaping the composition and functions of the gut microbiota. Studies with germ-free mice presented with immature lymphoid tissue, a reduction in intestinal lymphocytes and antimicrobial peptides which suggest an inappropriate development of their immune systems.[101] Reconstitution with intestinal microbes restored the aforementioned deficits in their immune systems.[101]

Dysbiosis induces impairment of the mucus barrier, accompanied by increased epithelial damage, bacterial translocation, goblet cell depletion, and host inflammation.[102,103] Generally, dysbiosis in IBD presents as reduced diversity, a decrease of SCFA-producing microbes, an increase in mucolytic bacteria, sulfate-reducing bacteria, and pathogenic bacteria.[104–106] Due to

this dysbiosis, the host mucosal barrier is compromised due to the lack of energy sources from SCFA for IEC production, growth, and differentiation, shift in regulatory T cell differentiation, degradation of mucus and bacterial translocation, increase of epithelial cells damage, induction of mucosal inflammation, and alteration in mucosal permeability.[107] Environmental factors, including diet and lifestyle, have a significant impact on the composition of the gut microbiota, influencing mucus homeostasis and the development of intestinal inflammation.[61] When the intestinal microbiota lacks dietary fiber, it can consume components of the protective mucus layer, leading to dysfunction of the barrier and increased susceptibility to pathogens and inflammation.[61] The relationship between the microbiota and the mucus barrier is integral, and it is essential to consider both aspects when studying the underlying mechanisms of inflammatory conditions like UC.

Furthermore, the role of the microbiome has been found to play a possible role in predicting disease course and response to therapy. In the study by Lee et al., a comprehensive approach involving metagenomics, metabolomics, and immune marker analysis revealed associations between microbial communities and the likelihood of responding to anti-cytokine or anti-integrin therapy for IBD.[108] The research emphasized the importance of microbial pathways, not just taxonomy, in influencing treatment outcomes. These pathways were validated by changes in serum metabolite levels and corresponding cytokine measurements. The study highlighted the significance of the gut microbiome in determining concentrations of luminal SCFAs and succinate, which play a role in shaping the course of inflammation.[108] Additionally, bile acid metabolism, particularly secondary bile acids, was identified as a determinant of response to anti-cytokine therapy in IBD.[108] Their findings also suggest the potential for developing targeted probiotics with specific anti-inflammatory functions or the ability to stimulate beneficial metabolic pathways for gut health.[108] The identified omics profiles could serve as targets for novel therapies and provide insights into the underlying mechanisms of these complex diseases.[108] Specifically, patients who were more likely to achieve remission with anti-cytokine therapy had a higher abundance of colonic butyrate-producing microbial species, promoting intestinal homeostasis (e.g., *Agathobaculum butyricproduces, Clostridium citroneae*). They also exhibited increased levels of succinate-consuming species such as *Phascolarctobacterium faecium*.[108] Conversely, among patients initiating anti-integrin therapy, remitters showed elevated abundances of *Bifidobacterium longum* and certain *Bacteroides*

species (e.g., *B. ovatus, B. stercoris*).[108] These findings underscore the intricate relationships between the gut microbiome and therapeutic responses in IBD, offering potential avenues for personalized treatment strategies.

1.3 Intestinal Permeability

1.3.1 Structure of the Intestinal Barrier

The intestinal epithelial barrier is a physicochemical and immunological barrier that guards against luminal antigens and enteric pathogens allowing the access of ions, fluids, and solutes.[52] Intestinal epithelial cells (IECs) prevent the invasion of pathogens through high proliferation rate, mucus secretion, tight junction formation, and innate immune response.[52] The intestinal barrier serves as a crucial defense mechanism, encompassing various elements that contribute to its function both as a physical barrier and an immunological defense boundary. The key components of the intestinal barrier include the outer mucus layer, specialized epithelial cells, and the inner lamina propria.[52] The outer mucus layer surrounds the gut microbiota, consisting of beneficial bacteria, and contains antimicrobial proteins (AMPs) and secretory immunoglobulin A (S-IgA) molecules. The mucus layer provides a protective shield against pathogens and aids in maintaining a balanced microbial environment.[52]

The central layer of the intestinal barrier is composed of a single layer of specialized IECs. These cells arise from pluripotent stem cells located at the base of the crypts.[44] Depending on specific transcription factors, these stem cells can differentiate into different cell types including goblet cells, Paneth cells, enteroendocrine cells, enterocytes, and microfold cells (M cells).[44] Goblet cells cover the surface of the intestinal epithelium with the mucus they secrete.[109] These cells play a vital role in the protection of the intestinal epithelium. In patients with UC, reduced goblet cell production and mucus layer thickness,[110,111] as well as altered mucus composition in terms of mucins, phosphatidylcholine, and glycosylation[112–116] has been well-documented. The mucus is composed of carbohydrates, lipids, water, but most importantly, antimicrobial peptides such as defensins which are produced by Paneth cells and secretory IgA.[109]

Paneth cells synthesize lysozyme and antimicrobial peptides such as defensins, which help combat pathogenic bacteria.[117] Furthermore, altered distribution and function of Paneth cells have been documented in IBD. Paneth cells typically reside in the bottom of the crypts of

Lieberkuhn in the small intestine.[109,117,118] In UC, Paneth cells have been found in the colonic mucosa, leading to the secretion of defensins even in the large intestine. [119] Ultimately, the role of Paneth cells may differ between the two disease phenotypes, as defensin expression is inducible by colonic inflammation in UC but reduced in patients with colonic CD.[120] The while the increased defensin secretion in UC may represent a physiological response to mucosal damage.[109,117,118]

Enteroendocrine cells produce enteric hormones involved in regulating various functions within the gut.[121] Enterocytes are responsible for absorbing water and nutrients from the intestinal lumen.[121] Finally, M cells are specialized cells involved in antigen sampling, allowing the immune system to monitor and respond to potential threats.[122] The inner layer of the intestinal barrier is known as the lamina propria, where cells of both innate and adaptive immunity reside. This includes innate immune cells such as natural killer cells, neutrophils, dendritic cells, and macrophages, as well as adaptive immune cells such as T cells and B cells.[123,124] These immune cells play crucial roles in defending against pathogens and maintaining immune homeostasis within the gut.[124] The mechanical integrity and regulation of paracellular permeability of ions and small molecules are maintained by three types of junctional complexes: tight junctions, adherens junctions, and desmosomes. Collectively, these components work together to form a complex and dynamic intestinal barrier, ensuring the protection and proper functioning of the gastrointestinal tract.[125–127]

1.3.2 Intestinal Permeability and "Leaky Gut"

Intestinal permeability (IP) refers to the functional property of the intestinal mucosal barrier that controls the passage of luminal contents and solutes across the intestinal surface.[44] Normal intestinal permeability allows for nutrient absorption and the coexistence of microbial symbionts of the host, while preventing luminal penetration of macromolecules and pathogens.[44] The purpose of the intestinal barrier is to reduce contact between luminal microbial contents and the mucosal immune system.[128] In healthy humans, it acts as a semi-permeable physical barrier allowing selective movement of nutrients while protecting the body from pathogenic invasion.[129] An impaired intestinal barrier can be associated with increased intestinal permeability, also known as "leaky gut," has been the focus of research as it appears to be a defining factor in the pathogenesis of IBD.[44,129] Various other conditions have been associated with altered intestinal

permeability. For instance, acute pancreatitis, multi-organ failure, major surgery, severe trauma, and critically ill patients exhibit documented changes in intestinal permeability.[130,131] These alterations may contribute to the high prevalence of Gram-negative sepsis and related mortality observed in critically ill individuals. Additionally, disturbances in the complex mechanism of intestinal permeability have been linked to the development of irritable bowel syndrome and metabolic dysfunction-associated fatty liver disease (MAFLD, formally known as non-alcoholic fatty liver disease, NAFLD).[132,133]

1.3.3 Factors that affect intestinal permeability

As mentioned previously, gut dysbiosis and reduced diversity play a role in increasing intestinal permeability by undermining the integrity of the mucosal barrier. Furthermore, genetics have been indicted in their involvement of gut dysbiosis and intestinal barrier function. The NOD2/CARD15 genotype has been shown to influence the composition of the gut microbiota in humans.[134,135] This dysbiosis may further exacerbate permeability dysfunction by disrupting the symbiotic relationship between the microbiota and the integrity of the mucosal barrier. In addition, genes associated with intestinal barrier homeostasis have been linked to susceptibility to IBD. This suggests a genetic predisposition, supported by the observation that up to 30% of first-degree relatives (FDRs) of CD patients exhibit altered small intestinal permeability.[136] There is a significant association between familial CD, NOD2/CARD15 variants, and altered permeability. The NOD2/CARD15 gene, involved in bacterial recognition, modulates innate and adaptive immune responses and is a key susceptibility locus for CD development.[134,135] Defects in bacterial recognition and processing have been documented in CD patients with specific genetic polymorphisms, particularly in pattern-recognition receptors like NOD2/CARD15 and genes involved in autophagy such as ATG16L1 and IRGM.[134,135] The lack of feedback between mutated NOD2/CARD15 expression and the gut microbiota can lead to the breakdown of tolerance in the intestinal mucosa.[134,135] Autophagy has also been linked to the regulation of tight junctions (TJs) through the degradation of a pore-forming claudin, further connecting autophagy to permeability.[137] However, some studies have not found a correlation between permeability and genetic polymorphisms, particularly in sporadic CD cases. Environmental factors also play a significant role in determining mucosal permeability, as evidenced by increased permeability observed in a proportion of spouses of CD patients.[138]

Recent studies have highlighted the importance of age[139] and smoking status,[140,141] rather than genotype, in FDRs.

Regardless of whether it is genetically determined or caused by environmental factors, impaired permeability disrupts the delicate balance between the mucosal barrier and luminal challenges. Innate immunity in IBD patients is unable to adequately counteract this disruption and instead responds with aberrant immune activation. However, genetics on their own do not take major claim over the onset of IBD. For example, genetically pre-disposed models of colitis do not develop IBD.[142,143] Therefore, there is an integral and primary role of microbes and environmental factors that cause intestinal inflammation.

There is a plethora of stressors that can increase human intestinal permeability beyond genetics and microbiota. Namely, diets such as the Western diet, high in saturated fatty acids and added sugar are associated with impaired intestinal barrier function. [86,144] The intestinal barrier system is compromised in response to long-term exposure to high-fat diets (HFD). HFD weaken the tightness of TJs by impairing the expression of tight junction proteins (TJPs),[145] allowing the entry of luminal contents into the lamina propria.[146] Translocated bile acids (BAs) and fatty acids contribute to oxidative stress and apoptosis in enterocytes, further compromising the tight seal of the lumen.[145,147] Moreover, fatty acid-induced unfolded protein response (UPR) stress in goblet cells can inhibit mucus secretion.[147] Combined with an increased load of hydrophobic BAs, this negatively affects the quantity and quality of mucus, facilitating the invasion of gut microbiota. Changes in the bacterial composition caused by the HFD promote the growth of pathogenic strains like *Desulfovibrio spp.*, which produce genotoxic hydrogen sulfide (H_2S) gas by deconjugating taurocholic acid (TCA).[148] Furthermore, mice fed HFD, presented a 100-fold decrease in mucin-degrading *A. muciniphila*.[40] The collection of these factors contributes to an inflammatory response that exacerbates the cycle of deterioration of the intestinal barrier, increasing the host susceptibility to gastrointestinal pathologies.

Beyond that, alcohol,[149] tobacco, and cannabis also play a role in increasing intestinal permeability. Other diet-independent factors include stress,[150] smoking,[141] age,[151] obesity,[152] medications such as non-steroidal anti-inflammatory drugs,[153] proton pump inhibitors,[154] and antibiotics, intensive exercise,[155] pregnancy,[156] severe trauma,[131] and other diseases such as Celiac disease[157] and chronic liver disease (i.e., non-alcoholic fatty liver disease).[158]

1.3.4 "Leaky Gut" and IBD

Several studies support the hypothesis that altered intestinal permeability may be an early event in the pathogenesis of IBD, preceding the onset of chronic intestinal inflammation. Hollander et al. was one of the first to identify increased intestinal permeability as a risk factor and etiological factor for CD.[159] This was observed as a two-fold increase in permeability of CD patients and their FDRs as compared to controls.[159] Later on, Turpin et al. analyzed urine samples from 1420 asymptomatic FDRs of individuals with CD and found that abnormal intestinal permeability (Lactulose: mannitol ratio (LMR) >0.03) was a notable risk factor for the onset of CD.[160] Notably, this increased intestinal permeability was identified to precede the diagnosis of CD by up to three years.[160] Additionally, Teshima et al. studied FDRs of patients with CD and found 30% to have increased intestinal permeability and 24% to have 3 or more small bowel ulcers using video capsule endoscopy.[161] Moreover, in a recent study by Leibovitzh et al.,[162] the gut microbiome of 3127 healthy FDRs of patients with CD was analyzed to investigate its association with gut barrier homeostasis. [162] They found that alterations in gut microbiome composition and functional pathways were linked to the integrity of the gut barrier. [162] Leibovitzh et al. identified 8 genera that were significantly associated with impaired gut barrier function (LMR >0.025). An increase in the relative abundance of *Colidextribacter, Streptococcus*, and *Bifidobacterium*, and a decrease in the prevalence *of Clostridia UCG 014, Adlercreutzia, Enterorhabdus,* Family XIII UCG 001*,* and *Clostridium sensu stricto I* were all linked to impaired gut barrier function. After adjustment for fecal calprotectin (FCP) levels >100 mg/g, α-diversity remained significantly lower in subjects with an LMR >0.025. [162] Furthermore, five of the eight taxa that were associated with gut barrier dysfunction (*Colidextribacter, Streptococcus, Bifidobacterium,* Family XIII UCG 001*,* and *Clostridia UCG 014*) maintained their significance after adjustment for FCP levels. [162]

Furthermore, the question whether mucosal barrier impairment and leaky gut is a cause or a response to intestinal inflammation has been widely debated. However, it has been established that increased intestinal permeability can exist during states of non-inflammation. Animal models of CD, such as IL-10 knockout mice and SAMP1/YitFc mice,[163] have also shown increased permeability prior to the onset of mucosal inflammation.[164] Increased intestinal permeability on its own is associated with inflammation and IBD pathology as demonstrated by Arrieta et al.[165] In this study, IL-10$^{-/-}$ mice received zonula occludens toxin pathway agonist AT-

1002 to increase their small intestinal permeability, which in turn exacerbated colitis.[165] In human studies, increased paracellular permeability has been observed in patients with quiescent IBD, even in the absence of endoscopic activity, and has been correlated with intestinal symptoms.[166] Also, Teshima et al. conducted an *ex vivo* study using Ussing chambers on colonic biopsies from CD patients to demonstrate a uniform increase in transepithelial conductivity, attributed to the downregulation of TJPs, despite minimal mucosal erosions.[136] Altogether, this data implicates leaky gut as an independent risk factor of CD.

In terms of UC, it's important to note that leaky gut is not universally observed in all individuals, and therefore has not been identified as a strong risk factor in the disease's pathogenesis. Büning et al. found that in UC remission, there is an observed increase in small intestinal permeability but not in colonic permeability.[167] This finding may potentially indicate a new risk factor for the development of extensive disease location in UC.[167] Since disease extent can vary among UC patients, the study suggested that increased intestinal permeability, even in clinical remission, might contribute to the risk of developing more extensive disease involving larger portions of the colon.[167] It's important to note that while this finding is intriguing, further research is needed to fully understand the relationship between increased intestinal permeability and disease extent in UC. It could potentially provide insights into the factors influencing disease progression and help in identifying individuals at higher risk for more severe forms of UC.[167] Furthermore, Alipour et al. showed that in pediatric patients with UC, the mucosal barrier in the non-inflamed ileum can be compromised, leading to reduced mucus secretion, increased bacterial penetration, immune response, and loss of bacterial diversity.[168] Similar microbial changes are observed in the absence of inflammation.[168] The findings suggest that pediatric UC may be mediated by a systemic mucosal barrier defect with abnormal bacterial colonization, which may precede and possibly promote inflammation.[168] These insights could lead to the development of targeted therapies of the microbiota for prevention and treatment of the disease.[168] However, this observation should be further explored in UC.

1.3.5 Tight Junction Proteins

IECs are mechanically attached by the junctional complexes of apical tight junctions and subjacent adherens junctions, which are collectively known as the apical junctional complex (AJC).[109,169,170] Desmosomes and gap junctions reside beneath the AJC and mediate intercellular

adhesion and crosstalk between adjacent IECs.[171,172] These structures also control paracellular transport of ions and small molecules between adjacent cells via passive transport.[173] IECs are arranged in a single layer of cells in invaginations known as crypts. IECs are also lined with villi in the small intestine.[173] Epithelial integrity is characterized by a 4–5-day turnover of cell shedding into the intestinal lumen at the surface and the proliferation of progenitor stem cells within the intestinal crypt to replace the loss of cells.[38] Disruption of intestinal barrier turnover contributes to invasion of luminal antigens and intestinal inflammation as seen in UC and CD.[174,175] Patients with IBD display several TJ abnormalities, such as reduced expression and redistribution of TJs and their constituents such as occludins, claudins, and junctional adhesion molecules (JAM), [174–177] leading to increased paracellular transport.[126,177,178] This is also observed in quiescent IBD and non-inflamed regions of the gut.[168] In vitro studies have shown that impaired barrier function is associated with TJPs disassembly from the apical membrane.[179,180] Furthermore, active UC patients present with altered TJ structure, disassembly of TJPs, and significantly increased transepithelial transport in atypical epithelial cells. [181] Additionally, TNF-α, a key mediator of inflammation in IBD, can modulate the transcription of TJPs, and anti-TNF-α agents have been found to improve intestinal permeability. However, TNF-α can also disrupt permeability by inducing apoptosis of enterocytes, increasing their shedding, and interfering with the redistribution of TJs.[182,183]

1.3.6 Claudins

Claudins are integral members of tight junctions, which act as cell-to-cell adhesion molecules.[184] There are currently twenty-seven of these tetraspan transmembrane proteins identified in mammals.[185] Claudins are known as “gate keepers” of the cell—that is, they are responsible for paracellular barrier functions and control the size and charge of molecules moving through the paracellular space.[184,186] Claudins have been classified as barrier-forming or channel-forming.[186,187] Barrier-forming or “tight/sealing” claudins are known to reduce permeability and are further categorized as non-charge and charge-selective claudins. While channel-/pore-forming claudins enhance permeability to allow for the selective transport of water and other molecules, therefore being described as “leaky” claudins.[187] “Tight” claudins include claudin -1, 3, 4, 5, 6, 8, 12, 18, 19[186,188–194] while “leaky” claudin-2 and -15 contribute to increase paracellular permeability to sodium and water[195,196]. The overall function of a certain claudin is

dependent on the complement of other claudins expressed within a tight junction.[197] Molecules can cross the tight junctions via two distinct size-selective and charge-selective paracellular pathways: the pore pathway and the leak pathway.[198,199] These can be distinguished by their selectivity and differential regulation by immune cells. However, permeability increases measured in most studies are secondary to epithelial damage, which allows non-selective flux via the unrestricted pathway.[200] For the purpose of this overview, claudin-1, -2, -3, -4, and -7 will be discussed.

Claudin-1 is barrier forming claudin, which is upregulated in UC.[201] Genetic studies have highlighted its importance in tight junction and barrier formation. Furuse et al., demonstrated the critical role of claudin-1 for trans-epidermal water loss in the barrier function.[186] Pope et al. demonstrated another function of claudin-1 in the regulation of intestinal epithelial homeostasis via regulation of Notch-signaling.[202] In short, upregulation of claudin-1 that is demonstrated in UC and colorectal cancer induced MMP-9 and p-ERK signaling to activate Notch-signaling, which then inhibits goblet cell differentiation. A reduction in goblet cell differentiation decreases *MUC2* expression leading to susceptibility to mucosal inflammation.[202] In IEC-18 monolayer cells treated with TNF-α , an increase in claudin-1 has been demonstrated, mostly residing in the cytoplasm instead of localizing in the TJ apical membrane, despite reduction in barrier function.[203] To explain this researchers have questioned the role of claudin-1 in needing another molecule to direct it to the appropriate location.[203] ITs expression is significantly upregulated in UC patients with colorectal carcinoma indicating its role in active disease along with active severity.[204] In human samples, observations of a significant increase in claudin-1 to occludin ratio in active UC compared to remission UC or healthy controls, suggests that TJPs may be correlated with disease state and the inflammatory process of UC.[203] This contrasts with CD patients who demonstrate TJ abnormality during recurring and remitting disease states.[203] In other studies, claudin-1 has been reported to be unchanged[176] or reduced.[205]

Claudin-2 is a charge-selective cation water channel predominately expressed in leaky epithelia and is upregulated in inflammatory conditions such as UC.[206] This claudin is 24.5 kDa in size and functions as an integral membrane protein with 230 amino acids, which consists of 4 transmembrane helices, 2 extracellular loops, a small intracellular loop, a short intracellular NH_2 terminus and a longer intracellular COOH terminus.[195,207] Ahmad et al. theorized that the increased expression of claudin-2 may be associated with an increase in colonocyte proliferation

and protection against colitis induced colonocyte death.[208] Schulzke et al. demonstrated that increased paracellular permeability was associated with an increase in claudin-2 leading to increased epithelial apoptosis.[177] Disruptions in intestinal permeability is multifactorial and occur in conjunction with downregulation and re-localization of claudin-1, -4, -7, and occludin, as well as increased apoptosis and epithelial lesions.[127,196,209] IL-13 has been identified as a key effector cytokine in the process of epithelial barrier dysfunction in UC.[205] IL-13 plays a role in increased intestinal permeability via the upregulation of claudin-2, induction of epithelial apoptosis, and inhibition of epithelial restitution processes ultimately resulting in the development of lesions and micro-erosions.[205] PI3K and STAT6 signalling pathways have also been identified as mechanisms, which regulate IL-13 induced claudin-2 expression.[205] For example, improvement in oxazolone-induced colitis is associated with a reduction in claudin-2 expression.[210] Furthermore, several mutations in transcription factors that regulate TJ protein expression have been indicted in the contribution to barrier dysfunction in patients with UC. Genome-wide association studies have identified Hepatocyte nuclear factor-4α (HNF-4α), a transcriptional regulator of TJPs, as a susceptibility locus for UC.[211] Patients with an HNF-4α mutation present with dysregulation of TJPs, changes in claudin expression, and increased intestinal permeability.[212,213]

Claudin-3 is a barrier forming claudin and is expressed liberally in the gastrointestinal tract. In rats, claudin-3 is expressed abundantly in the jejunum, ileum, and colon. However, expression in the colon is higher than elsewhere in the gut.[214] In humans, Claudin-3 is localized in TJs at the lateral cell membrane.[215] Claudin-3 has been reported to be reduced in active UC.[215] Milatz et al. determined that claudin-3 possesses the capability to modify TJ network and seal paracellular pathways against the passage of small ions of both charged or uncharged solutes.[194]

Claudin-4 is a barrier-forming claudin, specifically an anion channel. In UC, claudin-4 is shown to be reduced.[206] Although it is not as abundantly expressed as other claudins, mRNA and protein synthesis of claudin-4 have been detected in lung, renal and GIT tissues of rats, enteric neurons, and the human colon. In the human colon, claudin-4 is present in the lateral membrane, especially in the crypts and surface of enterocytes.[215] Based on cell models, claudin-4 functions as a barrier regulator and a chloride channel that bars sodium.[215] Claudin-4 relies on claudin-8 for proper assembly in TJs, and to function correctly as the anion selective paracellular pathway.[188]

Claudin-7 is a barrier-forming claudin, which is down-regulated in active UC.[206] Although claudin-7 is one of the most dominant claudins in the intestine,[216] its role has been ambiguous. A role of claudin-7 has recently begun to be identified. In 2020, Xing et al., showed that claudin-7 plays a critical role in intestinal epithelial stem cell function and regulation.[217] The mechanism occurs via control of Wnt/β-catenin signaling–dependent intestinal epithelial stem cell survival, self-renewal, and cell differentiation.[217] Wnt/β-catenin is a key regulator of the fate of stem cells.[217,218] Another Japanese study used *Vil1-cre; Cldn7 flox/flox* mice to elucidate the role of deletion of claudin-7 in the intestine.[219] They found that intestine specific claudin-7 KO mice developed colonic inflammation. Intestinal claudin-7 deficiency increases paracellular flux for small organic solutes across the colonic epithelial barrier, which plays a critical role in initiation of colonic inflammation.[219]

