Prevention of Inflammatory Bowel Disease by Broccoli-sourced and Microbially-produced Bioactives

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PREVENTION OF INFLAMMATORY BOWEL DISEASE BY BROCCOLI-SOURCED AND MICROBIALLY-PRODUCED BIOACTIVES.

By

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B.S. Husson University

A THESIS

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science (In Food Science and Human Nutrition)

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Inflammatory Bowel Diseases (IBD) are chronic, reoccurring, and debilitating conditions characterized by inflammation in the gastrointestinal tract, some of which can lead to more systemic complications and can include autoimmune dysfunction, a change in the taxonomic and functional structure of microbial communities in the gut, and complicated burdens in a person’s daily life. Like many diseases based in chronic inflammation, research on IBD has pointed towards a multifactorial origin involving factors of the host’s lifestyle, immune system, associated microbial communities, and environmental conditions. Too often, research focuses on just one aspect of IBD or uses one model with a narrow scope, that may result in unanticipated microbial changes, or that are not representative of genetic factors. This is reflected in the absence of genetic models in biochemical-centric research focused on the role of broccoli-metabolite sulforaphane (SFN) in preventing and treating IBD. To be an accurate reflection of IBD, research studies should expand their scope, for example by addressing the concepts of biogeographic specificity of both nutrient absorption and microbial community dynamics, or by using multiple research tools to better mimic the multiple presentations of IBD.

To date, no previous SFN or broccoli diet studies have used the IL-10-ko mouse model. With our study, we sought to cover this research gap by, first, proofing broccoli dietary measures in IL-10-ko mice that have a Crohn’s disease-like presentation of inflammation. We fed IL-10-ko mice either a broccoli diet or a control diet, initiated inflammation, and assessed that inflammation
using bodyweight gain, a disease activity index score, and immunohistology. All three of the parameters measured showed a consistent and marked reduction of inflammation in mice that were fed a broccoli diet. To assess the performance of this study, we also compared the bodyweight results of our novel IL-10-ko model to the results of an established dextran sulfate sodium (DSS) model of IBD. As expected, the broccoli diet prevented inflammation in the DSS model when compared to control diet fed mice. Excitingly, the IL-10 model had a much more pronounced effect on bodyweight gain, suggesting IL-10-ko mice may be an excellent environment for studying broccoli diet and SFN interactions with gut microbes.
DEDICATION

I would like to dedicate this thesis to my husband and his family, who have financially supported me throughout my graduate education and made working at graduate pay possible. I would also like to dedicate this thesis to my friends, who provided valuable feedback and proofreading on many assignments and graduate writings.
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GLOSSARY OF TERMS

Colorectal Cancer (CRC): a devastating cancer of the colon or rectum that is the 3rd leading cause of cancer related deaths.

Crohn’s Disease (CD): a form of IBD characterized by inflammation of several layers of the intestinal tissues which can occur along the length of the intestines, and which may have acute or chronic presentation.

Cytokines: peptides used for cell signaling which cannot cross the lipid bilayer membrane of eukaryotic cells and acts externally. A subclass is myokine, which are released by and have action in muscle tissue.

Dextran Sodium Sulfate (DSS): A salt and chemical colitogen that is widely used to induce colitis in mice.

Fecal Microbial Transplant (FMT): a transplant of fecal bacteria from the stool of a healthy donor to another individual. Often used for treatment of Clostridium difficile infection.

Gastrointestinal (GI): the digestive tract from the stomach to the colon.

Genome Wide Association Studies (GWAS): an observational study that involves a genome wide search for genetic variant trait associations in multiple individuals.

Glucoraphanin (GLR): the primary glucosinolate found in broccoli.

Glucosinolates (GSLs): a group of plant secondary compounds to protect against insect herbivory which contain nitrogen and sulfur, and which give cruciferous vegetables their distinct odor.

Inflammatory Bowel Diseases (IBD): a collection of symptoms indicating an immunological disorder of the gastrointestinal tract.
**Inflammatory Bowel Syndrome (IBS):** a collection of symptoms indicating a functional disorder of the gastrointestinal tract.

**Interleukin-10 (IL-10):** an anti-inflammatory cytokine which facilitates communication during immunoregulation and inflammation. Production of IL-10 downregulates Th1 and encourages B cell survival.

**Interleukin-10 knockout mice (IL-10-ko):** a mouse model which has had the entire IL-10 gene locus deleted through CRISPR/Cas9-mediated gene editing.

**Isothiocyanates (ITCs):** a group of byproducts of enzymatic conversation of GSLs, many of which confer protection to plants against insect herbivory but which also may act as anti-inflammatory compounds in mammals.

**Nuclear Factor Kappa B (NF-kB):** a protein complex that controls DNA transcription of a number of pro-inflammatory cytokines.