1.3.7 Occludin

Occludin was the first tight junction protein identified in 1963 by Farquhar and Palade.[171] It is a 65 kDa multidomain tetraspan protein that is localized to endothelial and epithelial tight junctions.[220] In tight junctions, occludin provides structural integrity to tissue in order to create highly polarized barriers with selective permeability to water, solutes, large molecules, and other cells.[221] The co-interaction with cytoplasmic adaptor proteins such as zonula occludens (ZO-1, -2, -3), 7H6, AF6, vinculin, and cingulin, mediate cytoskeletal tethering along with cell-to-cell partnering of transmembrane proteins such as other occludin proteins, claudins, and JAM-1, -2,and -3.[125] Its function is highly diverse with roles in differentiation, proliferation, migration, signal transduction, and gene expression.[221]

Occludin has demonstrated to be downregulated in UC.[181] In occludin-deficient embryonic cells, the cells differentiate into polarized epithelial cells with normal structural and functional tight junctions, leading to the speculation that occludin is necessary for the performance of tight junctions rather than their structure and function.[222] Further leading to the discovery of claudins.[222] Occludin-knockout mice have viable tight junctions, without renal or gastrointestinal disease,[223] However, males displayed testicular atrophy and sterility while females were not able to effectively suckle their young.[223] Both sexes did however develop chronic inflammation and hyperplasia of gastrointestinal epithelium, accompanied by a loss of parietal cells, chief cells, brain calcifications, osteopenia, and salivary glands defects.[223] In vivo

and in vitro studies of siRNA/micro-RNA knockout models of occludin demonstrated increased tight junction permeability in Caco-2 monolayers and in live mice undergoing intestinal perfusion.[224] In occludin depletion, there is a selective increase in macromolecular particles, elucidating the role of occludin in the leak pathway. Occludin deficiency also promotes ethanol-induced distribution of colonic epithelial barrier function in mice.[225]

In transgenic mice, the overexpression of occludin provides protection against the TNF-α induced increase in permeability of the leak pathway.[182] It is suspected that a post-transcriptional modification occurring at the second occludin promoter and transcription start site (exon 1a) enhances the sensitivity of TNF-α signaling.[226] This mechanism may explain how a reduction in occludin expression and colonic inflammation, as observed in UC, are correlated.[226]

1.3.8 Measuring Intestinal Permeability

Impairment of intestinal permeability may occur early in the development of IBD inflammation. Risk factors associated with disease relapse, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and stress, can induce inflammation through increased mucosal permeability. Measuring intestinal permeability is crucial as increased intestinal permeability acts as a risk factor of IBD development for healthy first-degree relatives of patients with CD and can predict the risk of relapse for those with CD. In patients with CD who are asymptomatic and have normal biochemical tests, an increased intestinal permeability further increases the risk of relapse by 8-fold.[227]

Multi-sugar-probe gut permeability tests are most utilized. Of those, the most common method for *in vivo* measurement of small intestinal permeability is the enteral administration of a lactulose and mannitol (L/M) test.[227] A L/M test is used to assess small intestinal permeability by measuring via urinary of excretion rates of lactulose and mannitol after oral administration of these sugars. Mannitol, a monosaccharide, with the molecular weight of 182Da demonstrates passive transcellular permeation and is almost completely absorbed without metabolism through the gut membrane.[227,228] Similar to mannitol, lactulose also is not metabolized in the small intestine and is filtered through kidney in its original form. However, as a disaccharide with a larger molecular weight of 342Da, lactulose is transported paracellularly, therefore, should not be absorbed in individuals with normal intestinal permeability function. It is minimally absorbed in the gastrointestinal tract, with less than 1% of the administered dose being absorbed and

excreted in urine.[229–231] Instead, lactulose reaches the colon intact, where it is primarily metabolized by saccharolytic microbiota in the proximal colon into lactic acid and acetic acid, leading to a reduction in intraluminal pH and blocking the absorption of intestinal ammonia.[232] This property makes it clinically useful in the treatment of hepatic encephalopathy.[232]

After the ingestion of a L/M solution, urine is collected after a 2-hour fasting period for 24 hours. The use of the L/M ratio was proposed to account for potential variations in the surface area of the intestinal mucosa among individuals.[233,234] By dividing the quantity of lactulose excreted by the quantity of mannitol excreted, the measurement is standardized and accounts for any differences in the intestinal permeability of subjects due to variations in the size or surface area of the gut and allows for a more accurate comparison between individuals.[233,234] The L/M ratio from the first six hours of urine collection is used to measure small intestinal permeability. L/M tests are used in clinical practice as they are non-invasive and have high sensitivity for detection of IBD.[227,228]

An important weakness of L/M tests are their inability to differentiate between increased intestinal permeability as the result of barrier dysfunction or the product of active inflammation.[128] Other sugars, such as sucrose, are used to evaluate the upper intestinal tract, reflecting the permeability of the stomach and proximal duodenum. Sucrose has also been shown to be correlated with fecal zonulin and FCP in UC remission.[235] Multisugar tests have been developed, including sucralose and erythritol, which allows for a functional assessment of the entire intestinal tract and expands the potential application to UC.[236]

Other means of measuring intestinal permeability include 51 Cr-EDTA, Ussing Chambers, and confocal laser endomicroscopy. Due to the complexity and invasiveness of 51 Cr-EDTA and Ussing Chambers, these methods are not suitable for use in humans.[44] Confocal laser endomicroscopy, is a novel imaging technique, which uses 1000-fold magnification to detect defects in the mucosal barrier. It can assess the ileocolon for active inflammation, mucosal health, and determine intestinal permeability. When used in the ileum, studies have shown this method to be an accurate way of demonstrating leaky gut in CD and UC.[237] It is also used in the diagnoses of GI tumors, Celiac disease, collagenous colitis, and irritable bowel syndrome.[238,239] With its high magnification capabilities, confocal laser endomicroscopy can detect cellular and subcellular changes, such as cell shedding, making it a powerful tool for imaging mucosal barrier defects in IBD.[151,239] Increased density of mucosal gaps after cell shedding has been observed in

the small bowel of CD patients and in macroscopically normal duodenum in both CD and UC.[239] These alterations may represent subclinical impairments of intestinal permeability that could potentially predict subsequent clinical relapse.[239] Confocal laser endomicroscopy has also been applied in UC patients, demonstrating that the occurrence of crypt architectural abnormalities can predict disease relapse in patients with apparent endoscopic remission.[237]

1.3.9 Inflammation and Tight Junction Protein Expression in UC

Trademark mucosal inflammation in IBD compromises the epithelial barrier; therefore, as tissue of the lamina propria is exposed to pathogenic bacteria and luminal contents, which contributes to an immune inflammatory response and defects in the barrier.[240,241] This barrier damage can be due to the alteration of TJ proteins along with pro-inflammatory factors migrating to the site of inflammation.[197] An alteration in claudin profiles in TJs are associated with disruption of the paracellular movement of fluids and solutes ultimately resulting in epithelial barrier dysfunction.[197] Increased expression of claudin-1, -2, and -18 as well as downregulation of claudin-3, -4, and -7 was reported in UC.[144,203,206,209,215]The increase in barrier vs. channel forming claudins affects ion movements in cells, which can also be reflected in symptoms such as diarrhea.[227,242]

Both CD and UCs share common features such as breaks in the epithelium, a reduction in tight junction strands, and glandular atrophy.[243–245] Patients with active CD often exhibit increased intestinal permeability, which is believed to be caused by epithelial damage, including apoptosis, erosion, and ulceration that occur during gut inflammation.[159,246,247] Inflammatory cytokines associated with gut inflammation can also affect epithelial permeability by altering the function of junctional complexes.[205,248,249]

Interestingly, impaired barrier function is not limited to active IBD but can also be observed in quiescent disease and even in first-degree relatives of CD patients.[138,250] Genetic studies have identified UC susceptibility loci related to defects in the epithelial barrier.[251,252] Studies on ileoanal pouch mucosa in pouchitis and in pouches with backwash ileitis prior to restorative proctocolectomy for UC have shown reduced barrier properties and increased bacterial translocation in pouches that have been functioning for an extended period. [253–255] Dysregulation of the epithelial barrier, including changes in paracellular permeability due to altered cell-to-cell junctions, may be a critical primary factor in the pathogenesis of IBD.[256]

In the context of UC, studies have revealed important findings regarding the epithelial barrier. Gitter et al. observed leaks from apoptotic foci in the sigmoid colon of patients with early UC, even when the epithelium appeared intact.[257] They also found a correlation between the degree of inflammation and increased conductance of the epithelium.[257] Another study measuring epithelial resistance in inflamed sigmoid colon samples from UC patients demonstrated an 80% reduction in epithelial resistance and a decrease in the depth of epithelial tight junctions.[241]

Research on the expression of claudins, a family of proteins involved in tight junctions, in UC patients has provided insights into barrier function. Studies have shown higher expression of claudin 2, which is associated with increased pore formation, in colonic samples from UC patients.[206,215,241]These increases in claudin 2 expression correlate with disease severity. Additionally, reductions in other tight junction proteins involved in tightening the junctions, such as claudin-3, -4, and -7, have been observed in UC patients.[206,215] However, there are conflicting findings regarding the expression of claudin-1 and occludin, with some studies reporting an increase in claudin-1:occludin ratio in UC patients and others showing a decrease in occludin expression.[203] Disease severity, as measured by the degree of inflammation, appears to be related to alterations in tight junction structure in UC.[203]

Table 1. Tight junction protein function and expression in UC adopted from Landy et al[256].

Tight Junction protein	Type of Claudin	Function	Expression in UC	
			Active	Inactive
Claudin-1	Pore-forming	- Improves epithelial tightness[186,258] - formation of TJ strands[259] - regulates trans-epidermal water loss	↑[203,209], ↔[176]	↔[209]
Claudin-2	Pore-forming[195,207]	- Initiates formation of TJ strands[259] - Water and cation selective (sodium) - Decreases barrier functions of claudin -1 and -4[260]	↑↑[176,209,215]	↔[209]
Claudin-3	Barrier forming	- Sealing claudin[194] - modify TJ network[194]	↓[215]	
Claudin-4	Barrier forming	- barrier regulator[127,261] - chloride channel that bars sodium to decrease paracellular conductance[261]	↓[206,215]	
Claudin-7	Barrier forming	- Wnt/β-catenin signaling–dependent intestinal epithelial stem cell survival, self-renewal, and cell differentiation	↓[206]	
Occludin	-	- Regulates paracellular activity[224] - Provides structural integrity[262] - Binds ZO-1[263] - Role in cellular adhesion[184]	↑,[264] ↔[209]	↓,[262,264,265]

Overall, the dysregulation of epithelial barrier function, characterized by changes in tight junction proteins, plays a significant role in the pathogenesis of inflammatory bowel diseases, particularly in UC. Further research is needed to fully understand the mechanisms underlying these alterations and their implications for disease progression and treatment.

1.4 Serum markers of bacterial translocation in IBD

1.4.1 Lipopolysaccharide and Lipopolysaccharide binding protein

Lipopolysaccharide (LPS) is an endotoxin derived from the outer membrane of Gram-negative bacteria. [266] Due to its nature, the gut is a major reservoir of this endotoxin, which also possesses the ability to act as a virulence factor. Based on the organism, LPS may have varied chemical composition, biological activity, and potency.[266] LPS has three major domains: the lipid A (an endotoxin) backbone, the core phosphorylated oligosaccharide, and the repeating oligosaccharides side chains.[267] LPS is often bound to LPS-binding protein (LBP), a host acute phase protein.[267–269] LPS-binding protein binds to CD14, and lipid A is recognized by host pattern recognition receptor Toll-like receptor 4 (TLR4). The TLR4-MD2 complex leads to the stimulation of nuclear factor-kappa B (NF-κB) by the MyD88 pathway ultimately resulting in the transcription of proinflammatory cytokines such as TNF chemokines, and major histocompatibility complex (MHC) receptors.[266,267] This inflammatory immune response is partially responsible for metabolic endotoxemia but is also critical to host recovery. For example, mice deficient in TLR4 are not able to regulate Gram-negative infection.[268] Furthermore, in humans TLR4 polymorphisms are associated with more severe Gram-negative infection.[269] Due to the role of LPS as a major inducer of inflammatory immune response, the connection between LPS and metabolic disease has been an area of interest.

Expansion of Gram-negative bacteria in the gut microbiota results in an increase in plasma LPS levels.[39] Disruptions in the microbiota can be due to heritability factors, diet, or environmental factors. High intestinal permeability is associated with an increase in Gram-negative bacteria (such as *Enterobacteriales*).[270] Meanwhile, there is also a negative correlation between plasma LPS and *Bifidobacterium* spp. While an increase in LPS causes an increase in endotoxemia, an increase in bifidobacteria due to prebiotic intake is related to a reduction in endotoxemia.[271] Bifidobacteria can reduce plasma endotoxin by improving barrier function thus inhibiting the translocation of bacteria and toxins. On the other hand, an increase in Gram-negative bacteria decreases gut barrier integrity and mucosal function to lead to an increase in plasma LPS levels.[267,272] UC patients with severe inflammation active disease demonstrated high LPS biosynthesis which is significantly lower in UC remission patients. [93] Overall, this data suggests that LPS and LPS-binding protein act as valid markers of intestinal permeability.

1.4.2 Lipoteichoic acid

Studies have reported that the presence of Gram-negative bacteria such as Bacteroides/Prevotella and Enterobacteriaceae in the colon is a qualifying risk factor for developing IBD.[273–275] Additionally, Gram-positive bacteria have also been shown to contribute to the development of colitis.[276] In a study by Nakanishi et al., they demonstrated that vancomycin-sensitive Gram-positive bacteria belonging to the Lachnospiraceace family (Lachnospiraceace bacterium *A4* and *Butyrivibrio firisolvens*) triggered colitis in C57BL/6 mice induced with DSS via monocyte/macrophage mobilization during disruption of colonic epithelial cells.[276]

Lipoteichoic acid (LTA) is a cell wall polymer containing alditol phosphate present in low G + C subdivision Gram-positive bacteria belonging to Firmicutes, such as *Bacillus subtilis*, *Staphylococcus aureus*, and *Listeria monocytogenes*.[277] LTA is shed during bacterial replication and antibiotic administration.[278] LTA binds to CD14 and Toll-like receptor 2 (TLR2), leading to the initiation of innate immune responses and further development of adaptive immunity.[277,279] Bound LTA can interact with antibodies and the complement cascade to induce a passive immune kill phenomenon.[277] TLR-2 has been shown to trigger the production of interleukin (IL)-6, IL-8, macrophage inflammatory protein (MIP)-1, MIP-2, and activate NF-kB.[279] It also triggers the release of neutrophils and macrophages of reactive oxygen and nitrogen species, acid hydrolases, highly cationic proteinases, bactericidal cationic peptides, growth factors, and cytotoxic cytokines, which may synergistically contribute to further cell damage.[277]

LTA occurs in varied chemical structures, leading to the classification of 5 types. Type I LTA has a simple unbranched polygylcerolphosphate backbone structure, while Type II-V LTA presents more complex structures. LTA in bacterial pathogens has the capacity to stimulate the pathways for the secretion of pro-inflammatory cytokines such as TNF-α, interleukin-1β,[278,280], IL-6, IL-8, [278,281,282] and nitric oxide.[280] Thus, LTA is considered the functional equivalent of the endotoxin LPS in Gram-positive bacteria, and shares many of its pathogenetic properties.[283] The main difference in the function of LPS and LTA is that LPS nanomolar range while LTA has been shown to present in the micromolar range.

The immune effects of LTA vary depending on the specific bacterial origin from which it is isolated. In male C57BL/6 mice with dextran sulfate sodium (DSS)-induced colitis, an abundance of Gram-positive bacteria was present at the site of injury and was associated with

loss of epithelial integrity in the colon.[284] The infiltration of Gram-positive bacteria in the mucosa of these DSS-induced experimental colitis mice, suggested that they might accelerate the occurrence of experimental colitis.[284] The results further suggested that Gram-positive bacteria induced intestinal inflammation through the muramyl dipeptide (MDP)-nucleotide-binding oligomerization domain-containing protein-2 (NOD2) pathway signaling pathway.[284] In another study with DSS-induced colitis mouse-model, commensal Gram-positive bacteria were shown to trigger the mobilization of inflammatory monocytes and macrophages into the colon.[285]

1.4.3 Pathological Changes in Ulcerative Colitis

There are several pathological changes that occur in UC such as gut microbiota dysbiosis, destruction of the intestinal epithelial barrier, a decrease in mucus secretion and SCFAs, and an increase in inflammatory factors set the stage for a compromised intestinal epithelial barrier. Most notably, a alterations in TJP such as ZO-1, occludin and claudins increase intestinal permeability and impair mucosal tissue in the intestine.[225,286] Furthermore, a change in the microbiota composition lead to an increase in flagellin and LPS which cumulatively promote pro-inflammatory factors.[282] As the barrier is compromised, it becomes easier for bacteria to invade the dense mucus layer, thus allowing harmful bacteria the opportunity to stimulate pro-inflammatory cytokines and recruit immune cells. These immune cells include dendritic cells, macrophages, Th cells, regulatory T cells, natural killer T cells which interact to stimulate an adaptive immune response.[287] For example, LPS is capable of inducing M1-type polarization in macrophages which causes the secretion of pro-inflammatory cytokines into the lamina propria.[282,283] Pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-9, IL-13, and IL-33 play a critical role in UC progression while anti-inflammatory cytokines such and TNF-β, IL-10, and IL-37 play an attenuating role in inflammation.[179]

1.4.4 Methods of Modulating Intestinal Permeability

Therapeutic agents commonly used in the management of IBD can induce and maintain mucosal remission not only through their immunomodulating effects but also via restoration of epithelial integrity and permeability. This has been demonstrated, for example, with anti-TNF-α drugs in CD.[8,108] Similarly, elemental diets have shown similar effects in CD, leading to

increased interest in dietary approaches incorporating immunomodulatory nutrients and probiotics.

The Western diet, characterized by its high fat and refined sugar content, is considered a risk factor for IBD development. It is believed to induce low-grade inflammation through gut dysbiosis and increased intestinal permeability.[288,289] Additionally, there is growing concern about the role of industrial food additives, such as emulsifiers, in promoting immune-related diseases.[290] Some additives have been shown to increase intestinal permeability by interfering with tight junctions, facilitating the passage of immunogenic antigens.[290] Conversely, certain fatty acids (such as propionate, acetate, butyrate, n-3 PUFA, and conjugated linoleic acid), amino acids (such as glutamine, arginine, tryptophan, and citrulline), and essential trace elements have demonstrated anti-inflammatory properties and the ability to restore mucosal permeability in experimental models of gut diseases.[291–294] However, their therapeutic efficacy, particularly in IBD, remains a subject of debate. Among them, butyrate, zinc, and probiotics have shown the strongest evidence in this regard.

Butyrate is a SCFA, and the main source of colonocyte energy,[35] produced during the dietary fibers fermentation by intestinal microbes such as *Ruminococcus spp., Eubacterium spp.,* and *Coprococcus spp.* These microbes have been found to stimulate mucus production and the expression of tight junctions *in vitro*. Furthermore, patients with diversion colitis exhibit low butyrate levels which is correlated to mild inflammation and a reduction in butyrate-producing bacteria.[295] Topical butyrate has shown efficacy in refractory distal UC.[296] Other fatty acids with similar properties, such as n-3 PUFA and phosphatidylcholine, have also been proposed as adjunctive therapies in IBD, although their use in clinical practice is still limited.

Zinc, an essential trace element for cell turnover and repair systems, is often deficient in inflammatory conditions and malnutrition.[297] Supplementation with zinc has been shown to restore intestinal permeability in CD patients, likely through its ability to modulate tight junctions in the small and large intestines[183,297].

Probiotics are live microorganisms that offer health benefits when consumed in sufficient amounts. They support gut health by balancing the intestinal microbiota and can be found in fermented foods, supplements, and dairy products.[294] The rationale for using probiotics in IBD lies in the dysbiosis characteristic of the disease. Numerous trials have investigated the efficacy of different species of probiotics in IBD, with conflicting results. Currently, the ones with

possible efficacy are *Escherichia coli* Nissle 1917*, Bifidobacterium, Lactobacillus rhamnosus GG*, and the multispecies VSL#3, which contains eight different probiotic strains.[298–301]However, their use is primarily limited to maintaining remission in UC, rather than treating active disease. The mechanisms of their effect in UC are not yet fully understood but may involve direct anti-inflammatory effects, strengthening of the mucosal barrier, and upregulation of tight junction proteins.[302] Probiotics have also shown beneficial effects in pouchitis by enhancing mucosal barrier function.[298] Another potential mechanism of action is the restoration of butyrate-producing bacteria, as IBD patients often have reduced bacterial species like *F. prausnitzii*.[74,96]

1.5 Prebiotics

1.5.1 Prebiotics in Ulcerative colitis

Apart from conventional medication, methods to reduce inflammation through modulation of the gut microbiome and reparation of the epithelial barrier have been of great interest. Prebiotics have been investigated for their role of conferring a benefit to the host through their fermentation by selective commensal microbes in the gut resulting in compositional and metabolic microbiota modulations/alterations.[302–305] Prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit [306]". These health benefits are not necessarily limited to the colon, but may also occur in the oral cavity, urogenital tract, lungs, and skin.[307] Typically, prebiotics were thought to be limited to non-digestible carbohydrate sources, such as fructo-oligosaccharides, galacto-oligosaccharides, resistant starches, pectin, arabinoxylan, and whole-grains, but an updated definition now includes non-carbohydrate sources, such as polyphenols and certain lipids [307].

Prebiotics confer benefit to the host through their fermentation by some commensal microbes in the gut resulting in compositional and metabolic modulations/alterations[302–304,308]. Prebiotics are a non-selective growth substrate, allowing the simultaneous growth of multiple beneficial strains, such as *F. prausnitzii*, *Roseburia* spp., *Eubacterium* spp., *Anaerostipes* spp., *Coprococcus* spp., *Bifidobacterium* spp. [271] Furthermore, prebiotics are synergistically co-metabolized by several distinct microbial groups such as butyrate-producing *F. prausnitzii* and acetate-producing *B. adolescentias* leading to more efficient co-fermentation [49]. However, it is important to note that not all non-digestible carbohydrates can be considered prebiotics. For

example, feeding IL-10 -/- knockout mice with dextrin fibers derived from corn resulted in microbiota shifts such as an increase of some Bacteroidetes families (*Porphyromonadaceae* and *Prevotellacea*) versus reduction of strict anaerobic Firmicutes (*Incertae Sedis XIV, Lachnospiraceae*, *Ruminococcaceae,* and *Lactobacillaceae*). These changes were also seen in conjunction with reduced pro-inflammatory pathways such as IL-12, IL-6, and chemokine ligand1 (CXCL1).[309] Despite these differences, these mice did not receive attenuation in colonic inflammation—or in other words, no health benefits to the host [309].

Arguably, a major function of prebiotics is their fermentation by commensal microbes into SCFAs. SCFAs are carboxylic acids that consist of two to six carbon atoms. Among SCFAs, acetate (C_2), propionate (C_3), and butyrate (C_4) are the most abundant.[174–176] SCFAs enhance mucus secretion, increase anti-microbial peptides, lower the pH of the colon to decrease oxygen concentration, and inhibit the growth of pathogenic anaerobes.[36,310,311] SCFAs also upregulate the expression of TJPs to maintain a healthy functional immune system and intestinal barrier[310,312] and reduce the production of putrefactive substances, such as ammonia, indole, branch-chain fatty acids, and phenol.[310] Butyrate acts as the main energy source for colonocytes. This SCFA is of particular interest in IBD, as it is significantly reduced in colonic cells leading to autophagy and energy deprivation.[35] Butyrate inhibits NF-κB activation via an increase in cytoplasmic nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) thus inhibiting pro-inflammatory cytokines and chemokines, such as interferon-γ (INF-γ), pro-inflammatory chemokine CXCL-8 (IL-8) in Caco-2 cells, and TNF-α.[311,313–315] A decrease in butyrate and other SCFAs, as seen in diversion colitis, causes mild inflammation.[295] Furthermore, butyrate has been shown to reverse inflammation-induced increase of claudin-1 proteins in vitro. [316] Butyrate was able to reduce claudin-2 expression in colonic epithelial cells,[317] proposing its role in barrier function via TJP regulation.[256]

Fructo-oligosaccharides (FOS) are formed by the polymerization of fructose units. They are not absorbed by the small intestine, but rather degraded in the colon by gut microbiota to primarily support the growth of *Lactobacillus* and *Bifidobacterium*. FOS has also been shown to improve symptoms of trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats via LAB stimulation and a reduction in myeloperoxidase (MPO) activity in mice. Additionally, FOS was effective at reducing the secretion of inflammatory cytokines such as IFN-γ, IL-17, and TNF-α.[318,319] Capitán-Cañadas et al. demonstrated that FOS increased expression of occludin and

reduced the IEC immune cells inflammation.[318] In a study by Liao et al, FOS and a synbiotics significantly improved expression of MUC2 and epithelial TJP such as ZO-1, occludin, and claudin-1.[320] Liao et al., also confirmed the roles of FOS and symbiotic protein in improving DSS-induced colitis and saw a reduction in expression of IL-6, TNF-α, and increased expression of TBOX-21.[320] T-BOX21 plays a role in TNF-α[275,321] and production in pro-inflammatory responses in the mucosal barrier. This study further saw an increased expression of IL-10, partially improved dysbiosis, and increased the abundance of anti-inflammatory bacteria such as *Faecalibacterium* and *Bifidobacterium*, while decreasing the abundance of pro-inflammatory microbes such as *Mucispirillum*.[320]

Table 2. Human clinical trials in UC treated with prebiotics and their effects on inflammatory biomarkers from oldest to most recent

Author	Design	Disease severity	n	Prebiotic	Length	Outcome
Welters et al.[322]	Crossover	Mild	20	24g/d Inulin	3 weeks, 4-week washout	- Significant reduction in endoscopic and histological inflammatory markers, increase fecal butyrate, decrease in fecal Bacteroides
Furrie et al[323]	RCT	Mild	18	oligofructose-enriched inulin at 12 g per day, and Bifidobacterium longum at 200 billion colony forming units per day	4 weeks	- Significant reduction in mucosal expression of proinflammatory cytokines (TNF-a, IL-1β), β -defensin - Decrease in inflammatory cells infiltrate and crypt cell abscess
Casellas et al.[324]	RCT	Mild-moderate	19	oligofructose-enriched inulin (12 g/day)	2 weeks	- Significant reduction in fecal calprotectin
Valcheva et al.[325]	RCT	Mild-moderate	25	7.5 g or 15 g oligofructose-enriched inulin	9 weeks	- increased *Bifidobacteriaceae* and *Lachnospiraceae*
Wilson et al. [326]	Open-label	Active UC	17	2.8 g/d Galactooligosaccharide	6 weeks	- *Bifidobacterium* and *Christensenellaceae* proportions increased in patients with milder disease
Valcheva et al. [327]	RCT	Clinical Remission	76	15g/d of either β-fructans (oligofructose and inulin; Synergy1/Prebiotin)	6 months	- Significant reduction in FCP increase during relapse

The most extensively tested prebiotic in human IBD is inulin-type β-fructans, which are FOS of different lengths. Inulin-type β–fructans are a class of prebiotics with a β-(2 → 1) linked fructose oligo- and polysaccharides.[328] Also known as inulin, and oligofructose/fructo-oligosaccharide, sources such as chicory root and agave contain a high amount of this prebiotic. A variety of commensal bacteria such as *Bifidobacteria, Lactobacillus, Streptococcus, Flavobacterium* possess the ability to ferment β-fructans.[328,329] In a study by Furrie *et al.,* one month of oral therapy with *Bifidobacterium longum* in combination with prebiotic inulin and FOS in active UC patients demonstrated a significant improvement in colonic inflammation.[330] In another study, concentrations of FCP were reduced in active UC patients who received inulin and FOS.[331] Finally, a pilot study by Valcheva et al. showed that the effect of inulin-type β-fructans on inflammation and colonic microbiome in active UC was dose-dependent with significant clinical efficacy at 15 g daily dose.[325] β-fructans increased fecal *Bifidobacteriaceae* and *Lachnospiraceae* abundance but these shifts were not correlated with improved disease scores.[325] In contrast, a significant increase in colonic butyrate production by β-fructans was further related to reduction in symptomatic disease activity, suggesting that prebiotic-induced alterations of gut microbiota metabolism are more important than compositional changes for the benefits in UC.[325]

Generally, FOS has been the most studied and utilized prebiotic, which lends to its recognition among government, institutions, food and natural products, medicine, cosmetics, and even animal feed. It has been shown to improve intestinal dysbiosis, SCFA production, and plays a role in the activation of immune responses.[318,320,321] In an animal study by Koleva et al, colitis was significantly reduced in HLA-B27 rats who were fed FOS through a reduction in chronic inflammation.[332] However, rats fed inulin only decrease inflammation in half of the animals.[332] Inulin was able to increase the total number of gut bacteria, Bacteroides-Prevotella-Polyphyromonas group, Bifidobacteria, and reduced Clostridium cluster IV.[332] In fecal samples, FOS independently increased Bifidobacterium spp., and mediated a decrease in Enterobacteriaceace and *C. difficile toxin B* in feces.[332] In a study by Valcheva et al. in patients with mild-moderate UC, ingestion of high-dose of inulin-type β-fructans significantly increased colonic butyrate production, which was negatively associated with Mayo score.[325] Furthermore, there was an increase in abundance of Bifidobacteriaceae and Lachnospiraceae.[325] This study theorized that only functional alteration (rather than structural) is required in gut microbiota to

display benefit in UC.[325] In a recent study by Armstrong et al, the differential role of prebiotic fiber was explored in healthy and disease individuals. In healthy individuals with a balanced gut microbiota and intact gut barrier, fermentation of certain fibers was found to enhance the barrier and reduce inflammation.[333] However, in conditions like IBD, where gut microbiota function is impaired, unfermented dietary fibers may promote inflammation due to direct effects or altered production of SCFA.[333] Further research is needed to determine if patients with IBD should avoid certain fibers when their gut microbiota is imbalanced, and fibers could be considered as additional therapy only after remission is achieved.[333]

Recent studies in animal models and in healthy volunteers have reported that β-fructans can improve intestinal barrier function, improved glucose metabolism and weight loss in metabolic syndrome. [325,334–336] However, in the context of UC it is unknown if the same prebiotic carbohydrates have a role in enhancing barrier integrity through a reduction in intestinal permeability, especially in remission.

1.6 Conclusion and Rationale

IBD refers to a group of chronic disorders characterized by inflammation of the gastrointestinal tract, which includes UC and CD. The etiology of IBD is not fully known, however; the combination of microbial dysbiosis and increased intestinal permeability is evident in many patients. Prebiotics have emerged as potential therapeutic agents in the management for increased intestinal permeability associated with UC. By selectively promoting the growth and function of beneficial gut bacteria, prebiotics can modulate the gut microbiota composition and promote a healthy microbial community. This, in turn, contributes to the restoration of the intestinal barrier function and/or the reduction of inflammation. Prebiotics have shown promising effects in improving mucosal barrier integrity, enhancing the production of short-chain fatty acids, and regulating immune responses in animal models for colitis. However, further research is needed to elucidate the specific mechanisms underlying the beneficial effects of prebiotics in UC and to determine the optimal dosage, duration, and specific types of prebiotics that can provide the greatest therapeutic benefits. Nonetheless, the evidence suggests that prebiotics hold great potential as a complementary, adjunct approach in the management of UC and the restoration of intestinal permeability.

1.7 Overview of Master's Work

The UC-Synergy trial,[327] a recent randomized-control trial, aimed to examine the safety, tolerability, and efficacy, and to define mechanisms underlying the activity of inulin-type β-fructans for use in prevention of UC relapse in patients in clinical remission with a documented relapse in the preceding 18 months. The result of this study showed that the consumption of β -fructans for 6 months by UC patients in remission was ineffective at improving clinical relapse rate or time to relapse but did reduce the overall severity of relapse by preventing subclinical inflammation and significantly reducing the fecal-calprotectin increase during symptomatic relapse.[327] **However, it is unknown whether reduction of severity of biochemical relapse mediated by β-fructans in UC patients is associated with improved barrier function and reduced intestinal permeability.**

1.7.1 Central Hypothesis and Aims

Background and Supporting Data: A recent randomized-control trial at the University of Alberta, initiated and led by Dr. L. Dieleman, aimed to assess the safety, tolerability, and efficacy of inulin-type β-fructans for relapse prevention in UC patients.[327] This study also explored possible mechanisms of prebiotics activity including microbiota shifts in composition and activity and host interactions (colonic transcriptomics and mucosal and peripheral cytokine secretion). Following the screening process, only UC patients with a documented relapse in the preceding 18 months, who at the time of screening presented with symptomatic and endoscopic inactive disease (total Mayo score ≤ 2, and endoscopic score of 0 or 1) were included. Clinical relapse was defined as total Mayo score ≥ 3; biochemical relapse was defined as endpoint Fecal calprotectin (FCP) ≥ 250 μg/mg, or an increase of ≥100 μg/mg from baseline in patients with already high FCP concentrations. The results from this study showed that the oral intake of β-fructans for 6 months by UC patients in clinical remission was ineffective at preventing clinical relapse (primary outcome) but did reduce the overall severity of relapse by preventing subclinical inflammation (secondary outcome). In addition, those patients randomized to β-fructans who relapsed had only a 3-fold median increase in FCP as opposed to the 17-fold median increase in FCP of the placebo arm relapsing patients ($p = 0.038$).[327]

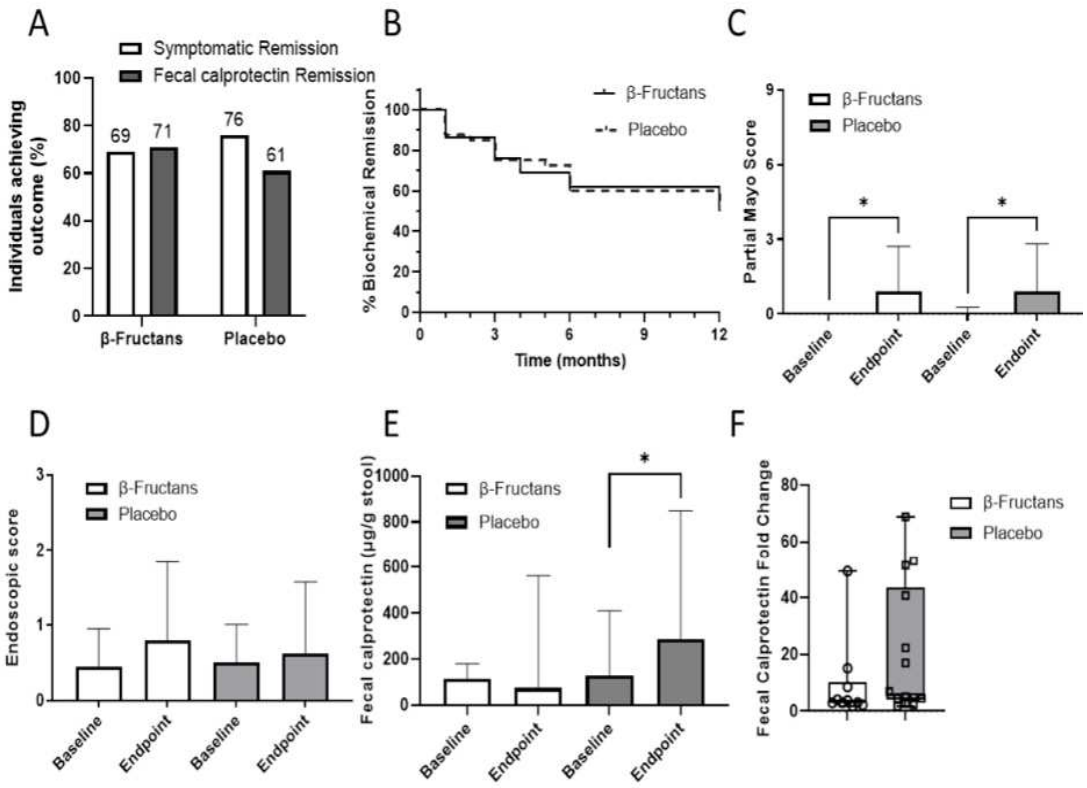

Figure 1. Clinical outcomes as measured in participants at baseline and endpoint from Valcheva et al.[327]

Microbiota analysis demonstrated that β-fructans intake was associated with significant changes in fecal microbiota α- and β-diversity. Fecal microbiota of UC patients on β-fructans was enriched with strict anaerobes involved in the prebiotic fermentation such as *Bifidobacterium longum, Coprococcus* spp., and *Faecalibacterium prausnitzii*. Meanwhile microbes associated with inflammation, such as *Mediterraneibacter* (formal *Ruminococcus gnavus* group), *Turicibacter* spp., and *Bilophila* spp. were significantly reduced following β-fructans intake. It was also confirmed that the prebiotics consumption induced significant increase in fecal butyrate and a decrease in valerate concentrations (Figure 2).

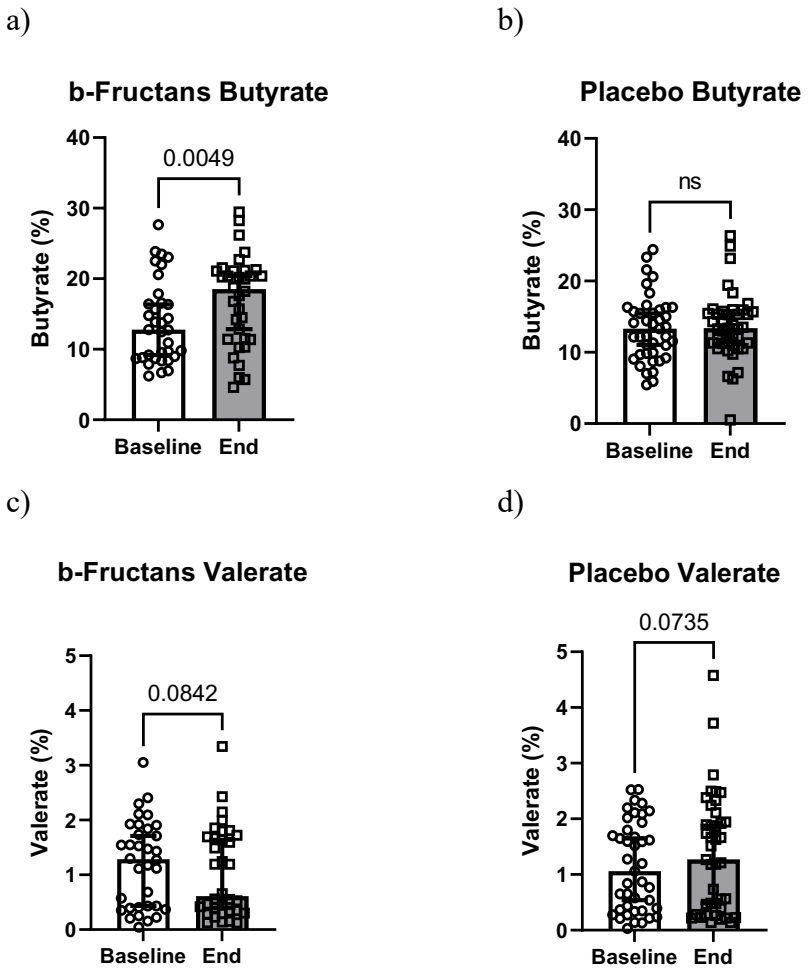

Figure 2. Changes in fecal short-chain fatty acids relative concentrations following a 6-month intervention with inulin-type β-fructans in UC patients with clinical remission.

Clinical data from our trial in UC patients suggest that β-fructans can be beneficial for UC patients in re-modeling the fecal microbiota composition and metabolic activity to reduce the severity of biochemical relapse.

Hypothesis: Based on the clinical findings, we hypothesize that β-fructans supplementation may enhance barrier integrity in UC patients with inactive disease.

Specific Aims: To test this hypothesis, we propose to:

a) Determine if β-fructans intake was associated with changes in epithelial tight junction proteins mRNA gene expression including *claudin-2* and *occludin*.

b) Measure serum concentrations of pathogen-associated patterns (PAMPs) such as LPS, LBP (for Gram-negative bacteria) and LTA (for Gram-positive bacteria) to assess changes in gut permeability.
c) Determine if intestinal permeability measurements were associated with inflammation (clinical relapse and fecal calprotectin) and/or microbial metabolites of prebiotics fermentation (fecal SCFAs).

CHAPTER II: THE EFFECT OF β-FRUCTANS ON MARKERS FOR INTESTINAL PERMEABILITY IN PATIENTS WITH ULCERATIVE COLITIS

2.1 Introduction

UC is a sub-type of IBD caused by a complex interplay of genetic, environmental, and microbial factors. Disruption of the microbiome along with the intestinal epithelial barrier allows the interaction of microorganisms with immune cells, causing a cascade of chronic inflammation in the colon. Inulin-type β-fructans are non-digestible carbohydrates that beneficially alter activity of gut microbiota and thus are classified as prebiotics.[13] β-Fructans, were also shown to reduce intestinal inflammation in rodent colitis models.[14] Their efficacy in managing inflammation in IBD, however, is not as well documented as the effect of probiotics and it remains disputed whether their activity relates to the stimulation of specific members of the intestinal microbiota, or metabolic alterations such as an increased production of short chain fatty acids (SCFAs).[12,13,15]

Intestinal permeability refers to functional property of the intestinal mucosal barrier that controls the interactions between the gut and gut microbes. An impaired intestinal barrier and increased intestinal permeability, also known as "leaky gut," has been the focus of research as it appears to be one of the defining factors in the pathogenesis of IBD.[44,129] Disruption of intestinal barrier turnover contributes to invasion of luminal antigens and subsequent chronic intestinal inflammation as seen in UC and CD.[176,177] IECs are mechanically attached by the junctional complexes TJs, adherence junctions, and desmosomes.[109,169,170] These structures also control paracellular transport of ions and small molecules between adjacent cells via passive transport. TJ proteins serve to protect against the external environment while also preventing the loss of solutes and water into the lumen. Patients with IBD display several TJ abnormalities, such as reduced expression and redistribution of TJs and their constituents such as occludin, claudins, and JAM, [174–177] leading to increased paracellular transport.[126,337] The increase in paracellular transport results in increased permeability to large molecules such as pathogens that induce an immune response which can both independently cause and perpetuate existing inflammation.

Occludin was the first tight junction transmembrane protein to be identified in IECs. As a major transmembrane protein of TJs, occludin exists as a tetraspan protein with two extracellular loops, and NH2- and COOH-terminal cytoplasmic domains. Occludin plays a role in paracellular activity, cellular adhesion, provides structural integrity, and binds to ZO-1. Occludin expression has been differentially expressed based on IBD state, severity, and activity. For example, occludin downregulation has been observed during remission while being increased in active UC.[264] Occludin reduction in patients with IBD has been proposed to act as a protective mechanism since it down regulates capase-3 which in turn leads to less apoptosis and attenuate colitis.[338]

Claudins are another type of TJ proteins known as "gate keepers" and characterized as channel-forming and barrier-forming. Claudin-2 is a charge-selective claudin for small cations and water that is expressed in low amounts in the crypts of the human colon. Proinflammatory cytokine IL-13, which is upregulated in UC, is shown to upregulate claudin-2 expression, increase epithelial restitution rate in UC which in turn increases intestinal permeability.

PAMPs produced by multiple bacterial species are recognized by pathogen recognition receptors (PRRs) and induce host cell innate immune responses. LTA and LPS, represent two major PAMPs molecules produced by Gram-positive and Gram-negative bacterial species, respectively. Both LTA and LPS can interact with many host factors or regulate intracellular signaling pathways to induce host immune response, therefore contributing to bacterial pathogenesis. Lipopolysaccharide (LPS) is typically present at levels that are not enough to activate macrophages unless it is accompanied by the accessory molecule called LPS-binding protein (LBP). Therefore, LBP serves as a valuable biomarker indicating the activation of innate immune responses to microbial products. Due to the role of LPS as a major inducer of inflammatory immune response, the connection between LPS and metabolic disease has been an area of interest. Expansion of Gram-negative bacteria in the gut microbiome results in an increase in plasma LPS levels, therefore, an increase in endotoxin production. [39]

LTA is a cell wall polymer present in Gram-positive bacteria.[277] Bound LTA can interact with antibodies and the complement cascade to induce a passive immune kill phenomenon.[277] TLR-2 has been shown to trigger the production of IL-6, IL-8, MIP-1, MIP-2, and activate NF-kB.[279] It also triggers the release of neutrophils and macrophages of reactive oxygen and nitrogen species, acid hydrolases, highly cationic proteinases, bactericidal cationic peptides, growth

factors, and cytotoxic cytokines, which may synergistically contribute to further cell damage.[277] Thus, LTA is considered the functional equivalent of the endotoxin lipopolysaccharide in Gram-positive bacteria, and shares many of its pathogenic properties.[283] In male C57BL/6 mice with DSS-induced colitis, an abundance of Gram-positive bacteria was present at the site of injury and was associated with loss of epithelial integrity in the colon.[284] The infiltration of Gram-positive bacteria in the mucosa of these DSS-induced experimental colitis mice, suggested that they might accelerate the occurrence of experimental colitis.[284]

Recent studies in animal models and in healthy volunteers have reported that β-fructans can improve intestinal barrier function, glucose metabolism and weight loss in metabolic syndrome.[325,334–336] However, in the context of UC it is unknown if the same prebiotic carbohydrates have a role in enhancing barrier integrity. Prebiotics are "a substrate that is selectively utilized by host microorganisms conferring a health benefit."[306] Depending on the structure, saccharide units, degree of polymerization, and linkages, different communities of bacteria can be stimulated.[339,340] Scott et al.[341] highlighted how different prebiotics exhibit varying levels of selectivity. For example, long-chain inulin was shown to be utilized by *Roseburia inulinivorans*, as opposed to *Bifidobacterium* spp.[341] Furthermore xylo-oligosaccharides (XOS) exhibits more selectivity as a growth substrate compared to fructo-oligosaccharides (FOS).[341]

Prebiotics are fermented into lactate and SCFAs (acetate, propionate, and butyrate). SCFAs enhance mucus secretion, increase anti-microbial peptides, lower the pH of the colon to decrease oxygen levels and inhibit the growth of pathogenic anaerobes.[312] These products of fermentation also modulate the expression of tight junction proteins to maintain a healthy functional immune system and intestinal barrier[310,312] as well as reduce production of putrefactive substance such as ammonia, indole, branch chain fatty acids, and phenol.[310] Butyrate is of particular interest in IBD, as it is significantly reduced in colonic cells leading to autophagy and energy deprivation.[35] Besides being the main energy source for colonocytes, butyrate also inhibits pro-inflammatory cytokines and chemokines, such as interferon-γ (INF-γ), pro-inflammatory chemokine CXCL-8 (IL-8) in Caco-2 cells, and TNF-α.[311,313–315]

Inulin-type β–fructans are a class of prebiotics with a β-(2 → 1) linked fructose oligo- and polysaccharides. Also known as inulin, and oligofructose/fructo-oligosaccharide, sources such as chicory root and agave contain a high amount of this prebiotic. A variety of commensal bacteria such as *Bifidobacterium, Lactobacillus, Streptococcus, Flavobacterium* possess the ability to

ferment β-fructans.[328,329] In a study by Furrie *et al.,* one month of oral therapy with *Bifidobacterium longum* in combination with prebiotic inulin and FOS in active UC patients demonstrated a significant improvement in colonic inflammation.[330] In another study, concentrations of FCP were reduced in active UC patients who received inulin and FOS.[331] Clinical data from our trial in UC patients suggest that β-fructans significantly reduced the severity of biochemical relapse. However, it is unknown whether these protective effects were related to a reduction in intestinal permeability. Therefore, we hypothesized that part of the protective effects of β-fructans could be mediated by enhancing intestinal barrier integrity.

2.2 Methodology

2.2.1 Study design

The proposed sub-study was designed to investigate the effect of β-fructans on indirect markers of intestinal permeability using samples obtained in the framework of clinical trial Study Pro00041938. Colonic biopsy samples and serum collected at baseline and endpoint from patients included in the Intention-to-Treat safety population were assessed. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was used to quantify tight junction proteins gene expression in colonic tissue mRNA. Enzyme-linked Immunosorbent Assay was performed on serum samples to determine changes in components of Gram-negative and Gram-positive bacteria. Finally, a statistical analysis was performed to determine the correlation between the aforementioned factors along with fecal calprotectin and fecal SCFA concentrations using gas chromatography measured in the primary analysis.