**Microbiome:** The collection of microorganism genomes found in a particular location or situation, that may include genomes from bacteria, archaea, fungi, protists, and viruses.

**Sulforaphane (SFN):** The primary bioactive hydrolysis product of glucoraphanin.

**T helper cells (Th cells):** T cells, such as Th1, that are important in the adaptive immune response.

**Ulcerative colitis (UC):** a form of IBD characterized by inflammation of the mucosal layer of intestinal epithelia mostly confined to the colon, and which may have acute or chronic presentation.
CHAPTER 1
INTERPLAY OF BROCCOLI/BROCCOLI SPROUT BIOACTIVES WITH GUT MICROBIOTA
IN REDUCING INFLAMMATION IN INFLAMMATORY BOWEL DISEASES

Abstract

Inflammatory Bowel Diseases (IBD) are chronic, reoccurring, and debilitating conditions characterized by inflammation in the gastrointestinal tract, some of which can lead to more systemic complications and can include autoimmune dysfunction, a change in the taxonomic and functional structure of microbial communities in the gut, and complicated burdens in a person’s daily life. Like many diseases based in chronic inflammation, research on IBD has pointed towards a multifactorial origin involving factors of the host’s lifestyle, immune system, associated microbial communities, and environmental conditions. Treatment currently exists only as palliative care, and seeks to disrupt the feedback loop of symptoms by reducing inflammation and allowing as much of a return to homeostasis as possible. Various anti-inflammatory options have been explored, and this review focuses on the use of diet as a holistic means of improving gut health. Specifically, we highlight the connection between the role of sulforaphane from cruciferous vegetables in regulating inflammation and in modifying microbial communities, and to break down the role they play in IBD.

Keywords: inflammatory bowel disease, Crohn’s Disease, ulcerative colitis, broccoli, broccoli sprouts, sulforaphane, glucoraphanin, gut microbiota, dietary bioactives.

Statement of Significance: This review highlights the importance of the interplay between broccoli sprout bioactives and gut microbiota and its important role in reducing inflammation in inflammatory bowel disease.
1 Introduction

Chronic inflammatory diseases involve interactions between a macroorganism host and its immune system, the microorganisms which associate with or encounter that host, and the environmental conditions of the host which include the collective exposures to chemical, microbial, or other stressors. Understanding these complex diseases and ways to circumvent them requires a holistic research framework [1,2]. Not only do inflammatory diseases contribute to dramatic morbidity and mortality, but they can create negative knock-on effects such as social or lifestyle restrictions in service to disease management [3,4], and an increased risk for a variety of cancers [5]. Inflammatory Bowel Diseases (IBD) are notable examples of chronic diseases which involve host-microbe-environment interactions in the gastrointestinal (GI) tract that can significantly disrupt daily life. Prolonged inflammation has also been linked to a higher risk of colorectal cancer (CRC) development [6–9].

There are two major presentations of IBD (Fig. 1). Ulcerative colitis (UC) is primarily limited to the colon, where inflammation occurs along the mucosal epithelial layer [10]. Crohn’s Disease (CD) is an autoimmune disorder where inflammation can occur along the entire length of the GI tract [7], can be transmural, and can be associated with additional complications such as fistulas and strictures. Some patients with IBD show overlapping pathologic features of UC and CD [11]. Both UC and CD patients experience fluctuations between active periods of inflammation and periods of reduced disease activity. Symptoms include abdominal pain, diarrhea, bloody stool, weight loss, and fatigue. In the gut, both presentations of IBD can involve compromised gut epithelial barrier function and alterations to the gut microbial communities, discussed in detail further in this review.
Figure 1. Primary Inflammatory Bowel Disease presentations, ulcerative colitis and Crohn’s disease, compared to a healthy colon. UC is limited to the colon and is characterized by pseudopolyps and haustra loss (smoothing and shortening of the colon). CD can occur anywhere in the GI tract and is characterized by fissures, muscle thickening, and “cobblestoning”, which is the unique bubbling of the interior wall.

IBD has burgeoning global prevalence, with the greatest incidence rates in the United States, Canada, the United Kingdom, and western European countries and increasing incidence rates in nations which are assimilating those diets or industrialization of their food systems and sociopolitical or cultural structure [12,13]. The United States Centers for Disease Control and Prevention estimates 1.3% of U.S. adults have reported a diagnosis for IBD, and the risk for IBD development is highest in populations with lifestyle situations that are associated with increased stress and reduced access to resources, such as lower education rates, higher unemployment rates, or those living in poverty [14]. Diagnosis usually occurs between the ages of 20 and 40,
and people diagnosed with IBD are more likely to have concurrent cardiovascular disease, cancer, arthritis, and kidney or liver disease, all of which typically have a chronic inflammation component. IBD poses a significant impact on the healthcare industry in the US. In 2015, health care expenditures for GI disorders totalled $135.9 billion, with IBD ranking in the top five most expensive, at $7.2 billion in the US. From 2006 to 2014, the annual number of visits to the emergency department for IBD increased by 52%, and the number of hospitalizations from 2005 to 2014 increased by 13%. In addition, 13% of IBD cases are readmitted to the hospital within 30 days [15].

Palliative treatment for IBD reduces symptoms, and often targets inflammation as a way to recover host homeostasis and a functional microbial community in the gut, and achieve an overall reduction in side effects. However, current over-the-counter and prescription drugs for inflammation may cause ulcers or other symptoms. The risk of developing colorectal cancer (CRC) increases with IBD duration [6–9]. Non-steroidal anti-inflammatory treatments and corticosteroids decrease risk of cancer incidence and mortality, and are widely available for the management of inflammatory diseases [16]. However, they have been associated with a variety of serious side effects, including stomach ulcers and a disordering of the microbial community structure in the gut, i.e. cause gut dysbiosis, and there is a growing need for non-steroidal treatment options that address chronic inflammation while preserving GI function and microbial communities.

Much is still unknown about IBD etiologies and development, however research has pointed towards a multifactorial origin. Environmental triggers for IBD include smoking, history of surgery, oral contraceptives, geography, socioeconomic status, infection, and diet [17,18], and host genetics and immune factors are also involved. Collectively, these factors are also known to alter host-associated microbial community structure and function, i.e. cause dysbiosis, which is a hallmark of IBD. Microbial-based therapeutics, such as probiotics or fecal microbial transplant (FMT) show promising results for resolving symptoms, but are often used as treatments rather
than preventatives. However, diet is a crucial source of plant-derived bioactives which could provide non-steroidal anti-inflammatory compounds as a preventative strategy, and many of these metabolites have been demonstrated to drive host-microbial homeostasis and gut health [19]. This review explores a burgeoning research area: using dietary bioactives as an IBD intervention by directly providing anti-inflammatory compounds or indirectly providing the precursors which attract functional microorganisms to create anti-inflammatory compounds.

2 Current Status of Knowledge

2.1 Genetics and Immune Modulation of IBD

IBD has long been associated with genetic risk factors. There is increased prevalence in relatives of affected individuals as well as higher rates of disease among monozygotic versus dizygotic twins [20]. With the sequencing of the human genome, genome-wide association studies (GWAS) have elucidated over 200 gene loci associated with IBD [21,22]. Many of these genes play key roles in the proper functioning of the immune system, and their altered expression impacts important functions such as autophagy (ATG16L1, IRGM); mucosal barrier function and pathogen recognition and clearance (NOD2); and T cell response (IL10, IL21) [23]. Defects stemming from genetic alteration can result in impairment of proper immune cell function, leading to aberrant immune responses such as release of pro-inflammatory factors and dampened anti-inflammatory responses, further discussed below.

Dysregulated inflammation of the GI tract is a hallmark feature of IBD pathogenesis. Under normal conditions, inflammation is part of the innate immune response to harmful stimuli including microbial infiltration of tissues and tissue damage. Inflammation acts to support the protective mucosal barrier between luminal microorganisms and the host epithelium [24,25], in an effort to neutralize the irritating stimulus and restore homeostasis. The dysregulated inflammatory and
immune response to damage and/or pathogens leads to the chronicity of inflammation seen in IBD patients.

Acute inflammation can be initiated by the host upon recognition of pathogen-associated molecular patterns (PAMPs) on or in microbial cells, and damage-associated molecular patterns (DAMPs) released from damaged host cells [26], by pattern recognition receptors (PRRs) [27]. PRRs, which include the family of Toll-like receptors (TLRs), are upregulated in the cells of IBD patients, and activate signaling cascades that lead to the recruitment of immune cells in gut tissues. Thus, IBD is marked by accumulations of CD4 and CD8 T lymphocytes at the disease site in attempts to ward off infections. Upon arrival, CD4 and CD8 may differentiate into T-helper cell types Th1 or Th2 [7]. Th1 and Th2 aggregation results in the increased production of proinflammatory cytokines, which coordinate the immune response. UC is reportedly driven by an aberrant Th2 response [28], while CD is linked to Th1 [29]. Acute inflammation becomes chronic when the target stimuli fails to be eliminated, and resolution pathways are dysregulated [5,24].