2.2.2 Study Pro00041938 clinical design

The clinical study design is described elsewhere.[327] Briefly, a total of 76 subjects of females and males between the ages of 18-65 years with clinically confirmed endoscopic remission (Mayo Clinical Score ≤ 2/12-point Mayo scale at baseline, endoscopic score 0 or 1) and recent clinical relapse within 18 months preceding the study were randomized to the β-fructans or placebo arm. Out of the 76 subjects, 35 were in the β-fructans group and 41 in the placebo group. Fecal samples, serum and plasma were collected at baseline, month 1, month 3 and at endpoint (Month 6 or relapse). Colonic biopsies (20-25 cm from the anal verge) were

collected at screening and endpoint (month 6 or relapse). All subjects who completed at least one follow-up study visit over the span of 6 months were included in the intention-to-treat analysis. The study protocol was approved by Health Research Ethics Board (Study ID Pro00041938) at the University of Alberta and Natural Health Directorate at Health Canada. The study is publicly accessible at the U.S. National Institute of Health database (clinicaltrials.gov identification number NCT02865707).

2.2.3 Participant characteristics

Inclusion criteria were treatment on stable doses of 5-aminosalicylic acid (5-ASA) for at least 2 weeks, azathioprine and/or biologics (infliximab, vedolizumab) for at least 2 months prior to screening, or no other medication for at least 1 week prior to the start of the trial, and negative tests for *Clostridoides difficile* toxin, stool pathogens, and pregnancy.

Exclusion criteria included use of oral or rectal steroids 4 weeks prior to the screening, topical 5-ASA, use of methotrexate or 6-mercaptopurine, use of antibiotics within 2 months, and use of anti-diarrheal agents within 3 days of the screening visit. Patients with significant chronic disorders (severe cardiac disease, renal failure, severe pulmonary disease, or severe psychiatric disorder), any active infection, or pregnancy were also excluded.

2.2.4 Sample size determination and power

In a previous pilot study in the Dieleman Lab involving patients with active UC, who were receiving stable 5-ASA treatment, the supplementation of β-fructans (Synergy1®) for a duration of 9 weeks showed a 77% positive response in the group receiving a daily dose of 15 g compared to a 33% response in the group receiving a lower dose of 7.5 g.[325] Based on the favorable outcome observed in that study, a dose of 15 g/day was used in the current study. The researchers anticipated a 30% difference in the incidence of UC patients experiencing clinical relapse within 6 months. To achieve a statistically significant result, a total of 84 patients (42 in each arm) was required.[327] This sample size would allow researchers to detect a difference of 30% with a power of 80% using a two-sided test at a significance level of $p=0.05$[327]. Considering an anticipated dropout rate of 20%, an overall sample size of 100 patients was proposed for this trial.[327]

2.2.5 Human LBP ELISA

Four Human LBP ELISA kits (Invitrogen, Thermo Fisher Scientific; Catalog number: EH297RB) were used for this study. The wash buffer was diluted 20x created using 20 mL of the provided Wash Buffer Concentrate in 380 mL of Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water. Then, the Assay Diluent was prepared in a 5-fold dilution with 15 mL of the Assay Diluent in 60 mL of Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water. The biotin conjugate was then prepared in two 50 mL centrifuge tubes. Samples were then diluted 1000-fold by diluting 0.5 uL serum in 49.95 uL of Assay diluent if tested in singlet, or 1 uL in 99 uL of Assay Diluent if performed in duplicate. The standards were then diluted 2.5-fold by adding 400 uL of diluted Assay Diluent to prepare the 200 ng/mL standard solution and mixed gently. 270 uL of diluted Assay diluent was pipetted into each tube and the reconstituted standard was used to create a 2.5-fold dilution series with the 8^{th} tube being blank. Finally, the Streptavidin solution was diluted 500-fold with the diluted Assay Diluent, where 30 uL of Streptavidin- HRP was diluted in 15 mL of Assay Diluent. The plate was then read using. The exact assay protocol was performed as described in Human LBP ELISA kit Product Information Sheet. Molecular Devices SpectraMax M3 plate reader was used to read the absorbance at 450 nm. SoftMax Pro version 5.4 was used to generate the standard curve using the recommended four parameter algorithm.

2.2.6 Human LPS ELISA

A competitive inhibition ELISA kit was used to determine the concentration of LPS in each sample (Abbexa, Cambridge, United Kingdom; Catalog number: abx514093). Samples were assayed using a 1/50 dilution. The exact assay protocol was performed as described in in the kit's information sheet. Molecular Devices SpectraMax M3 plate reader was used to read the absorbance at 450 nm. SoftMax Pro version 5.4 was used to generate the standard curve using the recommended four parameter algorithm.

2.2.7 Human LTA ELISA

Sandwich ELISA was used to determine the concentration of LTA in each sample (Abbexa, Cambridge, United Kingdom; Catalog number: abx257487). Samples were assayed using a 1/2.5 dilution. The exact assay protocol was performed as described in the kit's

information sheet. Molecular Devices SpectraMax M3 plate reader was used to read the absorbance at 450 nm. SoftMax Pro version 5.4 was used to generate the standard curve using the recommended four parameter algorithm.

2.2.8 Quantitative Polymerase Chain reaction

Primer Design

Primers for *claudin-2* and *occludin* were obtained from Ohura et al.[342] Next, primer accession numbers were verified using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST). Then, the forward and reverse primer sequences were aligned against the theoretical primer sets from Integrated DNA Technologies (IDT) to optimize primer alignment against the gene sequence of interest and ordered. Finally, the primers were tested in endpoint PCR six times to ensure optimization of PCR conditions and product amplification.

Table 3. Sequences of oligonucleotide primers used in RT-qPCR analysis

Gene	GenBank Accession Number	Primer Sequence (5'-3')		Product Size
		Forward	***Reverse***	
18S rRNA	M10098	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151 bp
claudin-2	NM_020384	CTCCCTGGCCTGCATTATC	ACCTGCTACCGCCACTCTG	91 bp
occludin	NM_002538	TCCAATGGCAAAGTGAATGA	CGCTGCTGTAACGAGGCT	213 bp

cDNA Synthesis

High-Capacity cDNA Reverse Transcription kits and Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems by Life Technologies, Waltham, USA) were used to convert RNA from colonic biopsies to first-strand cDNA for use in RT-qPCR. A total of 51 participants with colonic biopsies available both from baseline and end-of-study were included in tight junction protein analysis (β-fructans n = 25, placebo n = 26). mRNA extraction was previously performed by Valcheva et al.[327] For the current study, each RNA sample was first diluted to 200 ng prior to cDNA conversion. Table 4 describes the volume of each reagent added per RNA sample.

Table 4. cDNA synthesis Master Mix

Reagent	µL/Reaction
10X RT Buffer	2
25X dNTP Mix	0.8
10X RT Random primers	2
Reverse Transcriptase	1
Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water	4.2
mRNA	10

RT-qPCR Analysis

Following cDNA synthesis, each sample was assayed with RT- qPCR to determine the relative mRNA gene expression of *claudin-2* and *occludin* tight junction proteins. Table 5 outlines the concentration of each reagent used per RT-qPCR reaction. 96-well plates were used and assayed using CFX Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, United States).

Table 5. RT-qPCR Master Mix

Reagent	µM
Power SYBR Green PCR Master Mix	1X
Forward primer	0.8
Reverse primer	0.8
Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water	0.38
Template cDNA	0.8

Each 96- well plate contained baseline and end-of-study/Month 6 samples from 11 patients in duplicate probed with the gene of interest (e.g., *claudin-2* or *occludin*) along with a 18S rRNA as a housekeeping gene was analyzed using CFX Manager Software (version 3.1, Hercules, United States). 18S rRNA has historically been shown to be the most stable housekeeping gene therefore used for normalization in comparative analyses.[343] In this study 18S rRNA was repeatedly tested against *β-actin* and *GAPDH* gene expression. It repeatedly and most consistently gave a strong, stable signal with high specificity.

Cycling protocol was based on the recommended protocol outlined in Power SYBR Green PCR Master Mix Reagents kit (Catalog Number: 4367659, Applied Biosystems by Life

Technologies, Waltham, United States) and summarized in Table 6. Each sample was assayed in duplicate and two blank controls were added per primer on each plate. Intra-assay variability was also tested. Finally, relative gene expression was determined using the DDC_t Method.[344]

Table 6. RT-qPCR cycling protocol for tight junction protein gene expression analysis in colonic biopsies from UC patients

Phase	Temperature	Time	
AmpliTaq GoldÒ Polymerase Activation	95°C	10 minutes	
Denaturation	95°C	15 seconds	40 Cycles
Annealing/ Extension	60°C	60 seconds	
Final Extension	72°C	30 seconds	
Melting Peak	65°C to 95°C by 0.5°C	Every 5 seconds	

2.2.9 Statistical Analysis

Upon analyzing the normality of the data using a D'Agostino & Pearson test, Anderson-Darling test, and Shapiro-Wilk test, the data did not pass normality, therefore, non-parametric tests were conducted during statistical analysis. Groups were compared using a paired Wilcoxon t-test (two-tailed) analysis for baseline (M0) to endpoint (M6/end-of-study) PAMP serum concentration, Mann Whitney unpaired t-test, correlation for changes in serum PAMP concentration and changes in relative gene expression of *claudin*-2 and *occludin* between M0 to M6/end-of-study. Additionally, a nonparametric one-way ANOVA (Kruskal-Wallis test) analysis was conducted to compare gene-expression results between clinical/FCP response subgroups in both arms. Multivariate analyses were used to compare changes in total and individual fecal SCFAs, changes in PAMPs, and relative TJP mRNA gene expression. Finally, descriptive statistics were employed for each analysis. All statistical methods were computed using GraphPad Prism (version 10.0.2, San Diego, United States). A p-value <0.05 was considered significant and all numbers are expressed as median with interquartile range (IQR).

2.3 Results

2.3.1 Effect of 6-month β-fructans supplementation on relative gene expression of *occludin* and *claudin-2*

Results obtained from qPCR were calculated using the $2^{-\Delta\Delta Ct}$ method to determine the relative gene expression of *claudin-2* and *occludin* in mRNA derived from colonic biopsies from the endpoint sample (Month 6/end-of-study) relative to baseline (Month 0) sample. An 18S rRNA gene primer was used as a housekeeping gene to account for variations in cDNA yield and integrity. Results are expressed as relative gene expression (RQ) and show fold-change in gene expression from end to baseline. Statistical tests for normality confirmed that RQ results were within non-normal distribution (D'Agostino & Pearson test, Anderson-Darling test, and Shapiro-Wilk test, $p<0.01$ for all tests). As demonstrated in Figure 3, a non-parametric unpaired Mann-Whitney t-test did not show significant differences between the RQ (fold-change) in *occludin* gene expression between study arms (placebo median RQ 1.01, IQR: 0.79 – 1.92; β-fructans median RQ 1.17, IQR: 0.51 – 2.11, $p = 0.9034$). *Claudin-2* RQ showed a trend of higher gene expression in placebo group in comparison to β-fructans (placebo median RQ 1.69, IQR: 0.75 – 2.79; β-fructans median RQ 1.00, IQR: 0.26 – 2.70, $p = 0.0712$).

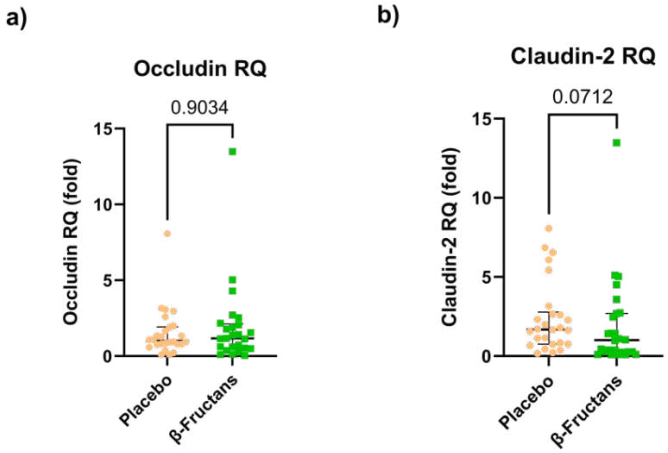

Figure 3. Relative gene expression (fold-change) of a) occludin and b) claudin-2 tight junction protein in colonic biopsies from UC patients treated with β-fructans or placebo.

To test if TJPs expression is associated with UC disease activity, we categorized the patients into those who stayed in biochemical remission (placebo *n*=16; β-fructans *n*=18) or

experienced biochemical relapse (placebo *n*=10; β-fructans *n*=7) based on biochemical (FCP) response. As demonstrated in Figure 4a and b, there was a trend in lower *occludin* and *claudin-2* gene expression particularly in β-fructans patients who remained in biochemical remission. We then grouped patients based on clinical disease activity (endpoint Mayo score ≥3).

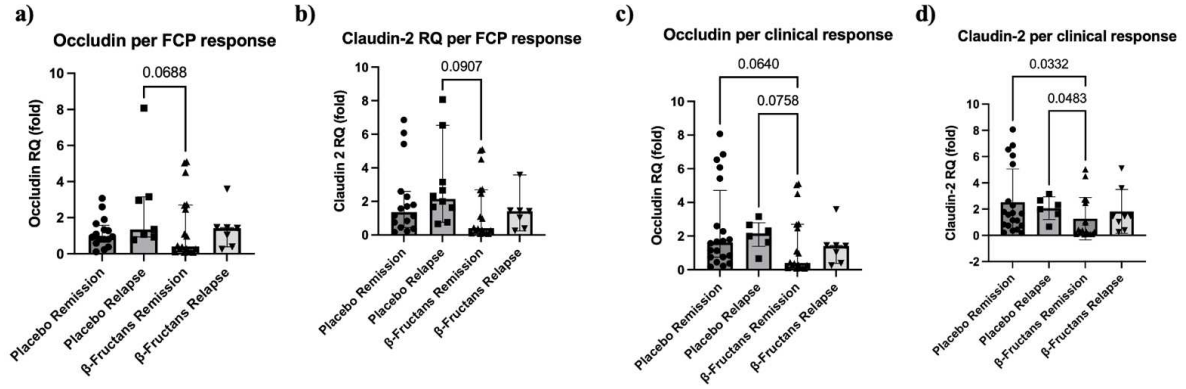

Figure 4. Differences in tight junction protein gene expression in UC patients with different degree of disease activity at endpoint treated with β-fructans or placebo for 6-months. One-way ANOVA (Kruskal-Wallis test) analysis of occludin and claudin-2 expression based on either clinical response or FCP in all subgroups. Vertical bars expressed as median and IQR, statistically significant differences p < 0.1 are shown above corresponding bars.

The patients were categorized into those who stayed in clinical remission (placebo *n*=20; β-fructans *n*=17) or experienced symptomatic relapse (placebo *n*=6; β-fructans *n*=9). Analysis with ANOVA test confirmed that patients who were treated with β-fructans and remained asymptomatic also showed considerable reduction in TJPs gene expression in both *occludin* and *claudin-2* (Figure 4c, d). This finding can suggest that β-fructans could be particularly effective in UC patients in remission by improving the tight junction function. Finally, to determine the correlation between the TJP mRNA gene-expression of *claudin-2* and *occludin*, a Spearman Rank test was used. This Spearman's Rank correlation revealed a positive correlation between *claudin-2* and *occludin* gene expression in the placebo (R^2=0.604, p=0.001) and β-fructans group (R^2=0.528, p=0.007).

2.3.2 Effect of 6-month β-fructans supplementation on concentrations of serum LBP, LPS, and LTA

After analyzing the tight junction proteins, we wanted to see if these differences in TJP mRNA gene expression were related to changes in bacterial translocation. Serum samples from

baseline and end-of-study/Month 6 were assayed by ELISA to quantify LBP, LPS, and LTA concentrations. These markers were suggested as an indirect surrogate markers of change in intestinal permeability and for bacterial translocation. We first examined the relationship between baseline (M0) and endpoint (M6/end-of-study) sample concentrations between groups using a Mann-Whitney test. At baseline there was a significant difference in serum LPS (EU/mL) concentration between treatment arms (Figure 5a; placebo median 11.0, IQR 9.82-12.3; β-fructans median 5.62, IQR 2.96-11.3, p= 0.0167). This difference remained significant at the end of treatment (Figure 5b; placebo median 11.6, IQR 9.00- 12.2; β-fructans median 4.91, IQR 3.30-10.7, p= 0.0190). This analysis can suggest that there may have been unequal randomization, which may complicate the PAMP analysis. The difference in LBP (ng/mL) at baseline (placebo median 18,732, IQR 13,233-37,114; β-fructans median 18,636, IQR 11,022-28,688, p= 0.4791) and Month 6/end-of-study (placebo median 16,831, IQR: 11,949-38,725; β-fructans median 20,823, IQR: 14,284-73,856, p=0.9752) was not significant between groups. Neither was the difference in LTA (pg/mL) at M0 (placebo median 119, IQR: 75.5-219; β-fructans median 128 IQR: 71.2-186; p= 0.7072) or M6/end-of-study (placebo median 126, IQR: 74.5-217; β-fructans median 136, IQR: 78.0-216, p = 0.9152) significant between groups.

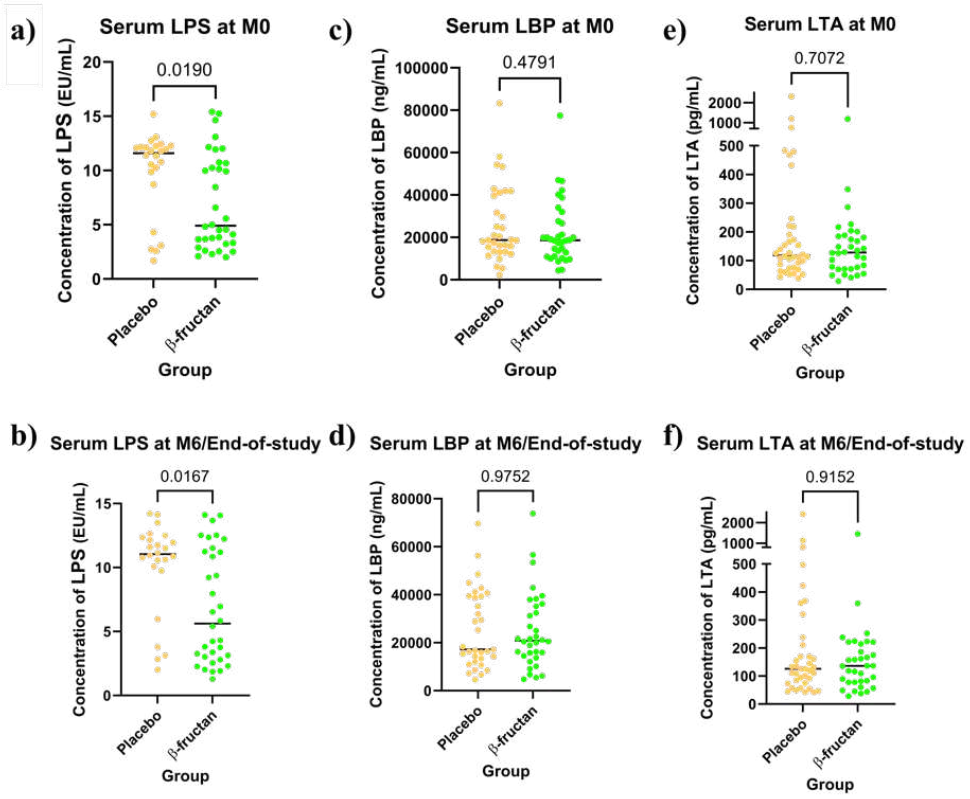

Figure 5. Difference between M0 and M6/end-of-study PAMP concentrations between β-fructans and placebo group.

We then examined the change in concentration of each PAMP over the study period within individuals. Table 7 summarizes the median and IQR values for each PAMP at M0 and M6/end-of-study. Using a paired t-test, we did not find any significant difference in the change in LBP serum concentration in the β-fructans (p=0.3092) or placebo (p=0.4939) group over the treatment course. Although the change in LPS showed a trend to be lower in the β-fructans group, there was no statistical significance in the β-fructans (p=0.3974) or placebo (p=0.6437) group. Similarly, as displayed in Table 7, LTA concentrations was not significantly changed in the β-fructans (p=0.1207) or placebo (p=0.2738) group over the course of the study. Finally, to account for the significant differences in the baseline LPS concentrations between groups, we assessed if the change in PAMPs differed between the β-fructans and placebo group (Table 7). Results from Mann-Whitney test showed no significant differences in the shifts of all markers for bacterial translocation (LBP: p=0.2149; LPS: p=0.3176; LTA: p=0.0633).

Table 7. Difference in serum concentrations of PAMPs over study period within groups and change in PAMPs concentration over study period between groups. Concentrations are shown as median and IQR.

	Placebo n= 26			*β-fructans n=25*			**ΔPAMPs between groups**
	M0	**M6/End**	**p-value**	**M0**	**M6/End**	**p-value**	
LBP (ng/mL)	18,732 (13,233-37,114)	16,831 (1,949-38,725)	0.4939	18,636 (11,022-28,688)	20,823 (14,284-73856)	0.3092	p=0.2149
LPS (EU/mL)	8.323 (2.744- 11.93)	7.686 (3.127- 11.60)	0.6437	4.912 (3.302- 10.70)	5.624 (2.957-11.31)	0.397	p=0.3176
LTA (pg/mL)	118.6 (75.53-218.7)	126.0 (74.53- 217.2)	0.2738	128.2 (71.22- 185.6)	136.2 (77.96- 215.8)	0.1207	p=0.0633

The results show that PAMP concentrations were not affected by β-fructans intervention. Since current data suggests that leaky gut is one of the potential pathological changes in UC, we then investigated if PAMP serum concentrations had an effect on mucosal inflammation. For this analysis, patients were divided into subgroups based on clinical disease activity response and FCP response, as we did during the TJP gene-expression analysis. Figure 6 shows the comparison in change of PAMP concentration per subgroup based on clinical and FCP response. There were no significant differences between subgroups. The closest p-value to significance was between placebo remission and β-fructans remission in change in LTA which was 0.0945.

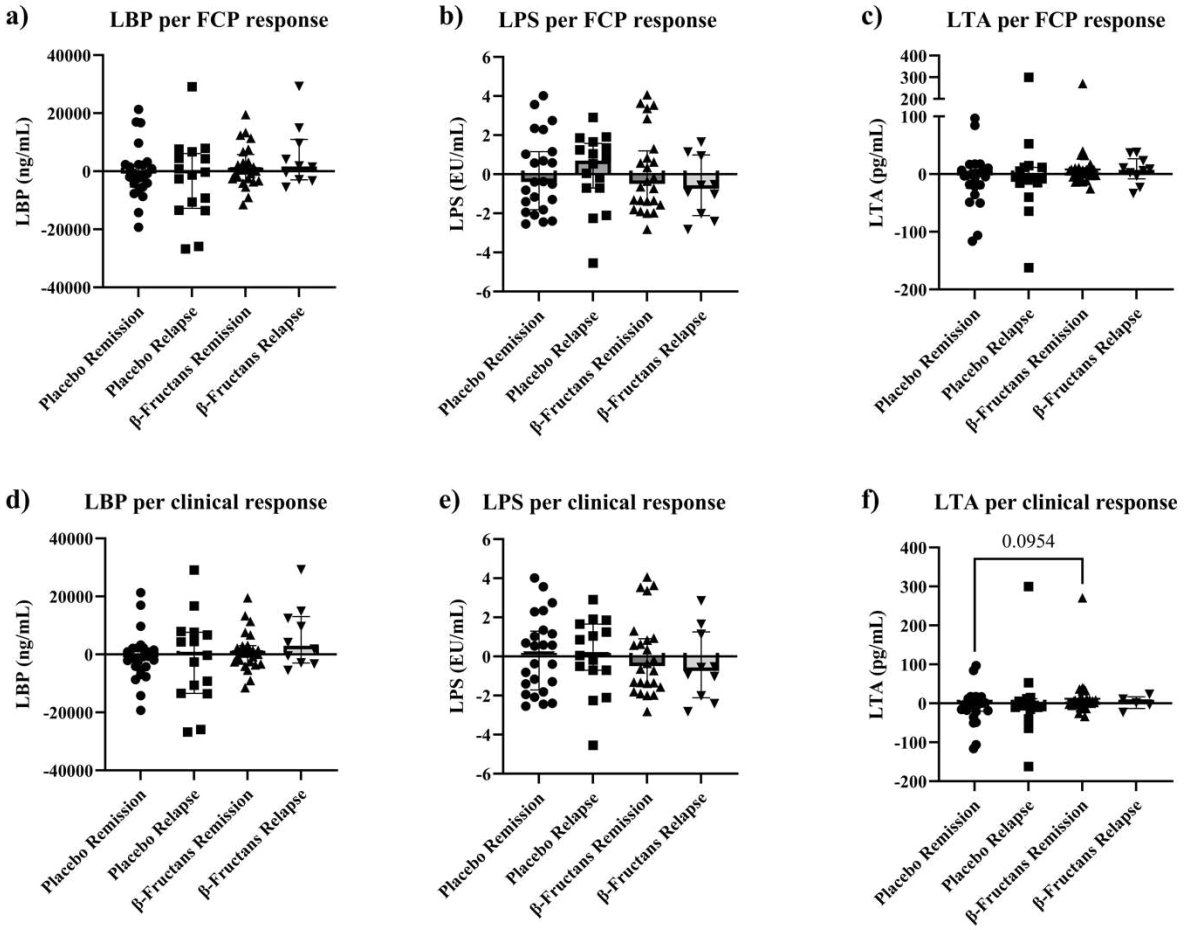

Figure 6. Differences in serum PAMP (LBP, LPS, and LTA) concentration in UC patients with different degree of disease activity treated with β -fructans or placebo for 6-months. One-way ANOVA (Kruskal-Wallis test) analysis of LBP, LPS, and LTA based on either clinical response or FCP in all subgroups. Vertical bars expressed as median and IQR, statistically significant differences (p< 0.1) are shown above corresponding bars.

There was no significant difference in PAMP serum concentration between patients in remission and those who experienced relapse based on both clinical and FCP response, and therefore, it could be suggested that intestinal permeability is not a primary mechanism initiating the pro-inflammatory response. To determine the extent of the effect of inflammatory markers on indirect markers of intestinal permeability, linear regression was used to identify the correlations between change in PAMP concentration and change in FCP. Table 8 reports the Spearman's Rank Correlation Coefficient (R^2) and p-value for each test. The only significant correlations were considered weak and occurred between ΔLBP and ΔFCP in the *β-fructans group (*R^2=0.1396, p=0.0295) as well as ΔLTA and ΔFCP in the placebo group *(*R^2=0.1601, p=0.0128).

Table 8. Spearman's correlation analysis between change serum concentrations of LBP, LPS, and LTA and change in FCP.

	Placebo ΔFCP (µg/g)		*β-fructans* ΔFCP (µg/g)	
	R^2	p-value	R^2	p-value
ΔLBP (ng/mL)	0.06074	0.1304	0.1396	0.0295
ΔLPS (EU/mL)	0.002009	0.7865	0.001220	0.8445
ΔLTA (pg/mL)	0.1601	0.0128	0.002953	0.7602

Due to the documented relationship between LPS and its binding protein, LBP, we were curious to see if there was a correlation between LBP and LPS in our population. Results from linear regression analysis indicated a lack of correlation in the LBP versus LPS serum concentrations (Figure 8) (R^2=0.010, p=0.407).

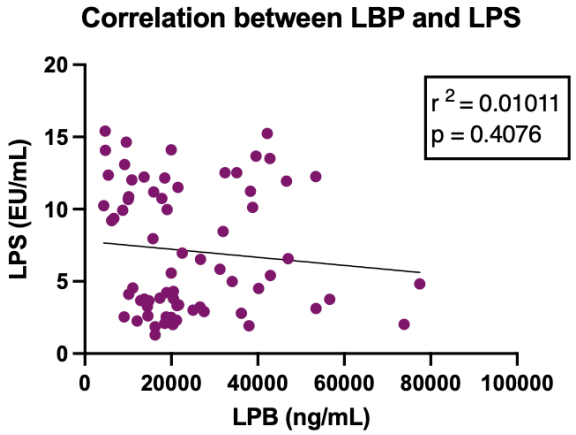

Figure 7. Correlation between LBP and LPS concentrations in all study participants

2.3.3 Multivariate analysis between relative gene expression of tight junction proteins and fold-change of fecal SCFAs

To determine the relative effect of variables related to intestinal permeability, inflammation, and production of microbial metabolites, a principal component analysis (PCA) was conducted. PCA analysis showed that PC1 defined 24.2% of the observed separation while the PC2 contributed 18.6%. As most of the patients were in remission, there was no obvious clustering. FCP and *occludin* had a negative association to relative butyrate concentrations in the placebo group. In addition, PAMPs and branched chain fatty acids were grouped in a single quadrant. In the case of β-fructans group, the PCA identified a different interface between

variables. Patients with high FCP also showed an increase in PAMPs but reduced gene expression for *occludin* and *claudin-2*, and none of the SCFAs were somewhat correlated to markers of intestinal permeability nor mucosal inflammation.

To confirm with individual assessment, we ran individual linear regression models to further explore the results of the multivariate analysis. It has been previously shown that the SCFAs acetate, butyrate, and propionate, produced by intestinal microbes, can regulate energy metabolism, cell differentiation and apoptosis and inflammation. Therefore, it was important to study if the observed effect of β-fructans on mucosal inflammation can be partly explained by interaction between microbial metabolites and the host tight junction proteins. A linear regression model was utilized to obtain a detailed analysis of the relationship between gene expression of colonic *claudin-2* with fecal total SCFA, acetate, propionate isobutyrate, butyrate, isovalerate, and valerate.

Table 9. Linear regression analysis of claudin-2 gene expression and SCFA in β-fructans /Placebo groups

	Placebo		***β-fructans***	
	R^2	**p-value**	**R^2**	**p-value**
Total SCFA	0.09255	0.1308	0.02608	0.4406
Acetate	0.005210	0.7260	0.001534	0.8525
Propionate	0.0001849	0.9474	0.01146	0.6106
Isobutyrate	0.006944	0.6857	0.0009289	0.8850
Butyrate	0.002578	0.8054	0.01350	0.5802
Isovalerate	0.001143	0.8697	0.0007586	0.8960
Valerate	0.4132	**0.0004**	0.03290	0.3856
FCP	7.343e-005	0.9669	0.5671	**<0.0001**

As seen in Figure 8, the relationship between *claudin-2* gene expression and valerate concentrations changes in placebo group was significant (R^2=0.4132, p=0.0004). The rest of the relationships between *claudin-2* and other SCFAs were insignificant (Table 10). In addition, the linear regression analysis model showed a significant relationship between *claudin-2* and FCP (R^2=0.5671, p=<0.0001) (Table 10).

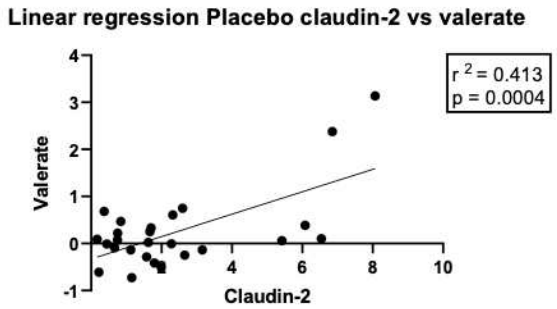

Figure 8. Linear regression analysis of Claudin-2 RQ and fecal valerate in Placebo patients

We then studied the relationship between *occludin* expression and SCFAs. Similarly, in placebo patients the *occludin* gene expression also showed strong positive association with fecal valerate (R^2=0.5844, p=<0.0001) as well as between *occludin* and FCP, based on linear regression (R^2=6696, p=<0.0001). As demonstrated in Table 11, the rest of the relationships between *occludin* and other SCFAs were insignificant.

Table 10. Linear regression analysis of occludin gene expression and various short-chain fatty acids in β-fructans/Placebo group

	Placebo		***β-fructans***	
	R^2	**p=value**	**R^2**	**p=value**
Total SCFA	0.07143	0.1869	4.281e-006	0.9922
Acetate	0.04439	0.3016	0.002299	0.8200
Propionate	0.04195	0.3155	0.01595	0.5474
Isobutyrate	0.01295	0.5799	0.03077	0.4016
Butyrate	0.02820	0.4122	0.01323	0.5841
Isovalerate	0.05513	0.2483	0.05207	0.2726
Valerate	0.5844	**<0.0001**	0.03187	0.3932
FCP	0.6696	**<0.0001**	0.04633	0.3015

2.3.4 Correlation between serum LPS, LBP, and LTA and change in FCP

As per the third aim of this study, we wanted to determine if the effect of β-fructans on intestinal permeability was associated with FCP. To do this, we first assessed the correlation between PAMP and FCP concentration at baseline and the same for PAMP and FCP concentration at end-of-study. As demonstrated in Table 11, inflammation determined by FCP had no significant correlation with serum LPB, LPS or LTA concentrations. It could be then

suggested that bacterial translocation and/or bacterial antigens systemic circulation were not directly associated with mucosal inflammation in UC.

Table 11. Linear regression analysis between baseline PAMPs and FCP and Month 6/End-of-study PAMPs and FCP

	Fecal calprotectin							
	Placebo				***β-fructans***			
	M0		**M6/End**		**M0**		**M6/End**	
	R2	**p-value**	**R2**	**p-value**	**R2**	**p-value**	**R2**	**p-value**
LBP (ng/mL)	0.05975	0.1337	0.05145	0.1650	0.01042	0.5656	0.01674	0.4659
LPS (EU/mL)	0.039	0.229	0.008	0.589	0.0271	0.351	0.022	0.398
LTA (pg/mL)	0.018	0.422	0.003	0.744	0.007	0.625	0.008	0.623

We then examined the correlation between change in PAMPs and change in FCP from baseline to end-of-study using the non-parametric Spearman rank correlation. There were no significant associations in the β-fructans group between change in FCP and change LBP (p= 0.0295, R^2= 0.1396), change in LPS (p= 0.844, R^2= 0.001) or change in LTA (p= 0.7602, R^2= 0.0029). Similarly, the placebo group also did not show any statistical differences in change in FCP and changes in LBP (p= 0.1304, R^2= 0.06074), LPS (p= 0.7865, R^2= 0.002), or LTA (p= 0.0128, R^2= 0.1601) serum concentrations.

2.3.5 Multivariate analysis of fecal SCFAs fecal calprotectin, PAMPs and TJPs gene expression

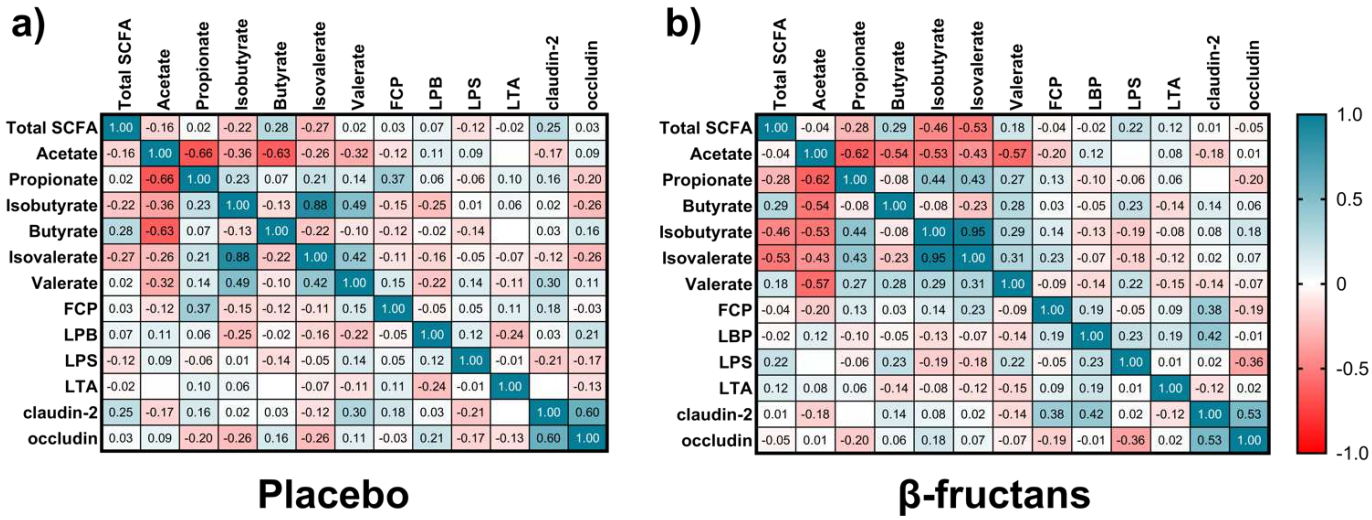

Figure 9. Heat map of Spearman correlation matrix including changes in fecal microbial metabolites (total and individual SCFAs), inflammation (determined by FCP), markers of bacterial translocation (LBP, LPS, and LTA), and TJP gene-expression (occludin and claudin-2)

Due to colonic fermentation of dietary fibres, it was important to analyze if changes in fecal microbial metabolites (total and individual SCFAs) and/or inflammation (determined by FCP) were associated with changes in markers for intestinal permeability. After conducting a multivariate analysis on these parameters, we found a positive correlation between propionate relative concentrations and change in FCP in the placebo group (Figure 9a: R^2=0.373, p=0.016)

In presence of β -Fructans, neither inflammation nor markers for permeability showed correlations to the microbial metabolites such as SCFA. This can be explained by the fact that even patients who experienced worsening in their symptoms still had improved microbial metabolism.

2.4 Discussion

During the UC-Synergy trial, a 6-month supplementation of β-fructans (oligofructose and inulin) did not prevent clinical relapse in patients with UC who were in remission.[327] However, β-fructans did lead to a significant reduction in the severity of biochemical or subclinical relapse when compared to placebo.[327] Additionally, this reduction was associated with increased levels of the anti-inflammatory metabolite butyrate and changes in the gut microbiota composition, induced by β-fructans.[327] In this post-study analysis of the Synergy trial, we aimed to investigate if the beneficial effect of prebiotic inulin-type β-fructans supplementation in inactive UC could be attributed to reduction in the colonic intestinal permeability. Increased paracellular permeability or so called "leaky gut" has been observed in IBD patients as early as 1982.[345]

The intestinal barrier is composed of multiple components such as the mucosal lining, and IECs which are bound together by tight junction proteins. Perturbation of the intestinal barrier compromises its permeability and allows for the passage of pathogens and luminal antigen contents, which may in turn trigger or potentiate inflammation in both gastrointestinal and systemic diseases. While an increase in circulating endotoxin has been previously reported in active UC [346], LPS levels during remission are significantly lower.[88] The current results demonstrate no significant changes in serum LPS and LBP concentrations neither within individuals from the same treatment, nor between treatments. This may indicate that the intestinal barriers in these UC patients in remission were not leaky enough to detect an increase in endotoxin.[243] On the same thread, the lack of changes in LBP could be expected as LBP has a concentration-dependent relationship with LPS.

LTA is a major cell-wall component and the functional equivalent of LPS in Gram-positive bacteria and an indicator of translocation of Gram-positive bacteria can induce the expression of inflammatory cytokines and mediators when derived from pathogenic microbes [277,280] These LTAs, similar to other pathogen derived PAMP, activate macrophages, leading to the release of various cytokines.[278] Although there was no difference in serum LTA concentration between the intervention and control group in this study, further analysis of the gut microbiota of these patients is warranted in order to interpret these results further in the context of Gram-positive bacteria changes in the gut microbiome of these patients.

The intestinal epithelial barrier is maintained by tight junctions, which consist of proteins including *claudin-2* and *occludin*. These junctions prevent the diffusion of proteins between

different cell membranes and control the movement of molecules and ions.[197] *Claudin-2* is highly expressed at birth and rapidly reduced onwards in healthy individuals. *Claudin-2* forms pores that allow the passage of cations and water.[195,207] Its expression in the human colon varies in different studies, with some showing no detection in normal samples and others indicating restricted expression in certain cell types. However, its upregulation in intestinal inflammation such as IBD, including active UC, has been well-documented. [205,209,347] The increased expression of *claudin-2* is likely linked to IL-13 mediated STAT6 activation.[210] Although the current study report only a trend toward overall reduced gene expression of *claudin-2* in β-fructans group, the gene suppression reached significance in those UC patients treated with β-fructans and remained in clinical and biochemical remission. Importantly, *claudin-2* gene suppression was not demonstrated in the placebo group remittent patients.

Similar to *claudin-2* gene expression, there was no statistically significant difference in *occludin* mRNA gene expression over the course of the study between both groups. Only patients who maintained in remission during β-fructans intake showed downregulation in *occludin* mRNA gene expression. This result supports previous reports of *occludin* expression studies showing a reduction of *occludin* mRNA expression in the colonic mucosa of remission versus relapse UC patients.[209,264,348 307] For example Yamamoto-Furusho et al. saw a significant difference in *occludin* gene expression levels between active and inactive UC patients, where remittent UC patients exhibited lower levels of *occludin* expression compared to those in relapse.[264] It should be pointed out that all studies compared individual samples collected at one timepoint and yet it has not being documented the dynamic in TJP expression during the course of disease development. To further support, a study by Kuo et al. demonstrated that epithelial *occludin* expression exacerbated DSS-induced colitis, while occludin KO mice were resistant to colitis development.[338] They also identified that mild inflammatory stimuli that trigger occludin downregulation also downregulate caspase-3 and, in turn, confer resistance to intrinsic and extrinsic pathway apoptosis promoting apoptotic resistance and preventing tissue damage.[338] The lack of consensus between trends in occludin expression in UC may allude to the ever-changing interactions between the barrier function, gut microbiota, and genetics. Furthermore, our study population consisted of participants with symptomatically inactive disease and the majority remained in remission. By analyzing the results of *occludin* and *claudin-2* gene expression in this study, they may indicate that this population had no differences in TJP integrity, therefore, it is

plausible to assume that there is no evidence of increased intestinal permeability and would not expect to see bacterial translocation of LPS, LBP, and LTA, as demonstrated in this study.

Furthermore, bacterial endotoxin has been shown to have a direct effect on the promotion of FCP expression, which is often associated with active inflammation. In this set of patients who were randomly assigned to receive oligofructose and inulin, there was only a 3-fold increase in median FCP levels compared to a 17-fold increase in the placebo group ($p=0.038$).[327] Thus, the idea to measure the correlation between inflammation, measured by FCP, and the changes in bacterial translocation and gene-expression was of importance. In this analysis of intestinal permeability, markers of endotoxemia (LPS, LBP, and LTA) did not seem to be correlated with FCP concentrations. This was confirmed in both the time point correlation analysis between PAMPs and FCP. These results do not support the expected positive association between bacterial translocation and colonic inflammation,[282,283] along with the expected positive association between abnormal TJP gene-expression and inflammatory markers. [209,215,347] Possible explanations includes low power as well as alternative driving factors of FCP such as BMI, disease severity/history, and diet.[349] Interestingly, the use of antibiotics and serotonin reuptake inhibitors significantly raised the relative risk (RR) of experiencing a flare-up (RR 3.321, 95% Cl 2.005 to 5.344, $p < 0.0001$).[327] Furthermore, the consumption of β-fructans led to a significant increase in anti-inflammatory fecal metabolites, including arabinose, L-arabitol, and 5-oxo-D-proline.[327]

SCFAs are carboxylic acids produced through gut microbial anaerobic fermentation. They act as ligands for G-protein-coupled receptors activating signaling cascades.[36,71] SCFAs can influence the inflammatory response and improve intestinal barrier integrity by enhancing tight junction proteins' functions.[36] As the main source of fuel for colonocytes, butyrate has an integral role in IEC function and integrity.[70,314] Butyrate's impact on the epithelial barrier involves upregulating TJPs through the activation of AMP-activated protein kinase.[350] Additionally, Valenzano et al.[351] found that butyrate treatment of Caco-2 cell monolayers led to a decrease of 90% in claudin-2 and an increase of 376% in claudin-7, suggesting its role in remodeling TJ and maturing barrier function. Moreover, butyrate has been shown to mitigate the negative effects of LPS on epithelial integrity by promoting claudin synthesis via activation of the Akt signaling pathway in IPEC-J2 cells.[352] Recently, acetate and propionate have been shown to sustain cell viability, reduce oxidative stress activity and improve intestinal barrier

function.[311,353] After studying the relationships between study gene expression of *claudin-2* and *occludin* and SCFAs, there were no significant relationships between these TJP genes and total SCFA, acetate, propionate, isobutyrate, butyrate, or isovalerate in either group. However, there was a significant relationship between TJP gene-expression RQ and valerate in the placebo group. Our previous work with HLA-B27 models demonstrated that valerate, a branched chain fatty acid, is product of protein fermentation.[332] This SFCA has also been validated as a marker of chronic colitis inflammation.[354] Therefore, as shown in Figure 2 a decrease in valerate from baseline to end of study further identifies a protective effect of β-fructans on colonic inflammation in UC. Reduction of fecal valerate concentrations was positively related to the trend of reduction in *claudin-2* expression in β-fructans group. Based on these observations, a possible effect of β-fructans intake on regulation of *claudin-2* gene expression in colon can be suggested. This effect is likely due to a direct suppression activity of the microbial metabolites produced in the carbohydrate fermentation and outcompeting protein fermentation pathways. Further analysis is needed to confirm if *claudin-2* gene expression can be also regulated by the specific microbiota shifts induced by β-fructans. Finally, regulation of *claudin-2* expression by β-fructans may contribute to enhanced intestinal barrier and thus partly explain the less severe biochemical relapse observed in this group.

UC is a mucosal disease that does not involve areas outside of the colon, except in the case of backwash ileitis.[355] However, backwash ileitis is associated with severe disease,[356] and since our population began the study in remission, we were able to assume that it did not play a role during analysis. Furthermore, the multivariate analysis did not reveal any significant correlations between serum PAMP concentration, changes in fecal SCFAs, and changes in FCP. Serum and plasma may not be valid matrices for identifying clinical outcome and intestinal permeability as many factors beyond the colon may affect them. For example, renal disease, diabetes, obesity, and liver disease can independently affect bacterial translocation rather than colonic intestinal barrier integrity alone. Therefore, matrices at the mucosal level may provide a more accurate picture of intestinal permeability as opposed to systemic factors. A recent study by Arango-González et al. investigated the link between intestinal permeability, circulating SCFAs, cardiometabolic health status (CMHS), and gut microbiota in a group of 116 Colombian adults.[357] The group found that intestinal fatty acid-binding protein (I-FABP), LBP, claudin-3, and SCFAs were not reliable biomarkers for linking intestinal permeability with cardiometabolic

health in these individuals.[357] However, they observed a potential association between poorly characterized peptides detected with the zonulin ELISA kit and improved cardiometabolic status and gut microbiota.[357] Therefore, further analyses related to fecal zonulin may be helpful in determining changes in intestinal permeability in this population.[357]

PAMPs such as LPS and LTA may more likely be a product of small intestinal permeability rather than colonic permeability.[358,359] Additionally, bacterial endotoxemia can be initiated and affected by multiple inflammatory conditions that do not include colonic inflammation, such as EIMs associated with IBD related to joint health. These EIMs can have genetic predisposition (such as HLA-B27 and NOD2/CARD15), rather than an exclusive result of UC inflammation.[360,361] Therefore, not seeing significant correlations between serum PAMPs and SCFA can be expected since SCFAs are fermented in the colon and have been shown to modulate colonic permeability rather than small bowel permeability. Teshima et al. supported this when they reported that 30% of healthy, asymptomatic first-degree relatives of patients with CD show elevated intestinal permeability, however a significant connection between small bowel ulceration observed through video capsule endoscopy (VCE) and increased intestinal permeability was not identified.[161] Interestingly, Deehan et al. showed that the expected molecular indicators of biological processes connecting the metabolic functions of the gut microbiome with host metabolism and immune response, such as TMAO, gut hormones, cytokines, and intestinal barrier integrity, remained unaffected by prebiotic supplementation and were not able to predict its effects.[362]

Naturally, we then focused on a multivariate analysis that was exclusive to factors that involved colonic factors such as SCFA, FCP, and TJP mRNA gene expression. These results were inconclusive as there were weak correlations between SCFAs, FCP, and TJP mRNA gene expression of *occludin* and *claudin-2*. Most studies investigating the effect of intestinal permeability have been conducted in Crohn's patients or IBD in general. When it comes to human studies of intestinal permeability in UC, research is not as robust with many studies being conducted in experimental models of colitis. Due to the differences in phenotypic disease manifestations, there are differences in barrier integrity, in general. It is not necessary the effects of different factors and mechanisms identified in CD to fully illustrate those in UC. For example, although mucin production is down regulated in both CD and UC, the mechanism for this effect is specific to the disease. In CD, although *Muc2* gene is expressed, downstream process of

protein unfolding is compromised, leading to misfolded mucin proteins.[55,106] In UC, the same down regulation is due to a lack of goblet cell production of mucin rather than a mutation in unfolding.[114] This effect of mucin production, secretion, and expression in UC is prominent in severe UC, as opposed to all CD patients.