Pro-inflammatory interleukin (IL) cell signaling peptides IL-6 and IL-8 are upregulated and found in higher concentrations in the plasma and serum of patients with IBD and CRC, which has been implicated in the development and maintenance of these diseases [30–33]. Each cytokine is part of a complex signaling cascade, thus there are numerous targets that could produce the aforementioned effects. For example, IL-6 is part of the IL-6/JAK/STAT3 pathway, where STAT3 is a transcription factor for IL-6, and STAT3 inhibition results in IL-6 downregulation. Glycoprotein 130 (gp130) activates phosphorylation of STAT3 which results in dimerization, translocation, and IL-6 gene transcription [32–37]. IL-6 signaling and IL-6 trans signaling can result in TLR activation of pro-inflammatory signaling pathways, resulting in cancerous cell survival and evasion of detection by immune cells. Thus, dietary bioactives such as sulforaphane, further discussed below, could act at multiple steps along the pathway to mitigate the inflammatory response.

IL-8 upregulation results in a pro-inflammatory response through increased neutrophil recruitment [24,30,38]. IL-8 transcription is dependent on the translocation of NF-κB p65 and p50
subunits across the nuclear membrane, and subsequent attachment at the IL-8 gene promoter [31]. Thus, inhibition of NF-κB results in downregulation of IL-8. The NF-κB subunits undergo nuclear translocation when TLR4 is activated at the cell surface and interacts with myeloid differentiation factor 88 (MyD88) [39,40], resulting in Bcl10 phosphorylation and combination with mucosa associated lymphoma translocation protein 1 (MALT1) [41]. The Bcl10/MALT1 complex ubiquitinates the IκB kinase-γ (IKKγ), which phosphorylates IκB, causing dissociation from the p65 and p50 subunits and subsequent translocation. Due to the complexity of the signaling cascades involved in the production of ILs and furthered by their presence, there are many possible targets for intervention when contemplating reducing inflammation. Relevant targets for reduction include pro-inflammatory cytokines, such as IL-6, IL-8, IL-12, IL-23 and IL-21, and targets for stimulation include anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β.

With over 200 identified risk loci [21,22], the genetics at play in IBD are complicated to say the least. GWAS studies have indicated that UC and CD share 110/163 loci, reflecting the clinical similarity seen in these disorders, but these studies have also found loci distinct to each pathology as well as some detrimental in CD but protective in UC [21,42]. Moreover, more than 50% of the loci associated with IBD are also associated with other immune-mediated diseases, namely ankylosing spondylitis, psoriasis, and primary sclerosing cholangitis, indicating potential genetic links between inflammatory diseases previously considered distinct [42]. However, it is clear that a person’s genes are not the only factor in determining their risk for IBD, as the identified susceptibility genes account for less than 25% of predicted heritability [23]. Also of note, most GWAS studies have been conducted in Caucasian or Asian populations, and the same genes have not been implicated in GWAS studies in other populations [43]. Therefore, it is paramount to examine the other factors at play in the gut to expand our understanding of IBD pathogenesis.
2.2 Gut Microbiota, Biogeography, and IBD

In addition to inflammation and other innate immune responses (e.g. mucus production), invasion of pathogenic bacteria into body tissues is prevented by commensal bacteria [44]. The taxonomic and functional structure of microbial communities found along the GI tract are highly dependent on the diet, health status, age, and microbial encounters of the host [45–47]. Specific organs and sites within organs foster different environmental conditions that can create spatial niches for microbial taxa, an ecological occurrence known as biogeography [48–50]. For example, pH changes dramatically along the GI tract and is linked to microbial diversity and density. IBD patients often have a less acidic GI tract [51] due to altered diet or treatments, and this may result in alterations to gut biogeography such as small intestinal bacterial overgrowth [52]. In particular, alterations in the mucosal-associated bacterial fraction are altered in IBD patients as compared to healthy participants [53,54].

Initial colonization of the human GI tract occurs at birth via exposures to environmental sources of microorganisms. However the most functional exposures have been linked to vaginal birth, breast feeding, and other sources of human-associated microbiota [55–57]. This first population of the gut is crucial in developing and maintaining epithelial barrier function [58], for example by stimulating epithelial cell division and growth, mucus production, angiogenesis (which supports epithelial cells), and neurogenesis (which supports gut motility). Early-life GI infections and disease have not been associated with IBD risk later in life [59], however, disruptions in the process of host-association and microbially-induced immune tolerance have been associated with IBD risk [60].

Importantly, microbial community diversity in the GI is much reduced in animal and human models of IBD, suggesting that more understanding may be gained by assessing microbial makeup at the species or strain levels, as key ecological roles could be left open as certain taxa are absent from the gut [61]. Patients with GI inflammation were found to have altered richness of microbial taxonomic profiles, however, literature on this phenomenon has inconsistent findings,
likely due to a combination of small sample sizes, nuances in methods, relying on fecal microbiota as a proxy for other GI populations, and the situation-specific trends of the complex human gut microbiome. It is not always clear which taxa may be contributing to inflammation and which are altered simply through changing environmental conditions in an inflamed gut. Symptoms may also be related to the loss of species associated with health benefits, such as UC patients with decreased abundance of Lactobacilli, Roseburia hominis and Faecalibacterium prausnitzii [62]. Conversely, CD patients have increased abundance in Faecalibacterium prausnitzii [63], as well as in Ruminococcus gnavus, and decreased abundance in Dialister invisus and Bifidobacterium adolescentis [64].