In addition, researchers must be wary in translating the results of animal studies of intestinal barrier functions to humans. Many animal models of UC and the effect of microbial agents on barrier function are limited to single mechanisms. The results of animal studies provide helpful context to mechanistic processes regarding barrier function, but they cannot entirely replicate human UC due to its polyphenotypic nature and multi-factorial pathogenesis which work in tandem to exert complex effects on barrier function.

The primary limitation of the study was whether this population had enough colonic inflammation to see a difference in bacterial translocation. Since each patient started in remission, it is possible that the differences in inflammation were not adequate to cause changes in intestinal permeability or bacterial translocation at the time of sampling. Another limitation of the study revolves around the study design. This study was not designed to investigate the role of intestinal permeability in the previous RCT. As with any study, the proposed study will have inherent disadvantage such as a vulnerability to bias and cofounding. For example, serum concentrations of LPS, LBP, and LTA may be affected by small intestinal permeability rather than colitis and colonic damage. Patients with UC have no small bowel inflammation and therefore, these markers for bacterial translocation may not be suitable in UC. Furthermore, measurement of intestinal permeability was limited to indirect markers of intestinal permeability instead of direct *in vivo* measurement such as a lactulose/mannitol test. Finally, this study did not include healthy controls as a comparison which may have had the ability to increase power and reduce bias. The limited number of subjects may have meant that this study was not powered to identify a difference in permeability, especially with all the other factors that affect permeability and natural patient/biological variability. Further studies are required to compare baseline occludin gene expression in remission UC to healthy controls, effect of diet, and its change in trajectory of TJP mRNA expression with disease progression including duration and severity.

2.5 Conclusions

To conclude, the main finding of this study was that β-fructans intake induced downregulation in *occludin* and *claudin-2* gene expression, especially in those patients who remained in clinical and biochemical remission. In addition, valerate showed a strong positive correlation to *claudin-2* and *occludin* expression in the placebo group. There were no significant differences in measures of bacterial translocation as measured by serum LBP, LPS, and LTA. These results suggest that higher-powered studies are required to confirm trends in prebiotic-induced reduction in claudin-2 expression to improve colonic inflammation in UC patients.

CHAPTER III: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

3.1 General Conclusions

The Synergy trial found that oral supplementation of inulin-type β-fructans for 6 months reduced biochemical relapse severity, as measured by FCP. In this secondary analysis, we were interested to know whether this positive effect was via enhancement of intestinal permeability. This study aimed to see if prebiotic supplementation could enhance intestinal permeability and that those changes could be seen through markers of bacterial translocation and tight junction protein gene expression. The main finding of the study was that the oral supplementation of inulin-type β-fructans for 6 months lead to the downregulation of tight junction protein mRNA gene expression of *occludin* and *claudin-2*, especially those who stayed in remission in the β-fructans group. Measurements of bacterial translocation, assessed by the serum markers LBP, LPS, and LTA, did not show significant differences between the intervention and placebo groups from the beginning to the end of the study. Multivariate analysis showed a positive relationship between mRNA gene expression in the placebo group and the SCFA valerate. Overall, the study suggests that oral supplementation with inulin-type β-fructans resulted in the downregulation of *occludin* and *claudin-2* mRNA gene expression especially in β-fructan remitters, which is related to reduced colonic inflammation. Therefore, the protective effects of the β-fructans supplementation on colitis reduction has a modulatory effect on indirect markers of intestinal permeability. Further studies are warranted to identify the mechanisms of these protective effects on colitis.

Future studies should focus on multi-center, longitudinal studies with an added dietary component (e.g., n-3 PUFAs, anti-inflammatory diet) to determine the combined effect of diet and dietary supplementation. Additionally, it may be beneficial to assess other TJPs along with valid intestinal permeability tests (such as sugar probe tests) to understand the relationship between change in intestinal permeability and various TJPs within this population under prebiotic supplementation. Finally, the accelerated drive towards personalized medicine would

be a topic of interest in the population to tailor therapy and supplementation based on genetic profile, dysbiosis, and individual lifestyle factors.

REFERENCES

1. Wilks, S. Morbid appearances in the intestine of Miss Bankes. *Med Times Gazette* **2**, 264–265 (1859).
2. TRUELOVE, S. & WITTS, L. Cortisone in ulcerative colitis; final report on a therapeutic trial. *Br Med J* **2**, 1041–1048 (1955).
3. Lane, W. A. Remarks ON THE RESULTS OF THE OPERATIVE TREATMENT OF CHRONIC CONSTIPATION. *Br Med J* **1**, 126–130 (1908).
4. Midura, E. F. *et al.* Risk factors and consequences of anastomotic leak after colectomy: A national analysis. *Dis Colon Rectum* **58**, 333–338 (2015).
5. Artinyan, A. *et al.* Prolonged postoperative ileus - Definition, risk factors, and predictors after surgery. *World J Surg* **32**, 1495–1500 (2008).
6. Kirchhoff, P., Clavien, P. A. & Hahnloser, D. Complications in colorectal surgery: risk factors and preventive strategies. *Patient Safety in Surgery 2010 4:1* **4**, 1–13 (2010).
7. Pariente, B. *et al.* Development of the Crohn's disease digestive damage score, the Lémann score. *Inflamm Bowel Dis* **17**, 1415–1422 (2011).
8. Baumgart, D. C. & Le Berre, C. Newer Biologic and Small-Molecule Therapies for Inflammatory Bowel Disease. *New England Journal of Medicine* **385**, 1302–1315 (2021).
9. Olén, O. *et al.* Colorectal cancer in ulcerative colitis: a Scandinavian population-based cohort study. *Lancet* **395**, 123–131 (2020).
10. Graham, D. B. & Xavier, R. J. Pathway paradigms revealed from the genetics of inflammatory bowel disease. *Nature 2020 578:7796* **578**, 527–539 (2020).
11. Yantiss, R. K. *et al.* Prognostic significance of superficial fissuring ulceration in patients with severe 'indeterminate' colitis. *Am J Surg Pathol* **30**, 165–170 (2006).
12. Warren, S. & Sommers, S. C. PATHOGENESIS OF ULCERATIVE COLITIS*.
13. SLOAN, W. Local complications of chronic ulcerative colitis based on the study of 2000 cases. *Proc Mayo Clin* **25**, 240–244 (1950).
14. A, B. *et al.* Differentiating ulcerative colitis from Crohn disease in children and young adults: report of a working group of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition and the Crohn's and Colitis Foundation of America. *J Pediatr Gastroenterol Nutr* **44**, 653–674 (2007).
15. Bousvaros, A., Antonioli, D., … R. C.-J. of pediatric & 2007, undefined. Differentiating ulcerative colitis from Crohn disease in children and young adults: report of a working group of the North American Society for Pediatric. *europepmc.org*.
16. Dignass, A., Eliakim, R., Magro, F., … C. M.-J. of C. & 2012, undefined. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. *academic.oup.com*.
17. Annese, V. *et al.* European evidence based consensus for endoscopy in inflammatory bowel disease. *J Crohns Colitis* **7**, 982–1018 (2013).
18. Riley, S. A., Mani, V., Goodman, M. J., Dutt, S. & Herd, M. E. Microscopic activity in ulcerative colitis: what does it mean? *Gut* **32**, 174–178 (1991).
19. Filmann, N. *et al.* Prevalence of anemia in inflammatory bowel diseases in european countries: a systematic review and individual patient data meta-analysis. *Inflamm Bowel Dis* **20**, 936–945 (2014).

20. Orchard, T. R., Wordsworth, B. P. & Jewell, D. P. Peripheral arthropathies in inflammatory bowel disease: their articular distribution and natural history. *Gut* **42**, 387–391 (1998).
21. Buran, T., Kılınç, S. G. M. & Kasap, E. Prevalence of Extraintestinal Manifestations of Ulcerative Colitis Patients in Turkey: Community-Based Monocentric Observational Study. *Clinical Medicine and Medical Research* **1**, 39–46 (2020).
22. Mintz, R., Feller, E. R., Bahr, R. L. & Shah, S. A. Ocular manifestations of inflammatory bowel disease. *Inflamm Bowel Dis* **10**, 135–139 (2004).
23. Magro, F. *et al.* Journal of Crohn's and Colitis. 649–670 (2017) doi:10.1093/ecco-jcc/jjx008.
24. Lunder, A. K. *et al.* Prevalence of Sclerosing Cholangitis Detected by Magnetic Resonance Cholangiography in Patients With Long-term Inflammatory Bowel Disease. *Gastroenterology* **151**, 660-669.e4 (2016).
25. Roussomoustakaki, M. *et al.* Genetic markers may predict disease behavior in patients with ulcerative colitis. *Gastroenterology* **112**, 1845–1853 (1997).
26. Satsangi, J., Silverberg, M. S., Vermeire, S. & Colombel, J. F. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* **55**, 749 (2006).
27. Lamb, C. A. *et al.* British Society of Gastroenterology consensus guidelines on the management of inflammatory bowel disease in adults. *Gut* vol. 68 s1–s106 Preprint at https://doi.org/10.1136/gutjnl-2019-318484 (2019).
28. Levine, A. *et al.* Pediatric modification of the Montreal classification for inflammatory bowel disease: The Paris classification. *Inflamm Bowel Dis* **17**, 1314–1321 (2011).
29. Schroeder, K. W., Tremaine, W. J. & Ilstrup, D. M. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* **317**, 1625–1629 (1987).
30. Lewis, J. D. *et al.* Use of the Non-invasive Components of the Mayo Score to Assess Clinical Response in Ulcerative Colitis. *Inflamm Bowel Dis* **14**, 1660 (2008).
31. Coward, S. *et al.* Past and Future Burden of Inflammatory Bowel Diseases Based on Modeling of Population-Based Data. *Gastroenterology* **156**, 1345-1353.e4 (2019).
32. Ng, S. *et al.* Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *thelancet.com*.
33. Windsor, J. W. & Kaplan, G. G. Evolving Epidemiology of IBD. *Current Gastroenterology Reports* vol. 21 Preprint at https://doi.org/10.1007/s11894-019-0705-6 (2019).
34. Al-Bawardy, B., Shivashankar, R. & Proctor, D. D. Novel and Emerging Therapies for Inflammatory Bowel Disease. *Front Pharmacol* **12**, 569 (2021).
35. Donohoe, D. R. *et al.* The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. *Cell Metab* **13**, 517–526 (2011).
36. Den Besten, G. *et al.* The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* **54**, 2325–2340 (2013).
37. Cani, P. D. *et al.* Microbial regulation of organismal energy homeostasis. *Nature Metabolism* vol. 1 34–46 Preprint at https://doi.org/10.1038/s42255-018-0017-4 (2019).
38. Goethel, A., Croitoru, K. & Philpott, D. J. The interplay between microbes and the immune response in inflammatory bowel disease. *J Physiol* **596**, 3869–3882 (2018).

39. Cani, P. D. *et al.* Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. *Diabetes* **56**, 1761–1772 (2007).
40. Everard, A. *et al.* Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc Natl Acad Sci U S A* **110**, 9066–9071 (2013).
41. Dabke, K., Hendrick, G. & Devkota, S. The gut microbiome and metabolic syndrome. *J Clin Invest* **129**, 4050–4057 (2019).
42. Turner, J. R. Intestinal mucosal barrier function in health and disease. *Nature Reviews Immunology 2009 9:11* **9**, 799–809 (2009).
43. France, M. M. & Turner, J. R. The mucosal barrier at a glance. *J Cell Sci* **130**, 307 (2017).
44. Michielan, A. & D'Incà, R. Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis, Clinical Evaluation, and Therapy of Leaky Gut. *Mediators of Inflammation* vol. 2015 Preprint at https://doi.org/10.1155/2015/628157 (2015).
45. Proctor, L. Priorities for the next 10 years of human microbiome research. *Nature* **569**, 623–625 (2019).
46. Lam, W. W. L., Sun, K., Zhang, H. & Au, S. W. N. Crystal Structure of Flagellar Export Chaperone FliS in Complex With Flagellin and HP1076 of Helicobacter pylori. *Front Microbiol* **11**, 515806 (2020).
47. Colomb, F. *et al.* Epithelial mucins and bacterial adhesion. doi:10.1039/9781849739986-00596.
48. Birchenough, G. M. H., Nystrom, E. E. L., Johansson, M. E. V. & Hansson, G. C. A sentinel goblet cell guards the colonic crypt by triggering Nlrp6-dependent Muc2 secretion. *Science* **352**, 1535–1542 (2016).
49. Rios-Covian, D., Gueimonde, M., Duncan, S. H., Flint, H. J. & De Los Reyes-Gavilan, C. G. Enhanced butyrate formation by cross-feeding between Faecalibacterium prausnitzii and Bifidobacterium adolescentis. *FEMS Microbiol Lett* **362**, (2015).
50. Fagundes, R. R. *et al.* Inulin-grown Faecalibacterium prausnitzii cross-feeds fructose to the human intestinal epithelium. *Gut Microbes* **13**, (2021).
51. Chatzidaki-Livanis, M., Geva-Zatorsky, N., Comstock, L. E. & Hooper, L. V. Bacteroides fragilis type VI secretion systems use novel effector and immunity proteins to antagonize human gut Bacteroidales species. *Proc Natl Acad Sci U S A* **113**, 3627–3632 (2016).
52. Fang, J. *et al.* Slimy partners: the mucus barrier and gut microbiome in ulcerative colitis. *Experimental & Molecular Medicine 2021 53:5* **53**, 772–787 (2021).
53. Johansson, M. E. V., Holmén Larsson, J. M. & Hansson, G. C. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *Proc Natl Acad Sci U S A* **108**, 4659–4665 (2011).
54. Li, H. *et al.* The outer mucus layer hosts a distinct intestinal microbial niche. *Nat Commun* **6**, 1–13 (2015).
55. Paone, P. & Cani, P. D. Mucus barrier, mucins and gut microbiota: the expected slimy partners? *Gut* **69**, 2232–2243 (2020).
56. Jostins, L. *et al.* Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491**, 119–124 (2012).
57. Van Der Post, S. *et al.* Structural weakening of the colonic mucus barrier is an early event in ulcerative colitis pathogenesis. *Gut* **68**, 2142–2151 (2019).
58. Korem, T. *et al.* Growth dynamics of gut microbiota in health and disease inferred from single metagenomic samples. *Science* **349**, 1101 (2015).

59. Zeevi, D. *et al.* Structural variation in the gut microbiome associates with host health. *Nature 2019 568:7750* **568**, 43–48 (2019).
60. Huttenhower, C. *et al.* Structure, function and diversity of the healthy human microbiome. *Nature 2012 486:7402* **486**, 207–214 (2012).
61. Rothschild, D. *et al.* Environment dominates over host genetics in shaping human gut microbiota. *Nature* **555**, 210–215 (2018).
62. Jakobsson, H. E. *et al.* Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by Caesarean section. *Gut* **63**, 559–566 (2014).
63. Rodríguez, J. M. *et al.* The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb Ecol Health Dis* **26**, (2015).
64. Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K. & Knight, R. Diversity, stability and resilience of the human gut microbiota. *Nature* **489**, 220–230 (2012).
65. Sepehri, S., Kotlowski, R., Bernstein, C. N. & Krause, D. O. Microbial diversity of inflamed and noninflamed gut biopsy tissues in inflammatory bowel disease. *Inflamm Bowel Dis* **13**, 675–683 (2007).
66. Wang, P. X., Deng, X. R., Zhang, C. H. & Yuan, H. J. Gut microbiota and metabolic syndrome. *Chin Med J (Engl)* **133**, 808 (2020).
67. Tottey, W. *et al.* Colonic Transit Time Is a Driven Force of the Gut Microbiota Composition and Metabolism: In Vitro Evidence. *J Neurogastroenterol Motil* **23**, 124 (2017).
68. Byrne, C. S., Chambers, E. S., Morrison, D. J. & Frost, G. The role of short chain fatty acids in appetite regulation and energy homeostasis. *Int J Obes (Lond)* **39**, 1331 (2015).
69. Smith, P. M. *et al.* The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* **341**, 569–573 (2013).
70. Chang, P. V., Hao, L., Offermanns, S. & Medzhitov, R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci U S A* **111**, 2247–2252 (2014).
71. Tang, Y., Chen, Y., Jiang, H., Robbins, G. T. & Nie, D. G-protein-coupled receptor for short-chain fatty acids suppresses colon cancer. *Int J Cancer* **128**, 847–856 (2011).
72. Round, J. L. *et al.* The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* **332**, 974–977 (2011).
73. Geuking, M. B. *et al.* Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* **34**, 794–806 (2011).
74. Sokol, H. *et al.* Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proceedings of the National Academy of Sciences* **105**, 16731–16736 (2008).
75. Ochoa-Repáraz, J. *et al.* A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. *Mucosal Immunol* **3**, 487–495 (2010).
76. Atarashi, K. *et al.* Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature 2013 500:7461* **500**, 232–236 (2013).
77. Minot, S. *et al.* Rapid evolution of the human gut virome. *Proc Natl Acad Sci U S A* **110**, 12450–12455 (2013).
78. Wang, J., Gao, Y. & Zhao, F. Phage–bacteria interaction network in human oral microbiome. *Environ Microbiol* **18**, 2143–2158 (2016).

79. Reyes, A. *et al.* Viruses in the faecal microbiota of monozygotic twins and their mothers. *Nature* **466**, 334–338 (2010).
80. Norman, J. M., Handley, S. A. & Virgin, H. W. Kingdom-agnostic metagenomics and the importance of complete characterization of enteric microbial communities. *Gastroenterology* **146**, 1459–1469 (2014).
81. Fettweis, J. M. *et al.* Differences in vaginal microbiome in African American women versus women of European ancestry. *Microbiology (Reading)* **160**, 2272–2282 (2014).
82. Li, J. *et al.* An integrated catalog of reference genes in the human gut microbiome. *Nature Biotechnology 2014 32:8* **32**, 834–841 (2014).
83. Turnbaugh, P. J. *et al.* The Human Microbiome Project. *Nature* vol. 449 804–810 Preprint at https://doi.org/10.1038/nature06244 (2007).
84. Wang, J. *et al.* A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature 2012 490:7418* **490**, 55–60 (2012).
85. Fan, Y. & Pedersen, O. Gut microbiota in human metabolic health and disease. *Nature Reviews Microbiology* vol. 19 55–71 Preprint at https://doi.org/10.1038/s41579-020-0433-9 (2021).
86. Cani, P. D. *et al.* Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* **57**, 1470–1481 (2008).
87. Sumida, K. *et al.* Circulating Microbiota in Cardiometabolic Disease. *Front Cell Infect Microbiol* **12**, 892232 (2022).
88. Knox, N. C., Forbes, J. D., Peterson, C. L., Van Domselaar, G. & Bernstein, C. N. The Gut Microbiome in Inflammatory Bowel Disease: Lessons Learned from Other Immune-Mediated Inflammatory Diseases. *American Journal of Gastroenterology* **114**, 1051–1070 (2019).
89. Swanson, K. S. *et al.* Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *The ISME Journal 2011 5:4* **5**, 639–649 (2010).
90. Boudeau, J., Glasser, A. L., Masseret, E., Joly, B. & Darfeuille-Michaud, A. Invasive ability of an Escherichia coli strain isolated from the ileal mucosa of a patient with Crohn's disease. *Infect Immun* **67**, 4499–4509 (1999).
91. Zhang, X. J. *et al.* Palmatine ameliorated murine colitis by suppressing tryptophan metabolism and regulating gut microbiota. *Pharmacol Res* **137**, 34–46 (2018).
92. Qiu, X., Zhang, M., Yang, X., Hong, N. & Yu, C. Faecalibacterium prausnitzii upregulates regulatory T cells and anti-inflammatory cytokines in treating TNBS-induced colitis. *J Crohns Colitis* **7**, e558–e568 (2013).
93. Zhu, S. *et al.* Composition and diverse differences of intestinal microbiota in ulcerative colitis patients. *Front Cell Infect Microbiol* **12**, (2022).
94. Zhu, S. *et al.* Composition and diverse differences of intestinal microbiota in ulcerative colitis patients. *Front Cell Infect Microbiol* **12**, (2022).
95. Chen, W., Liu, F., Ling, Z., Tong, X. & Xiang, C. Human Intestinal Lumen and Mucosa-Associated Microbiota in Patients with Colorectal Cancer. *PLoS One* **7**, e39743 (2012).
96. Sokol, H. *et al.* Low Counts of Faecalibacterium prausnitzii in Colitis Microbiota. *Inflamm Bowel Dis* **15**, 1183–1189 (2009).
97. Smith, F. M. *et al.* A characterization of anaerobic colonization and associated mucosal adaptations in the undiseased ileal pouch. *Colorectal Disease* **7**, 563–570 (2005).

98. Sokol, H., Seksik, P., Furet, J. & Firmesse, O. Low Counts of Faecalibacterium prausnitzii in Colitis Microbiota. *academic.oup.com*.
99. Verma, R., Verma, A. K., Ahuja, V. & Paul, J. Real-time analysis of mucosal flora in patients with inflammatory bowel disease in India. *J Clin Microbiol* **48**, 4279–4282 (2010).
100. Lloyd-Price, J. *et al.* Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* **569**, 655–662 (2019).
101. Mazmanian, S. K., Cui, H. L., Tzianabos, A. O. & Kasper, D. L. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. *Cell* **122**, 107–118 (2005).
102. Rigottier-Gois, L. Dysbiosis in inflammatory bowel diseases: the oxygen hypothesis. *ISME J* **7**, 1256–1261 (2013).
103. Ribaldone, D. G. *et al.* Adalimumab therapy improves intestinal dysbiosis in Crohn's disease. *J Clin Med* **8**, (2019).
104. Kushkevych, I., Dordević, D. & Vítězová, M. Possible synergy effect of hydrogen sulfide and acetate produced by sulfate-reducing bacteria on inflammatory bowel disease development. *J Adv Res* **27**, 71–78 (2021).
105. Yu, S. *et al.* Paneth Cell-Derived Lysozyme Defines the Composition of Mucolytic Microbiota and the Inflammatory Tone of the Intestine. *Immunity* **53**, 398-416.e8 (2020).
106. Png, C. W. *et al.* Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. *American Journal of Gastroenterology* **105**, 2420–2428 (2010).
107. Conte, M. Lo *et al.* Alterations of the intestinal mucus layer correlate with dysbiosis and immune dysregulation in human Type 1 Diabetes. *EBioMedicine* **91**, 104567 (2023).
108. Lee, J. W. J. *et al.* Multi-omics reveal microbial determinants impacting responses to biologic therapies in inflammatory bowel disease. *Cell Host Microbe* **29**, 1294-1304.e4 (2021).
109. Antoni, L., Nuding, S., Wehkamp, J. & Stange, E. F. Intestinal barrier in inflammatory bowel disease. *World J Gastroenterol* **20**, 1165–1179 (2014).
110. Pullan, R. D. *et al.* Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut* **35**, 353–359 (1994).
111. Chen, S. J., Liu, X. W., Liu, J. P., Yang, X. Y. & Lu, F. G. Ulcerative colitis as a polymicrobial infection characterized by sustained broken mucus barrier. *World Journal of Gastroenterology : WJG* **20**, 9468 (2014).
112. Clamp, J. R., Fraser, G. & Read, A. E. Study of the carbohydrate content of mucus glycoproteins from normal and diseased colons. *Clin Sci (Lond)* **61**, 229–234 (1981).
113. Braun, A. *et al.* Alterations of Phospholipid Concentration and Species Composition of the Intestinal Mucus Barrier in Ulcerative Colitis: A Clue to Pathogenesis. (2009) doi:10.1002/ibd.20993.
114. Larsson, J. M. H. *et al.* Altered O-glycosylation profile of MUC2 mucin occurs in active ulcerative colitis and is associated with increased inflammation. *Inflamm Bowel Dis* **17**, 2299–2307 (2011).
115. Raouf, A. H. *et al.* Sulphation of colonic and rectal mucin in inflammatory bowel disease: reduced sulphation of rectal mucus in ulcerative colitis. *Clin Sci* **83**, 623–626 (1992).