In addition to these nuances, species-level competition in the gut can have life-stage-specific benefits or detriments. For example, exposure to microbial diversity [65] and even non-pathogenic strains of Escherichia coli early in life can prime the immune system [66], yet UC and CD patients have been reported to have an increased abundance of pathogenic strains of E. coli during disease flare-ups [62,67]. This shift may be triggered by low-fiber/high-fat diets which are known to shift host metabolites, as well as entice E. coli and other pathobionts to the gut, which further shifts host-microbial interactions, increases inflammation, and results in a slightly high oxygen content along the epithelial wall which could result in the creation of cell-damaging reactive oxygen species [68]. Similarly, infection with certain strains of Helicobacter pylori, best known for causing ulcerative infections in the stomach and intestines, is associated with a large reduction in IBD risk as its presence triggers more immune monitoring [69]. However, H. pylori is also found in patients with IBD, and is commonly used to trigger CD-like symptoms in immune-deficient mouse models.

Complicated species-level antagonism can remodel gut communities [70]. Bacteroides fragilis has been identified as a gut commensal and early-life colonizer [71], and exhibits competition against other Bacteroides species in the gut. However, if unseated as the dominant species, B. vulgatus will increase in abundance and become dominant, and its proteases and
other activities can induce colitis in mouse models [72]. Bacteroides spp. have a complicated relationship with human health, and differences in microbial genome and phenome are associated with disease [73] and colorectal cancer risk [74]. FMT has been used to “reset” the gut microbiota in a number of GI conditions, by providing a diverse and functional community from a healthy donor to a patient. FMT can impact intestinal permeability, the production of short-chain fatty acids (especially butyrate) and secondary bile acids, compete against pathogenic strains, and more, and has been used successfully to treat UC and CD. A systematic review of controlled trials found that UC patients had only a ~20% FMT success rate, while CD patients experienced a ~60% FMT success rate [75].

2.3 Dietary Factors and IBD Risk

It is now evident that diet is playing a major role in the increasing prevalence of IBD. The highest incidences of IBD are in the United States and Europe, where the diet has increasingly shifted toward high quantities of animal protein and fat, sugar, and highly processed foods, and low quantities of plant-based foods - referred to as a Westernized Diet. High amounts of animal protein, fat, and sugar have been associated with increased risk for developing IBD. In addition, as countries have Westernized their diets, they have also seen an increase in the rates of IBD [23,4]. The link between diet and IBD risk is well established in mouse models, as well as in humans where it is also recognized that diet is often accompanied by other social/lifestyle or environment co-factors.

Diet is a crucial source of plant-derived bioactives which could provide a non-steroidal anti-inflammatory effect, and many of these play an important role in driving microbial homeostasis and gut health [19]. For example, dietary fibers are crucial to short-chain fatty acid (e.g. butyrate) production which can support intestinal cell health and function and increase gut motility, and high fiber diets have been found to improve fecal markers of dysbiosis in patients
with UC [76]. Another commonly researched plant metabolite group is polyphenols, which have been found to decrease inflammation, improve microbial abundance and diversity, and reduce oxidative stress [77–79]. There is a growing need for treatment options that address chronic inflammation while preserving GI function and promoting the stability of commensal microbial communities. Studies have shown that Westernized diets are detrimental to the gut microbiota diversity, and can lead to a pre-diabetic state, and changes in mucosal integrity, similar to that seen in IBD [23,80]. Therefore, positive changes in diet, achieved by incorporating more plant-based foods containing anti-inflammatory metabolites, may be a promising route in the prevention and treatment of IBD.

2.4 Targeting IBD with Broccoli Sprout Bioactives

Cruciferous vegetables are known for being rich in bioactives including fibers, flavonoids, and other antioxidants [81,82]. Epidemiological studies have shown that diets containing cruciferous vegetables, such as broccoli and broccoli sprouts, have inflammation-reducing benefits, are associated with lower cancer risk, and have specifically been shown to be an effective treatment in IBS, UC, and CD [83,84]. High consumption of cruciferous vegetables has been associated with decreased serum levels of pro-inflammatory cytokines, including IL-1β, TNFα, and IL-6 [85,86], as well as the inhibition of NFκB [86,87]. More specifically, the glucosinolates (GSLs) that are uniquely abundant in cruciferous vegetables can be metabolized into a variety of compounds with dramatically different actions and activity levels. GSLs are nitrogen- and sulfur-containing compounds which give cruciferous vegetables their distinct odor, which acts as a detractant against generalist herbivorous insects but an attractant for specialists [88]. GSLs and many of their byproducts have well-demonstrated anti-insect activities [88], yet are cited to have numerous health benefits in humans.
Figure 2. Glucoraphanin hydrolysis. A. GLR hydrolysis in the presence of myrosinase upon damage to the broccoli plant. Epithiospecifier protein preferentially converts GLR to SFN-nitrile. B. GLR hydrolysis has been demonstrated by gut bacteria in the colon of mammals. Low pH environments favor conversion to SFN-nitrile.