116. Gersemann, M., Wehkamp, J. & Stange, E. F. Innate immune dysfunction in inflammatory bowel disease. *Journal of Internal Medicine* vol. 271 421–428 Preprint at https://doi.org/10.1111/j.1365-2796.2012.02515.x (2012).
117. Cunliffe, R. *et al.* Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disease. *Gut* **48**, 176–185 (2001).
118. Müller, C. A., Autenrieth, I. B. & Peschel, A. Innate defenses of the intestinal epithelial barrier. *Cellular and Molecular Life Sciences* **62**, 1297–1307 (2005).
119. Simmonds, N., Furman, M., Karanika, E., Phillips, A. & Bates, A. W. H. Paneth cell metaplasia in newly diagnosed inflammatory bowel disease in children. *BMC Gastroenterol* **14**, 93 (2014).
120. Wehkamp, J. *et al.* Inducible and Constitutive β-Defensins Are Differentially Expressed in Crohn's Disease and Ulcerative Colitis. *Inflamm Bowel Dis* **9**, 215–223 (2003).
121. Gunawardene, A. R., Corfe, B. M. & Staton, C. A. Classification and functions of enteroendocrine cells of the lower gastrointestinal tract. *Int J Exp Pathol* **92**, 219 (2011).
122. Gebert, A., Rothkötter, H. J. & Pabst, R. M cells in Peyer's patches of the intestine. *Int Rev Cytol* **167**, 91–159 (1996).
123. Marshall, J. S., Warrington, R., Watson, W. & Kim, H. L. An introduction to immunology and immunopathology. *Allergy, Asthma and Clinical Immunology* **14**, 1–10 (2018).
124. Zaeem Cader, M. & Kaser, A. Recent advances in inflammatory bowel disease: mucosal immune cells in intestinal inflammation. *Gut* **62**, 1653–1664 (2013).
125. Aijaz, S., Balda, M. S. & Matter, K. Tight junctions: molecular architecture and function. *Int Rev Cytol* **248**, 261–298 (2006).
126. Lee, S. H. Intestinal Permeability Regulation by Tight Junction: Implication on Inflammatory Bowel Diseases. *Intest Res* **13**, 11 (2015).
127. Mees, S. T. *et al.* Expression of tight and adherens junction proteins in ulcerative colitis associated colorectal carcinoma: upregulation of claudin-1, claudin-3, claudin-4, and β-catenin. *Int J Colorectal Dis* **24**, 361–368 (2009).
128. Peterson, L. W. & Artis, D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol* **14**, 141–153 (2014).
129. Martini, E., Krug, S. M., Siegmund, B., Neurath, M. F. & Becker, C. Mend Your Fences: The Epithelial Barrier and its Relationship With Mucosal Immunity in Inflammatory Bowel Disease. *Cell Mol Gastroenterol Hepatol* **4**, 33–46 (2017).
130. Juvonen, P. O., Alhava, E. M. & Takala, J. A. Gut Permeability in Patients with Acute Pancreatitis. *http://dx.doi.org/10.1080/003655200453683* **35**, 1314–1318 (2009).
131. Faries, P. L., Simon, R. J., Martella, A. T., Lee, M. J. & Machiedo, G. W. Intestinal permeability correlates with severity of injury in trauma patients. *J Trauma* **44**, 1031–1036 (1998).
132. Ray, K. Leaky guts: intestinal permeability and NASH. *Nature Reviews Gastroenterology & Hepatology 2015 12:3* **12**, 123–123 (2015).
133. Piche, T. *et al.* Impaired intestinal barrier integrity in the colon of patients with irritable bowel syndrome: involvement of soluble mediators. *Gut* **58**, 196–201 (2009).
134. D'Incà, R. *et al.* Increased intestinal permeability and NOD2 variants in familial and sporadic Crohn's disease. *Aliment Pharmacol Ther* **23**, 1455–1461 (2006).
135. Buhner, S. *et al.* Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation? *Gut* **55**, 342–347 (2006).

136. Teshima, C. W., Dieleman, L. A. & Meddings, J. B. Abnormal intestinal permeability in Crohn's disease pathogenesis. *Ann N Y Acad Sci* **1258**, 159–165 (2012).
137. Nighot, P. K., Hu, C. A. A. & Ma, T. Y. Autophagy enhances intestinal epithelial tight junction barrier function by targeting claudin-2 protein degradation. *J Biol Chem* **290**, 7234–7246 (2015).
138. Peeters, M. *et al.* Clustering of increased small intestinal permeability in families with Crohn's disease. *Gastroenterology* **113**, 802–807 (1997).
139. Halfvarson, J. *et al.* Age determines the risk of familial inflammatory bowel disease—A nationwide study. *Aliment Pharmacol Ther* **56**, 491–500 (2022).
140. Berkowitz, L. *et al.* Impact of cigarette smoking on the gastrointestinal tract inflammation: Opposing effects in Crohn's disease and ulcerative colitis. *Frontiers in Immunology* vol. 9 Preprint at https://doi.org/10.3389/fimmu.2018.00074 (2018).
141. Verschuere, S. *et al.* Cigarette smoking alters epithelial apoptosis and immune composition in murine GALT. *Laboratory Investigation* **91**, 1056–1067 (2011).
142. Madsen, K. L. *et al.* Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. *Inflamm Bowel Dis* **5**, 262–270 (1999).
143. Kennedy, R. J. *et al.* Interleukin 10-deficient colitis: new similarities to human inflammatory bowel disease. *Br J Surg* **87**, 1346–1351 (2000).
144. Poritz, L. S. *et al.* Loss of the tight junction protein ZO-1 in dextran sulfate sodium induced colitis. *J Surg Res* **140**, 12–19 (2007).
145. Gulhane, M. *et al.* High Fat Diets Induce Colonic Epithelial Cell Stress and Inflammation that is Reversed by IL-22. *Sci Rep* **6**, (2016).
146. Rohr, M. W., Narasimhulu, C. A., Rudeski-Rohr, T. A. & Parthasarathy, S. Negative Effects of a High-Fat Diet on Intestinal Permeability: A Review. *Adv Nutr* **11**, 77–91 (2020).
147. Heazlewood, C. K. *et al.* Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med* **5**, 0440–0460 (2008).
148. Wang, B. *et al.* A High-Fat Diet Increases Gut Microbiota Biodiversity and Energy Expenditure Due to Nutrient Difference. *Nutrients* **12**, 1–20 (2020).
149. Parlesak, A., Schäfer, C., Schütz, T., Bode, J. C. & Bode, C. Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease. *J Hepatol* **32**, 742–747 (2000).
150. Vanuytsel, T. *et al.* Psychological stress and corticotropin-releasing hormone increase intestinal permeability in humans by a mast cell-dependent mechanism. *Gut* **63**, 1293–1299 (2014).
151. Fritscher-Ravens, A. *et al.* Confocal endomicroscopy shows food-associated changes in the intestinal mucosa of patients with irritable bowel syndrome. *Gastroenterology* **147**, 1012-1020.e4 (2014).
152. Genser, L. *et al.* Increased jejunal permeability in human obesity is revealed by a lipid challenge and is linked to inflammation and type 2 diabetes. *J Pathol* **246**, 217–230 (2018).
153. Washio, E. *et al.* Proton Pump Inhibitors Increase Incidence of Nonsteroidal Anti-Inflammatory Drug-Induced Small Bowel Injury: A Randomized, Placebo-Controlled Trial. *Clinical Gastroenterology and Hepatology* **14**, 809-815.e1 (2016).

154. Cohen, M. E. *et al.* Prophylaxis for Stress Ulcers With Proton Pump Inhibitors Is Not Associated With Increased Risk of Bloodstream Infections in the Intensive Care Unit. *Clinical Gastroenterology and Hepatology* **15**, 1030-1036.e1 (2017).
155. van Wijck, K. *et al.* Physiology and pathophysiology of splanchnic hypoperfusion and intestinal injury during exercise: Strategies for evaluation and prevention. *Am J Physiol Gastrointest Liver Physiol* **303**, 155–168 (2012).
156. Mokkala, K. *et al.* Gut Microbiota Richness and Composition and Dietary Intake of Overweight Pregnant Women Are Related to Serum Zonulin Concentration, a Marker for Intestinal Permeability. *J Nutr* **146**, 1694–1700 (2016).
157. Gan, J. *et al.* A case for improved assessment of gut permeability: a meta-analysis quantifying the lactulose:mannitol ratio in coeliac and Crohn's disease. *BMC Gastroenterol* **22**, 1–19 (2022).
158. Volynets, V. *et al.* Nutrition, intestinal permeability, and blood ethanol levels are altered in patients with nonalcoholic fatty liver disease (NAFLD). *Dig Dis Sci* **57**, 1932–1941 (2012).
159. Hollander, D. *et al.* Increased intestinal permeability in patients with Crohn's disease and their relatives: A possible etiologic factor. *Ann Intern Med* **105**, 883–885 (1986).
160. Turpin, W. *et al.* Increased Intestinal Permeability Is Associated With Later Development of Crohn's Disease. *Gastroenterology* **159**, 2092-2100.e5 (2020).
161. Teshima, C. W. *et al.* Increased Intestinal Permeability in Relatives of Patients With Crohn's Disease Is Not Associated With Small Bowel Ulcerations. *Clin Gastroenterol Hepatol* **15**, 1413-1418.e1 (2017).
162. Leibovitzh, H. *et al.* Altered Gut Microbiome Composition and Function Are Associated With Gut Barrier Dysfunction in Healthy Relatives of Patients With Crohn's Disease. *Gastroenterology* **163**, 1364-1376.e10 (2022).
163. Olson, T. S. *et al.* The primary defect in experimental ileitis originates from a nonhematopoietic source. **203**, 541–552 (2006).
164. Olson, T. S. *et al.* The primary defect in experimental ileitis originates from a nonhematopoietic source. *Journal of Experimental Medicine* **203**, 541–552 (2006).
165. Arrieta, M. C., Madsen, K. L., Field, C. J. & Meddings, J. B. Increasing small intestinal permeability worsens colitis in the IL-10-/- mouse and prevents the induction of oral tolerance to ovalbumin. *Inflamm Bowel Dis* **21**, 8–18 (2015).
166. Vivinus-Nébot, M. *et al.* Functional bowel symptoms in quiescent inflammatory bowel diseases: role of epithelial barrier disruption and low-grade inflammation. *Gut* **63**, 744–752 (2014).
167. Büning, C. *et al.* Increased Small Intestinal Permeability in Ulcerative Colitis: Rather Genetic than Environmental and a Risk Factor for Extensive Disease? *Inflamm Bowel Dis* **18**, 1932–1939 (2012).
168. Alipour, M. *et al.* Mucosal Barrier Depletion and Loss of Bacterial Diversity are Primary Abnormalities in Paediatric Ulcerative Colitis. *J Crohns Colitis* **10**, 462–471 (2016).
169. Groschwitz, K. R. & Hogan, S. P. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* **124**, 3–22 (2009).
170. Roda, G. *et al.* Intestinal epithelial cells in inflammatory bowel diseases. *World J Gastroenterol* **16**, 4264–4271 (2010).
171. Farquhar, M. G. & Palade, G. E. JUNCTIONAL COMPLEXES IN VARIOUS EPITHELIA. *Journal of Cell Biology* **17**, 375–412 (1963).

172. Tsukita, S., Furuse, M. & Itoh, M. Multifunctional strands in tight junctions. *Nature Reviews Molecular Cell Biology 2001 2:4* **2**, 285–293 (2001).
173. Koch, S. & Nusrat, A. The Life and Death of Epithelia During Inflammation: Lessons Learned from the Gut. *https://doi.org/10.1146/annurev-pathol-011811-120905* **7**, 35–60 (2012).
174. Vetrano, S. *et al.* Unique Role of Junctional Adhesion Molecule-A in Maintaining Mucosal Homeostasis in Inflammatory Bowel Disease. *Gastroenterology* **135**, 173–184 (2008).
175. Edelblum, K. L. & Turner, J. R. The tight junction in inflammatory disease: communication breakdown. *Current Opinion in Pharmacology* vol. 9 715–720 Preprint at https://doi.org/10.1016/j.coph.2009.06.022 (2009).
176. Zeissig, S. *et al.* Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* **56**, 61–72 (2007).
177. Krug, S. M., Schulzke, J. D. & Fromm, M. Tight junction, selective permeability, and related diseases. *Semin Cell Dev Biol* **36**, 166–176 (2014).
178. Salim, S. Y. & Söderholm, J. D. Importance of disrupted intestinal barrier in inflammatory bowel diseases. *Inflammatory Bowel Diseases* vol. 17 362–381 Preprint at https://doi.org/10.1002/ibd.21403 (2011).
179. Bruewer, M. *et al.* Proinflammatory Cytokines Disrupt Epithelial Barrier Function by Apoptosis-Independent Mechanisms. *The Journal of Immunology* **171**, 6164–6172 (2003).
180. Bruewer, M. *et al.* Interferon-γ induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. *The FASEB Journal* **19**, 923–933 (2005).
181. Kucharzik, T., Walsh, S. V., Chen, J., Parkos, C. A. & Nusrat, A. Neutrophil Transmigration in Inflammatory Bowel Disease Is Associated with Differential Expression of Epithelial Intercellular Junction Proteins. *Am J Pathol* **159**, 2001–2009 (2001).
182. Marchiando, A. M. *et al.* Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. *J Cell Biol* **189**, 111–126 (2010).
183. Barollo, M. *et al.* Antioxidative potential of a combined therapy of anti TNFα and Zn acetate in experimental colitis. *World Journal of Gastroenterology : WJG* **17**, 4099 (2011).
184. Tsukita, S. & Furuse, M. The structure and function of claudins, cell adhesion molecules at tight junctions. *Ann N Y Acad Sci* **915**, 129–135 (2000).
185. Mineta, K. *et al.* Predicted expansion of the claudin multigene family. (2011) doi:10.1016/j.febslet.2011.01.028.
186. Furuse, M. *et al.* Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* **156**, (2002).
187. Mrsny, R. J. *et al.* A key claudin extracellular loop domain is critical for epithelial barrier integrity. *Am J Pathol* **172**, 905–915 (2008).
188. Hou, J., Renigunta, A., … J. Y.-P. of the & 2010, undefined. Claudin-4 forms paracellular chloride channel in the kidney and requires claudin-8 for tight junction localization. *National Acad Sciences* **107**, 18010–18015 (2010).
189. Amasheh, S. *et al.* Contribution of claudin-5 to barrier properties in tight junctions of epithelial cells. *Cell Tissue Res* **321**, 89–96 (2005).

190. Alexandre, M. D., Jeansonne, B. G., Renegar, R. H., Tatum, R. & Chen, Y. H. The first extracellular domain of claudin-7 affects paracellular Cl- permeability. *Biochem Biophys Res Commun* **357**, 87–91 (2007).
191. Alexandre, M. D., Lu, Q. & Chen, Y. H. Overexpression of claudin-7 decreases the paracellular Cl- conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. *J Cell Sci* **118**, 2683–2693 (2005).
192. Hou, J., Gomes, A. S., Paul, D. L. & Goodenough, D. A. Study of claudin function by RNA interference. *J Biol Chem* **281**, 36117–36123 (2006).
193. Konrad, M. *et al.* Mutations in the tight-junction gene claudin 19 (CLDN19) are associated with renal magnesium wasting, renal failure, and severe ocular involvement. *Am J Hum Genet* **79**, 949–957 (2006).
194. Milatz, S. *et al.* Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes. *Biochim Biophys Acta* **1798**, 2048–2057 (2010).
195. Amasheh, S. *et al.* Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J Cell Sci* **115**, 4969–4976 (2002).
196. Rosenthal, R. *et al.* Claudin-15 forms a water channel through the tight junction with distinct function compared to claudin-2. *Acta Physiol (Oxf)* **228**, (2020).
197. Garcia-Hernandez, V., Quiros, M. & Nusrat, A. Intestinal epithelial claudins: expression and regulation in homeostasis and inflammation. doi:10.1111/nyas.13360.
198. van Itallie, C. M. *et al.* The density of small tight junction pores varies among cell types and is increased by expression of claudin-2. *J Cell Sci* **121**, 298–305 (2008).
199. Van Itallie, C. M., Fanning, A. S., Bridges, A. & Anderson, J. M. ZO-1 Stabilizes the Tight Junction Solute Barrier through Coupling to the Perijunctional Cytoskeleton. *Mol Biol Cell* **20**, 3930 (2009).
200. Chanez-Paredes, S. D., Abtahi, S., Kuo, W. T. & Turner, J. R. Differentiating between tight junction-dependent and tight junction-independent intestinal barrier loss in vivo. *Methods Mol Biol* **2367**, 249 (2021).
201. Stio, M., Retico, L., Annese, V. & Bonanomi, A. G. Vitamin D regulates the tight-junction protein expression in active ulcerative colitis Vitamin D regulates the tight-junction protein expression in active ulcerative colitis. *Scandinavian Journal of Gastroenterology* **51**, 1193–1191 (2016).
202. Pope, J. L. *et al.* Claudin-1 regulates intestinal epithelial homeostasis through the modulation of Notch-signalling. *Gut* **63**, 622–634 (2014).
203. Poritz, L. S., Harris, L. R., Kelly, A. A. & Koltun, W. A. Increase in the tight junction protein claudin-1 in intestinal inflammation. *Dig Dis Sci* **56**, 2802–2809 (2011).
204. Resnick, M. B., Konkin, T., Routhier, J., Sabo, E. & Pricolo, V. E. Claudin-1 is a strong prognostic indicator in stage II colonic cancer: a tissue microarray study. *Modern Pathology 2005 18:4* **18**, 511–518 (2004).
205. Heller, F. *et al.* Interleukin-13 Is the Key Effector Th2 Cytokine in Ulcerative Colitis That Affects Epithelial Tight Junctions, Apoptosis, and Cell Restitution. *Gastroenterology* **129**, 550–564 (2005).
206. Oshima, T., Miwa, H. & Joh, T. Changes in the expression of claudins in active ulcerative colitis. *J Gastroenterol Hepatol* **23**, S146–S150 (2008).
207. Rosenthal, R. *et al.* Claudin-2, a component of the tight junction, forms a paracellular water channel. *J Cell Sci* **123**, 1913–1921 (2010).

208. Ahmad, R. *et al.* Targeted colonic claudin-2 expression renders resistance to epithelial injury, induces immune suppression, and protects from colitis. *Mucosal Immunol* **7**, 1340–1353 (2014).
209. Weber, C. R., Nalle, S. C., Tretiakova, M., Rubin, D. T. & Turner, J. R. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation. *Lab Invest* **88**, 1110–1120 (2008).
210. Rosen, M. J. *et al.* STAT6 Deficiency Ameliorates Severity of Oxazolone Colitis by Decreasing Expression of Claudin-2 and Th2-Inducing Cytokines. *The Journal of Immunology* **190**, 1849–1858 (2013).
211. Babeu, J. P. & Boudreau, F. Hepatocyte nuclear factor 4-alpha involvement in liver and intestinal inflammatory networks. *World Journal of Gastroenterology : WJG* **20**, 22 (2014).
212. Ahn, S. H. *et al.* Hepatocyte nuclear factor 4α in the intestinal epithelial cells protects against inflammatory bowel disease. *Inflamm Bowel Dis* **14**, 908–920 (2008).
213. Zhong, W., Zhao, Y., McClain, C. J., Kang, Y. J. & Zhou, Z. Inactivation of hepatocyte nuclear factor-4α mediates alcohol-induced downregulation of intestinal tight junction proteins. *Am J Physiol Gastrointest Liver Physiol* **299**, G643 (2010).
214. Markov, A. G., Veshnyakova, A., Fromm, M., Amasheh, M. & Amasheh, S. Segmental expression of claudin proteins correlates with tight junction barrier properties in rat intestine. *J Comp Physiol B* **180**, 591–598 (2010).
215. Prasad, S. *et al.* Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. *Lab Invest* **85**, 1139–1162 (2005).
216. Fujita, H. *et al.* Differential expression and subcellular localization of claudin-7, -8, -12, -13, and -15 along the mouse intestine. *Journal of Histochemistry and Cytochemistry* **54**, 933–944 (2006).
217. Xing, T. *et al.* Tight Junction Protein Claudin-7 Is Essential for Intestinal Epithelial Stem Cell Self-Renewal and Differentiation. *Cell Mol Gastroenterol Hepatol* **9**, 641 (2020).
218. Pai, S. G. *et al.* Wnt/beta-catenin pathway: Modulating anticancer immune response. *J Hematol Oncol* **10**, 1–12 (2017).
219. Tanaka, H. *et al.* Intestinal deletion of Claudin-7 enhances paracellular organic solute flux and initiates colonic inflammation in mice. *Gut* **64**, 1529–1538 (2015).
220. Furuse, M. *et al.* Occludin: A Novel Integral Membrane Protein Localizing at Tight Junctions. *Journal of Cell Biology993) 123 (6): 1777–1788* **123**, 1777–1788 (1993).
221. Cummins, P. M. Occludin: One Protein, Many Forms. *Mol Cell Biol* **32**, 242–250 (2012).
222. Saitou, M. *et al.* Occludin-deficient Embryonic Stem Cells Can Differentiate into Polarized Epithelial Cells Bearing Tight Junctions. *J Cell Biol* **141**, 397–408 (1998).
223. Saitou, M. *et al.* Complex Phenotype of Mice Lacking Occludin, a Component of Tight Junction Strands. *Mol Biol Cell* **11**, 4131 (2000).
224. Buschmann, M. M. *et al.* Occludin OCEL-domain interactions are required for maintenance and regulation of the tight junction barrier to macromolecular flux. *Mol Biol Cell* **24**, 3056–3068 (2013).
225. Mir, H. *et al.* Occludin deficiency promotes ethanol-induced disruption of colonic epithelial junctions, gut barrier dysfunction and liver damage in mice. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1860**, 765–774 (2016).

226. Mankertz, J. *et al.* Gene expression of the tight junction protein occludin includes differential splicing and alternative promoter usage. *Biochem Biophys Res Commun* **298**, 657–666 (2002).
227. Rao, A. S. *et al.* Urine sugars for in vivo gut permeability: validation and comparisons in irritable bowel syndrome-diarrhea and controls. *Am J Physiol Gastrointest Liver Physiol* **301**, (2011).
228. Wang, L. *et al.* Methods to determine intestinal permeability and bacterial translocation during liver disease. *J Immunol Methods* **421**, 44–53 (2015).
229. Sequeira, I. R., Lentle, R. G., Kruger, M. C. & Hurst, R. D. Standardising the Lactulose Mannitol Test of Gut Permeability to Minimise Error and Promote Comparability. *PLoS One* **9**, e99256 (2014).
230. Müller, M., Walker-Smith, J., Shmerling, D. H., Curtius, H. C. & Prader, A. Lactulose: A gas-liquid chromatography method of determination and evaluation of its use to assess intestinal mucosal damage. *Clinica Chimica Acta* **24**, 45–49 (1969).
231. Carulli, N., Salvioli, G. F. & Manenti, F. Absorption of lactulose in man. *Digestion* **6**, 139–145 (1972).
232. Engevik, M. A. *et al.* Bifidobacterium dentium Fortifies the Intestinal Mucus Layer via Autophagy and Calcium Signaling Pathways. *mBio* **10**, (2019).
233. Arrieta, M. C., Bistritz, L. & Meddings, J. B. Alterations in intestinal permeability. *Gut* **55**, 1512–1520 (2006).
234. Hollander, D. Scandinavian Journal of Gastroenterology The Intestinal Permeability Barrier: A Hypothesis as to Its Regulation and Involvement in Crohn's Disease. *Alterations in intestinal permeability* **27**, 721–726 (1992).
235. Wegh, C. A. M. *et al.* Clinical Study Intestinal Permeability Measured by Urinary Sucrose Excretion Correlates with Serum Zonulin and Faecal Calprotectin Concentrations in UC Patients in Remission. (2019) doi:10.1155/2019/2472754.
236. Hoshiko, H. *et al.* An Observational Study to Evaluate the Association between Intestinal Permeability, Leaky Gut Related Markers, and Metabolic Health in Healthy Adults. (2021) doi:10.3390/healthcare9111583.
237. Buda, A. *et al.* Confocal laser endomicroscopy for prediction of disease relapse in ulcerative colitis: A pilot study. *J Crohns Colitis* **8**, 304–311 (2014).
238. Lim, L. G. *et al.* Confocal endomicroscopy identifies loss of local barrier function in the duodenum of patients with Crohn's disease and ulcerative colitis. *Inflamm Bowel Dis* **20**, 892–900 (2014).
239. Liu, J. J. *et al.* Mind the gaps: confocal endomicroscopy showed increased density of small bowel epithelial gaps in inflammatory bowel disease. *J Clin Gastroenterol* **45**, 240–245 (2011).
240. Hering, N. A., Fromm, M. & Schulzke, J. D. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. *J Physiol* **590**, 1035–1044 (2012).
241. Schmitz, H. *et al.* Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterology* **116**, 301–309 (1999).
242. Di Leo, V. *et al.* Lactulose/mannitol test has high efficacy for excluding organic causes of chronic diarrhea. *Am J Gastroenterol* **98**, 2245–2252 (2003).
243. Bruewer, M., Samarin, S. & Nusrat, A. Inflammatory bowel disease and the apical junctional complex. *Ann N Y Acad Sci* **1072**, 242–252 (2006).