GSLs can be metabolized into isothiocyanates (ITCs), a group of dietary bioactive metabolites with anti-inflammatory and anti-cancer activities [85,86,89–92]. When in the presence of plant-derived myrosinase enzymes, GSLs are hydrolyzed into glucose and an unstable molecule, thiohydroxamate-O-sulphanate, which is immediately broken down into ITCs (Fig. 2A). Which ITC product is formed depends on the GSL side chain, the pH of the environment, and the specifier proteins present [93]. Myrosinase and GSLs are housed in separate compartments in the plant, and come together when the plant is damaged by herbivory/mastication, crushing, or
heating. Epithiospecifier protein (ESP) is also present in the plant cell cytoplasm along with myrosinase, and converts GSL to ITC-nitrile, an alternative conversion product which is biologically inactive in humans [94]. ESP conversion of GSLs is more efficient, and therefore rates of conversion to ITC-nitrile are higher in raw broccoli plants than in cooked preparations (Fig. 2A). ESP is inactivated by low heat, which allows primary myrosinase conversion in mildly heated broccoli [95]. Myrosinase is denatured in the presence of high temperatures, and cooking vegetables results in significantly lower concentrations of ITCs in the vegetables as GSLs are left intact [96,97].

Of recent special interest, and one of the most well-studied ITCs, is sulforaphane (SFN) [98]. SFN is the primary hydrolysis product of glucoraphanin (GLR, also known as 4-methylsulfinylbutyl glucosinolate), which is the primary GSL found in mature broccoli, and at 15 times greater quantities in broccoli sprouts [88,99,100]. SFN has been shown to operate through inflammation pathways by inhibiting NF-κB, thereby downregulating pro-inflammatory cytokines important to IBD development and maintenance [101–104]. SFN inhibition of NF-κB translocation has been established, however the underlying mechanisms are yet to be elucidated. It has been theorized that SFN inhibits NF-κB by interacting with Keap1, which inhibits polyubiquitination and binding of Nrf2 and thereby allows Nrf2 nuclear accumulation [101]. Nrf2 activity is inversely linked to NF-κB activity, perhaps due to competition for CREB binding protein (CBP) [102,103]. Additionally, it is possible that SFN plays a direct role in inactivation of NF-κB by interacting with Cysteine residues crucial to NF-κB activation [104]. Securing of these thiol groups leads to impairment of redox-sensitive DNA binding, ultimately preventing NF-κB from gene attachment. Mechanisms for STAT3 phosphorylation inhibition by SFN are also poorly understood. However, proposed mechanisms include inhibition of STAT3-dependent luciferase reporter activity, and modification of miR-124-3p expression [91,105].

Recent studies suggest the interaction between cruciferous vegetables, primarily broccoli and broccoli sprouts, and gut microbiota could contribute to stronger protective effects against GI
inflammatory diseases and/or inflammation-associated tumorigenesis. Some studies have proposed that SFN is metabolized by GI bacteria [106,107], while SFN has also been shown to affect the composition of the gut microbiota, provide protective effects against dysbiosis, improve barrier function, and reduce the severity of IBD-like colitis in mouse models as discussed in detail further along in this review. As the gut microbiota contribute to the generation of broccoli sprout-derived bioactives [106,108–111], alterations in the gut microbiota may have significant impact on both the bioavailability and efficacy of broccoli sprout-derived bioactives (Fig. 2B).

2.5 Gut Microbiota-Mediated Metabolism of Glucosinolates

Mammals, including rats, mice and humans, do not produce digestive enzymes analogous to myrosinase for converting GSLs to ITCs [106,108,112,113]. Animal studies have been used to further investigate this conversion. In rats, SFN metabolites appeared in the urine after oral administration of purified GLR [114]. Consistently, one of our previous studies showed that mice fed a diet of steamed broccoli sprouts, which contained the highest level of GLR and minimal amount of SFN as compared to other preparations (raw or mild-heat-treated), have plasma and tissue SFN levels similar to mice fed steamed broccoli sprouts with active myrosinase added back in prior to consumption [111]. Germ-free rats failed to hydrolyze GSLs from Brussels sprouts diet [112]. Collectively, these data indicate a second conversion method occurring in the GI tract, where cecal and colonic microbiota have been found to have myrosinase-like hydrolases, and which have been demonstrated to convert GLR to SFN both in vivo and in vitro [100,106].

Animal studies also suggest that intact GSLs can reach the colon [109] and that the cecum/colon is the site of GSL conversion to the bioactive ITCs [106,109]. There is direct evidence of GLR hydrolysis in the rat cecal lumen in situ and absorption of the resulting SFN across the cecal enterocytes [106]. ITCs were detected in the mesenteric plasma 2 hours after the injection of GLR to the rat cecum, as compared to 15 minutes after the direct injection of SFN,
indicating a conversion process of GLR to SFN in cecum [106]. The ex vivo incubation in the study supported the role of cecal microbiota in GLR hydrolysis as well, though ITC nitriles were the main hydrolytic products at the low pH environment [106].

There is a lot of indirect evidence to support the important role of human gut microbiota in metabolizing GSLs, however, not much clarity has been achieved for specific microorganisms responsible for converting GSLs to ITCs. In humans, urinary ITC excretion after consumption of cooked broccoli decreased dramatically in participants who were pretreated with oral antibiotics and bowel cleansing [108], supporting an important role for the gut microbiota in the metabolism of GSLs to its bioactive forms when plant-derived myrosinase is inactivated by cooking. Despite the inactivation of myrosinase in cooked broccoli, studies have found low amounts of SFN and other GLR hydrolysis products in the plasma and urine of people who have consumed a cooked broccoli meal [115,116]. One crossover study found a delayed appearance of ITCs in the plasma and urine from people consuming the dietary supplement of broccoli sprouts that lack myrosinase [117]. In addition, population studies continue to show an ITC-related reduction in cancer risk in the US, where the majority of consumers prefer cooked broccoli [118], supporting the idea that this might be a physiologically-relevant process.

Some gut bacteria have been found to be capable of ITC-nitrile formation in acidic conditions [119], and furthermore, the microbial community structure influences the rate of ITC-nitriles formed. Wu et al. assessed GSL hydrolysis in mice fed broccoli and sinigrin, and found that bacteria in the families Clostridiaceae, Lachnospiraceae, and Porphyromonadaceae were positively associated with myrosinase activity [120]. Beta-glucosidase activity has been correlated with the abundance of members of the Lachnospiraceae and Ruminococcaceae families that potentially have glycoside hydrolases [121,122]. Several microorganisms isolated from the mammalian gut appear to have myrosinase-like glycoside hydrolases that can cleave the glycoside moiety from GSLs, and there is evidence for GLR hydrolysis to SFN by cecal microbiota both ex vivo and in vivo [123–125]. Some bacterial species isolated from the human gut appear
to have myrosinase-like enzymes that cleave the glycoside moiety from GSLs when incubated with GSLs in vitro, such as *Escherichia coli*, *Bacteroides thetaotaomicron*, *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus* spp., *Peptostreptococcus* spp., and *Bifidobacterium* spp. [109,123,124,126,127]. *Lactiplantibacillus plantarum* and *Lactococcus lactis* were capable of metabolizing GLR and glucoerucin into SFN nitrile and erucin nitrile [128]. *Lactobacillus agilis* was shown to convert sinigrin to allyl ITC [123].

### 2.6 Modulation of Gut Microbiota by Sulforaphane/Broccoli/Broccoli Sprouts

Epidemiological studies have investigated the impact of high fat/high protein and plant-based diets on microbial abundance and diversity [129]. Diets which are high in fats and sodium are associated with increased *Bacteroides*-genus-dominant enterotype [129]. In contrast, plant-based diets and plant-heavy diets result in increased *Prevotella*-genus-dominant enterotype [129]. Bacterial makeup changes rapidly in response to antibiotic treatment, smoking, lack of exercise, dietary changes, and dietary additives and contaminants such as pesticides and heavy metals [130]. Sudden and abnormal changes in the abundance and diversity of gut microbiota have been associated with symptoms such as bloating and stomach pain, and can result in an over active immune response to commensal bacteria [131].

Many studies have suggested the critical role of plant-based myrosinase in ensuring maximal bioavailability of ITCs, and the insufficiency of gut microbiota-based myrosinase as the primary method of ITC production [117]. The study by Clark *et al* found that subjects taking a broccoli sprout supplement with no active myrosinase, and thus conversion of GSLs to ITCs was primarily dependent on the subject’s gut microbiota, had SFN metabolite levels 5 times lower than those subjects consuming fresh broccoli sprouts [117]. However, other research has shown that prolonged exposure of the gut microbiota to GLR from 8 to 24 hours increased the formation of SFN [132], and feeding rats with 10% freeze-dried broccoli for 1 or 2 weeks resulted in improved
ability of cecal microbiota to hydrolyze GLR ex vivo [99]. These studies suggest changes in gut microbiota with exposure to GLR or broccoli feeding that promote SFN formation.