244. Weber, C. R. & Turner, J. R. Inflammatory bowel disease: is it really just another break in the wall? *Gut* **56**, 6 (2007).
245. Clayburgh, D. R., Shen, L. & Turner, J. R. A porous defense: the leaky epithelial barrier in intestinal disease. *Lab Invest* **84**, 282–291 (2004).
246. Munkholm, P. *et al.* Intestinal permeability in patients with Crohn's disease and ulcerative colitis and their first degree relatives. *Gut* **35**, 68–72 (1994).
247. Hollander, D. Crohn's disease--a permeability disorder of the tight junction? *Gut* **29**, 1621–1624 (1988).
248. Arrieta, M. C., Madsen, K., Doyle, J. & Meddings, J. Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse. *Gut* **58**, 41–48 (2009).
249. Zolotarevsky, Y. *et al.* A membrane-permeant peptide that inhibits MLC kinase restores barrier function in in vitro models of intestinal disease. *Gastroenterology* **123**, 163–172 (2002).
250. D'Incà, R. *et al.* Intestinal permeability test as a predictor of clinical course in Crohn's disease. *Am J Gastroenterol* **94**, 2956–2960 (1999).
251. Anderson, C. A. *et al.* Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nat Genet* **43**, 246–252 (2011).
252. Barrett, J. C. *et al.* Genome-wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region. *Nat Genet* **41**, 1330–1334 (2009).
253. Kroesen, A. J., Dullat, S., Schulzke, J. D., Fromm, M. & Buhr, H. J. Permanently increased mucosal permeability in patients with backwash ileitis after ileoanal pouch for ulcerative colitis. *Scand J Gastroenterol* **43**, 704–711 (2008).
254. Amasheh, S. *et al.* Inflamed pouch mucosa possesses altered tight junctions indicating recurrence of inflammatory bowel disease. *Int J Colorectal Dis* **24**, 1149–1156 (2009).
255. Merrett, M. N. *et al.* Intestinal permeability in the ileal pouch. *Gut* **39**, 226 (1996).
256. Landy, J. *et al.* Tight junctions in inflammatory bowel diseases and inflammatory bowel disease associated colorectal cancer. *World J Gastroenterol* **22**, 3117–3126 (2016).
257. Gitter, A. H., Wullstein, F., Fromm, M. & Schulzke, J. D. Epithelial barrier defects in ulcerative colitis: Characterization and quantification by electrophysiological imaging. *Gastroenterology* **121**, 1320–1328 (2001).
258. Angelow, S. & Yu, A. S. L. Claudins and paracellular transport: An update. *Curr Opin Nephrol Hypertens* **16**, 459–464 (2007).
259. Morita, K., Furuse, M., Fujimoto, K. & Tsukita, S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proceedings of the National Academy of Sciences* **96**, 511–516 (1999).
260. Turksen, K. & Troy, T. C. Barriers built on claudins. *J Cell Sci* **117**, 2435–2447 (2004).
261. Van Itallie, C., Rahner, C. & Anderson, J. M. Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. *J Clin Invest* **107**, 1319–1327 (2001).
262. Feldman, G. J., Mullin, J. M. & Ryan, M. P. Occludin: Structure, function and regulation. *Adv Drug Deliv Rev* **57**, 883–917 (2005).
263. Fanning, A. S., Jameson, B. J., Jesaitis, L. A. & Anderson, J. M. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem* **273**, 29745–29753 (1998).

264. Yamamoto-Furusho, J. K., Mendivil-Rangel, E. J. & Fonseca-Camarillo, G. Differential Expression of Occludin in Patients with Ulcerative Colitis and Healthy Controls. *Inflamm Bowel Dis* **18**, E1999–E1999 (2012).
265. Kucharzik, T., Walsh, S. V., Chen, J., Parkos, C. A. & Nusrat, A. Neutrophil Transmigration in Inflammatory Bowel Disease Is Associated with Differential Expression of Epithelial Intercellular Junction Proteins. *Am J Pathol* **159**, 2001–2009 (2001).
266. Reife, R. A. *et al.* Porphyromonas gingivalis lipopolysaccharide is poorly recognized by molecular components of innate host defense in a mouse model of early inflammation. *Infect Immun* **63**, 4686–4694 (1995).
267. Raetz, C. R. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles. *scherichia coli and Salmonella: cellular and molecular biology 1* 1035–1063 (1996).
268. Faber, J. *et al.* A toll-like receptor 4 variant is associated with fatal outcome in children with invasive meningococcal disease. *Acta Paediatr* **98**, 548–552 (2009).
269. Agnese, D. M. *et al. Human Toll-Like Receptor 4 Mutations but Not CD14 Polymorphisms Are Associated with an Increased Risk of Gram-Negative Infections*. https://academic.oup.com/jid/article/186/10/1522/832295.
270. Pedersen, C. *et al.* Fecal Enterobacteriales enrichment is associated with increased in vivo intestinal permeability in humans. *Physiol Rep* **6**, e13649–e13649 (2018).
271. Scott, K. P., Martin, J. C., Duncan, S. H. & Flint, H. J. Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, in vitro. *FEMS Microbiol Ecol* **87**, 30–40 (2014).
272. Moreira, A. P. B., Texeira, T. F. S., Ferreira, A. B., Do Carmo Gouveia Peluzio, M. & De Cássia Gonçalves Alfenas, R. Influence of a high-fat diet on gut microbiota, intestinal permeability and metabolic endotoxaemia. *British Journal of Nutrition* vol. 108 801–809 Preprint at https://doi.org/10.1017/S0007114512001213 (2012).
273. Elinav, E. *et al.* NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* **145**, 745–757 (2011).
274. Bloom, S. M. *et al.* Commensal Bacteroides species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease. *Cell Host Microbe* **9**, 390–403 (2011).
275. Garrett, W. S. *et al.* Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell* **131**, 33–45 (2007).
276. Nakanishi, Y., Sato, T. & Ohteki, T. Commensal Gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization. *Mucosal Immunol* **8**, 152–160 (2015).
277. Ginsburg, I. Role of lipoteichoic acid in infection and inflammation. *Lancet Infectious Diseases* vol. 2 171–179 Preprint at https://doi.org/10.1016/S1473-3099(02)00226-8 (2002).
278. Keller, J. F. *et al.* Toll-like receptor 2 activation by lipoteichoic acid induces differential production of pro-inflammatory cytokines in human odontoblasts, dental pulp fibroblasts and immature dendritic cells. *Immunobiology* **215**, 53–59 (2010).
279. Oliveira-Nascimento, L., Massari, P. & Wetzler, L. M. The role of TLR2 ininfection and immunity. *Frontiers in Immunology* vol. 3 Preprint at https://doi.org/10.3389/fimmu.2012.00079 (2012).

280. Boveri, M. *et al.* Highly purified lipoteichoic acid from gram-positive bacteria induces in vitro blood–brain barrier disruption through glia activation: Role of pro-inflammatory cytokines and nitric oxide. *Neuroscience* **137**, 1193–1209 (2006).
281. Kim, H. J. *et al.* Lipoteichoic acid and muramyl dipeptide synergistically induce maturation of human dendritic cells and concurrent expression of proinflammatory cytokines. *J Leukoc Biol* **81**, 983–989 (2007).
282. Rashidi, N. *et al.* Lipopolysaccharide- and Lipoteichoic Acid-mediated Pro-inflammatory Cytokine Production and Modulation of TLR2, TLR4 and MyD88 Expression in Human Endometrial Cells. *J Reprod Infertil* **16**, 72 (2015).
283. Finney, S. J., Leaver, S. K., Evans, T. W. & Burke-Gaffney, A. Differences in lipopolysaccharide- and lipoteichoic acid-induced cytokine/chemokine expression. *Intensive Care Med* **38**, 324–332 (2012).
284. Luo, X. *et al.* Paeoniflorin ameliorates experimental colitis by inhibiting gram-positive bacteria-dependent MDP-NOD2 pathway. *Int Immunopharmacol* **90**, (2021).
285. Nakanishi, Y., Sato, T. & Ohteki, T. Commensal Gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization. *Mucosal Immunol* **8**, 152–160 (2015).
286. Noth, R. *et al.* Increased intestinal permeability and tight junction disruption by altered expression and localization of occludin in a murine graft versus host disease model. *BMC Gastroenterol* **11**, 1–9 (2011).
287. Lee, Y. K. & Mazmanian, S. K. Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science* vol. 330 1768–1773 Preprint at https://doi.org/10.1126/science.1195568 (2010).
288. Volynets, V. *et al.* Intestinal Barrier Function and the Gut Microbiome Are Differentially Affected in Mice Fed a Western-Style Diet or Drinking Water Supplemented with Fructose. *J Nutr* **147**, 770–780 (2017).
289. Martinez-Medina, M. *et al.* Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut* **63**, 116–124 (2014).
290. Bancil, A. S. *et al.* Food Additive Emulsifiers and Their Impact on Gut Microbiome, Permeability, and Inflammation: Mechanistic Insights in Inflammatory Bowel Disease. *J Crohns Colitis* **15**, 1068–1079 (2021).
291. Lerner, A. & Matthias, T. Changes in intestinal tight junction permeability associated with industrial food additives explain the rising incidence of autoimmune disease. *Autoimmun Rev* **14**, 479–489 (2015).
292. Serino, M. *et al.* Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. *Gut* **61**, 543–553 (2012).
293. Hildebrandt, M. A. *et al.* High-Fat Diet Determines the Composition of the Murine Gut Microbiome Independently of Obesity. *Gastroenterology* **137**, 1716-1724.e2 (2009).
294. Hill, C. *et al.* The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* **11**, 506–514 (2014).
295. Tominaga, K. *et al.* Evaluation of intestinal microbiota, short-chain fatty acids, and immunoglobulin a in diversion colitis. *Biochem Biophys Rep* **25**, 100892 (2021).
296. Vernia, P. *et al.* Topical butyrate improves efficacy of 5-ASA in refractory distal ulcerative colitis: results of a multicentre trial. *Eur J Clin Invest* **33**, 244–248 (2003).

297. Bao, B. *et al.* Zinc decreases C-reactive protein, lipid peroxidation, and inflammatory cytokines in elderly subjects: a potential implication of zinc as an atheroprotective agent. *Am J Clin Nutr* **91**, 1634–1641 (2010).
298. Mimura, T. *et al.* Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* **53**, 108 (2004).
299. Bibiloni, R. *et al.* VSL#3 Probiotic-Mixture Induces Remission in Patients with Active Ulcerative Colitis. *Official journal of the American College of Gastroenterology | ACG* **100**, (2005).
300. Malchow, H. A. Crohn's disease and Escherichia coli: a new approach in therapy to maintain remission of colonic Crohn's disease? *J Clin Gastroenterol* **25**, 653–658 (1997).
301. Fujimori, S. *et al.* High dose probiotic and prebiotic cotherapy for remission induction of active Crohn's disease. *Journal of Gastroenterology and Hepatology (Australia)* **22**, 1199–1204 (2007).
302. Wasilewski, A., Zielińska, M., Storr, M. & Fichna, J. Beneficial effects of probiotics, prebiotics, synbiotics, and psychobiotics in inflammatory bowel disease. *Inflammatory Bowel Diseases* vol. 21 1674–1682 Preprint at https://doi.org/10.1097/MIB.0000000000000364 (2015).
303. Lichtenstein, L., Avni-Biron, I. & Ben-Bassat, O. Probiotics and prebiotics in Crohn's disease therapies. *Best Practice and Research: Clinical Gastroenterology* vol. 30 81–88 Preprint at https://doi.org/10.1016/j.bpg.2016.02.002 (2016).
304. Kumari, R. *et al.* Probiotics, prebiotics, and synbiotics: Current status and future uses for human health. in *New and Future Developments in Microbial Biotechnology and Bioengineering* 173–190 (Elsevier, 2020). doi:10.1016/b978-0-12-820528-0.00012-0.
305. Scaldaferri, F. *et al.* Gut microbial flora, prebiotics, and probiotics in IBD: Their current usage and utility. *BioMed Research International* vol. 2013 Preprint at https://doi.org/10.1155/2013/435268 (2013).
306. Gibson, G. R. *et al.* Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol* **14**, 491–502 (2017).
307. Valcheva, R. & Dieleman, L. A. Prebiotics: Definition and protective mechanisms. *Best Practice and Research: Clinical Gastroenterology* vol. 30 27–37 Preprint at https://doi.org/10.1016/j.bpg.2016.02.008 (2016).
308. Scaldaferri, F. *et al.* Gut microbial flora, prebiotics, and probiotics in IBD: Their current usage and utility. *Biomed Res Int* **2013**, (2013).
309. Valcheva, R. *et al.* Soluble Dextrin Fibers Alter the Intestinal Microbiota and Reduce Proinflammatory Cytokine Secretion in Male IL-10–Deficient Mice. *J Nutr* **145**, 2060–2066 (2015).
310. Koh, A., De Vadder, F., Kovatcheva-Datchary, P. & Bäckhed, F. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. *Cell* vol. 165 1332–1345 Preprint at https://doi.org/10.1016/j.cell.2016.05.041 (2016).
311. Tedelind, S., Westberg, F., Kjerrulf, M. & Vidal, A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. *World J Gastroenterol* **13**, 2826–2832 (2007).
312. Makki, K., Deehan, E. C., Walter, J. & Bäckhed, F. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. *Cell Host and Microbe* vol. 23 705–715 Preprint at https://doi.org/10.1016/j.chom.2018.05.012 (2018).

313. Rodríguez-Cabezas, M. E. *et al.* Intestinal anti-inflammatory activity of dietary fiber (Plantago ovata seeds) in HLA-B27 transgenic rats. *Clinical Nutrition* **22**, 463–471 (2003).
314. Segain, J.-P. *et al.* Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease. *Gut* **47**, 397 (2000).
315. Weng, M., Walker, W. A. & Sanderson, I. R. Butyrate regulates the expression of pathogen-triggered IL-8 in intestinal epithelia. *Pediatr Res* **62**, 542–546 (2007).
316. Devriese, S. *et al.* Activity in Patients with Ulcerative Colitis Correlates with Aberrant Claudin-1 Expression. *J Crohns Colitis* **11**, 229–236 (2017).
317. Daly, K. & Shirazi-Beechey, S. P. Microarray analysis of butyrate regulated genes in colonic epithelial cells. *DNA Cell Biol* **25**, 49–62 (2006).
318. Capitán-Cañadas, F. *et al.* Fructooligosaccharides exert intestinal anti-inflammatory activity in the CD4+ CD62L+ T cell transfer model of colitis in C57BL/6J mice. *Eur J Nutr* **55**, 1445–1454 (2016).
319. Capitán-Cañadas, F. *et al.* Prebiotic oligosaccharides directly modulate proinflammatory cytokine production in monocytes via activation of TLR4. *Mol Nutr Food Res* **58**, 1098–1110 (2014).
320. Liao, M. *et al.* Fructooligosaccharide supplementation alleviated the pathological immune response and prevented the impairment of intestinal barrier in DSS-induced acute colitis mice. *Food Funct* **12**, 9844–9854 (2021).
321. Cherbut, C., Michel, C. & Lecannu, G. The Prebiotic Characteristics of Fructooligosaccharides Are Necessary for Reduction of TNBS-Induced Colitis in Rats. *J Nutr* **133**, 21–27 (2003).
322. Welters, C. F. M. *et al.* Effect of dietary inulin supplementation on inflammation of pouch mucosa in patients with an ileal pouch-anal anastomosis. *Dis Colon Rectum* **45**, 621–627 (2002).
323. Furrie, E. *et al.* Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* **54**, 242–249 (2005).
324. Casellas, F. *et al.* Oral oligofructose-enriched inulin supplementation in acute ulcerative colitis is well tolerated and associated with lowered faecal calprotectin. *Aliment Pharmacol Ther* **25**, 1061–1067 (2007).
325. Valcheva, R. *et al.* Inulin-type fructans improve active ulcerative colitis associated with microbiota changes and increased short-chain fatty acids levels. *Gut Microbes* **10**, 334–357 (2019).
326. Wilson, B. *et al.* Prebiotic galactooligosaccharide supplementation in adults with ulcerative colitis: Exploring the impact on peripheral blood gene expression, gut microbiota, and clinical symptoms. *Nutrients* **13**, (2021).
327. Valcheva, R. *et al.* Double blind placebo-controlled trial for the prevention of ulcerative colitis relapses by β-fructan prebiotics: efficacy and metabolomic analysis. (2022) doi:10.1101/2022.01.16.22269376.
328. Mitmesser, S. & Combs, M. *The Microbiota in Gastrointestinal Pathophysiology Prebiotics: Inulin and Other Oligosaccharides*. (2017).
329. Fraberger, V., Call, L. M., Domig, K. J. & D'Amico, S. Applicability of yeast fermentation to reduce fructans and other FODMAPs. *Nutrients* **10**, (2018).

330. Furrie, E. *et al.* Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* **54**, 242–249 (2005).
331. CASELLAS, F. *et al.* Oral oligofructose-enriched inulin supplementation in acute ulcerative colitis is well tolerated and associated with lowered faecal calprotectin. *Aliment Pharmacol Ther* **25**, 1061–1067 (2007).
332. Koleva, P., Ketabi, A., Valcheva, R., Gänzle, M. G. & Dieleman, L. A. Chemically Defined Diet Alters the Protective Properties of Fructo-Oligosaccharides and Isomalto-Oligosaccharides in HLA-B27 Transgenic Rats. *PLoS One* **9**, e111717 (2014).
333. Armstrong, H. K. *et al.* Unfermented β-fructan Fibers Fuel Inflammation in Select Inflammatory Bowel Disease Patients. *Gastroenterology* **164**, 228–240 (2023).
334. Anderson, J. L. *et al.* Dietary intake of inulin-type fructans in active and inactive Crohn's disease and healthy controls: a case–control study. *J Crohns Colitis* **9**, 1024–1031 (2015).
335. Wang, Y. *et al.* Structural characterization of water-soluble polysaccharide from Arctium lappa and its effects on colitis mice. *Carbohydr Polym* **213**, 89–99 (2019).
336. Russo, F. *et al.* Inulin-enriched pasta improves intestinal permeability and modifies the circulating levels of zonulin and glucagon-like peptide 2 in healthy young volunteers. *Nutrition Research* **32**, 940–946 (2012).
337. Salim, S. Y. & Söderholm, J. D. Importance of disrupted intestinal barrier in inflammatory bowel diseases. *Inflammatory Bowel Diseases* vol. 17 362–381 Preprint at https://doi.org/10.1002/ibd.21403 (2011).
338. Kuo, W. T. *et al.* Inflammation-induced Occludin Downregulation Limits Epithelial Apoptosis by Suppressing Caspase-3 Expression. *Gastroenterology* **157**, 1323–1337 (2019).
339. Van De Wiele, T., Boon, N., Possemiers, S., Jacobs, H. & Verstraete, W. Inulin-type fructans of longer degree of polymerization exert more pronounced in vitro prebiotic effects. *J Appl Microbiol* **102**, 452–460 (2007).
340. Le Bastard, Q. *et al.* The effects of inulin on gut microbial composition: a systematic review of evidence from human studies. *European Journal of Clinical Microbiology and Infectious Diseases* **39**, 403–413 (2020).
341. Scott, K. P., Martin, J. C., Duncan, S. H. & Flint, H. J. Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, in vitro. *FEMS Microbiol Ecol* **87**, 30–40 (2014).
342. Ohura, K., Nishiyama, H., Saco, S., Kurokawa, K. & Imai, T. Establishment and Characterization of a Novel Caco-2 Subclone with a Similar Low Expression Level of Human Carboxylesterase 1 to Human Small Intestine. *Drug Metabolism and Disposition* **44**, 1890–1898 (2016).
343. Bas, A., Forsberg, G., Hammarström, S. & Hammarström, M.-L. Utility of the Housekeeping Genes 18S rRNA, b-Actin and Glyceraldehyde-3-Phosphate-Dehydrogenase for Normalization in Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis of Gene Expression in Human T Lymphocytes. *Scandinavian Journal of Immunology* **59**, 566–573 (2004).
344. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408 (2001).

345. Pearson, A. D. J., Eastham, E. J., Laker, M. F., Craft, A. W. & Nelson, R. Intestinal permeability in children with Crohn's disease and coeliac disease. *Br Med J (Clin Res Ed)* **285**, 20–21 (1982).
346. Pastorelli, L. *et al.* Procoagulatory state in inflammatory bowel diseases is promoted by impaired intestinal barrier function. *Gastroenterol Res Pract* **2015**, (2015).
347. Luettig, J., Rosenthal, R., Barmeyer, C. & Schulzke, J. D. Claudin-2 as a mediator of leaky gut barrier during intestinal inflammation. *https://doi.org/10.4161/21688370.2014.977176* **3**, (2015).
348. Tan, Y., Guan, Y., Sun, Y. & Zheng, C. Correlation of Intestinal Mucosal Healing and Tight Junction Protein Expression in Ulcerative Colitis Patients. *Am J Med Sci* **357**, 195–204 (2019).
349. Mendall, M. A., Chan, D., Patel, R. & Kumar, D. Faecal calprotectin: factors affecting levels and its potential role as a surrogate marker for risk of development of Crohn's Disease. *BMC Gastroenterol* **16**, (2016).
350. Peng, L., Li, Z. R., Green, R. S., Holzman, I. R. & Lin, J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J Nutr* **139**, 1619–1625 (2009).
351. Valenzano, M. C. *et al.* Remodeling of Tight Junctions and Enhancement of Barrier Integrity of the CACO-2 Intestinal Epithelial Cell Layer by Micronutrients. *PLoS One* **10**, (2015).
352. Yan, H. & Ajuwon, K. M. Butyrate modifies intestinal barrier function in IPEC-J2 cells through a selective upregulation of tight junction proteins and activation of the Akt signaling pathway. *PLoS One* **12**, (2017).
353. Saleri, R. *et al.* Effects of different short-chain fatty acids (SCFA) on gene expression of proteins involved in barrier function in IPEC-J2. *Porcine Health Manag* **8**, 1–13 (2022).
354. Premraj, N., Valcheva, R. & Dieleman, L. A. A230 ISOMALTODEXTRIN DOSE-DEPENDENTLY REDUCES COLITIS DEVELOPMENT IN HLA-B27 RAT COLITIS MODEL WITH ASSOCIATED CHANGES TO GUT MICROBIOME COMPOSITION AND SHORT CHAIN FATTY ACID PRODUCTION. *J Can Assoc Gastroenterol* **4**, 275–276 (2021).
355. Haskell, H. *et al.* Pathologic features and clinical significance of 'backwash' ileitis in ulcerative colitis. *American Journal of Surgical Pathology* **29**, 1472–1481 (2005).
356. Heuschen, U. A. *et al.* Backwash ileitis is strongly associated with colorectal carcinoma in ulcerative colitis. *Gastroenterology* **120**, 841–847 (2001).
357. Arango-González, A. *et al.* Putative intestinal permeability markers do not correlate with cardiometabolic health and gut microbiota in humans, except for peptides recognized by a widely used zonulin ELISA kit. *Nutrition, Metabolism and Cardiovascular Diseases* **33**, 112–123 (2023).
358. Guo, S., Al-Sadi, R., Said, H. M. & Ma, T. Y. Lipopolysaccharide Causes an Increase in Intestinal Tight Junction Permeability in Vitro and in Vivo by Inducing Enterocyte Membrane Expression and Localization of TLR-4 and CD14. *Am J Pathol* **182**, 375–387 (2013).
359. Williams, J. M. *et al.* A mouse model of pathological small intestinal epithelial cell apoptosis and shedding induced by systemic administration of lipopolysaccharide. *DMM Disease Models and Mechanisms* **6**, 1388–1399 (2013).
360. Reveille, J. D. The genetic basis of spondyloarthritis. *Ann Rheum Dis* **70**, i44–i50 (2011).

361. Turner, M. J. *et al.* HLA-B27 Misfolding in Transgenic Rats Is Associated with Activation of the Unfolded Protein Response. *The Journal of Immunology* **175**, 2438–2448 (2005).
362. Deehan, E. C. *et al.* Elucidating the role of the gut microbiota in the physiological effects of dietary fiber. *Microbiome* **10**, (2022).