Specifically, a number of studies have assessed the impacts of SFN and SFN-enriched broccoli diets on the abundance and diversity of the gut microbiota. Both broccoli and SFN diets have been associated with protection against dysbiosis. Mice given SFN have increased Bacteroidetes to Firmicutes ratios [120,133,134], with reported increases in abundance and richness of Proteobacteria, Bacteroides, Clostridium spp. (some of which are renamed to Clostridioides difficile) and butyrate-producing bacteria [135]. Furthermore, mice fed raw broccoli experienced increases in Bacteroidetes and decreases in Proteobacteria and Desulfovibrionaceae [120]. SFN and SFN-enriched broccoli have also been associated with decreases in Desulfovibrionaceae [79] and Mucispirillum schaedleri [135], which are associated with pro-inflammatory activity in the GI tract. Jun et al assessed SFN-mediated microbial changes in an aging mouse model, and found that old mice fed an SFN diet experienced increases in microbial diversity, shifting the microbiota of old mice, and ultimately increasing the similarities between old and young mice [133]. They additionally noted an increase in Allobaculum, a bacterial genus associated with increased epithelial function. Zhang et al assessed SFN modification of the gut microbiome in a DSS mouse model of UC. SFN significantly protected against community modification by DSS, and compared to DSS groups without SFN, SFN groups experienced decreases in the abundance of bacterial families Erysipelototrichaeae and Campylobacteraceae, both families associated with elevated intestinal inflammation or raised risk of IBD, as well as increases in Bacteroidales_S24-7 candidate family and Rikenellaceae, which have been associated with protection of intestinal health or against intestinal inflammation [134].

Recently, studies have investigated SFN as an antibacterial agent. SFN has a dose-dependent effect against both gram-negative and gram-positive bacteria. However, SFN is more effective against gram-negative bacteria such as E. coli and Salmonella enterica, than it is against gram-positive bacteria [136–138]. Additionally, SFN has been found to inactivate urease
produced by *Helicobacter pylori* [139], and modify production of nitric oxide in human patients with *H. pylori* infection [140], potentially leading to an inability to colonize the GI tract by this bacteria.

Importantly, dietary intervention is an equitable and in-demand strategy, as it creates a palliative care prevention and treatment strategy which is more affordable and accessible than a purified-supplement or medical treatment. The purified inactive GSLs and active ITC compounds are prohibitively expensive, but broccoli sprouts may be grown or easily found in groceries and markets. While purified compounds represent important research and treatment options, a whole-food solution would ensure a wider adoption of this strategy and greater public health impact in the general community. Moreover, dietary interventions which are paired with other healthy-lifestyle improvements are shown to have a greater impact on reducing chronic diseases [141], and built environment infrastructure (e.g. grocery stores, public transportation) which reduces the burden of accessing healthy resources and thus improves fiber intake during dietary interventions [142]. At present, conflicting study results and the complicated nature of IBD, diet, and lifestyle have precluded the ability to make clear diet recommendations for all IBD patients. However, as diet can be a trigger for symptoms, most IBD patients report self-imposed dietary restrictions as they attempt to manage their own symptoms [143]. While we cannot make recommendations for treatment, we can make recommendations based on harm-reduction, and diet needs to be part of a holistic treatment plan, to encourage patients to make dietary choices that promote commensal bacteria colonization and avoid choices which inadvertently exacerbate gut microbiome shifts.

### 3 Conclusions

Our data support microbial conversion of GLR to SFN and its subsequent systemic absorption and excretion into the urine in mice (manuscript under review). We also observed
remarkably higher levels of SFN in the colon tissue, as compared to the upper and lower small intestine and plasma (manuscript under review). However, the structural and functional complexity of the gut microbiota make mechanistic studies inconclusive. Studies on identifying specific bacterial populations that are capable of metabolizing GSL precursors are inconclusive, partly due to the limitation of the assay methods. In addition, the majority of these in vitro studies did not support ITCs as the major product of hydrolysis by these bacteria species. As gut microbiota are an extremely complicated system, better experimental methods need to be developed to accurately and efficiently study gut microbiota-mediated metabolism of GSLs. It is possible that the conversion of GSLs to ITCs is not achieved by one bacteria species, instead multiple players are involved in the process in a certain way. For example, an in vitro model has been used to mimic the human gut environment, in which SFN and SFN nitrile were detected in the culture medium that contained human gut microflora and purified GLR [144].

Another important consideration for human studies is that each individual’s gut microbiota is different, influenced by genetic and environmental factors such as diet and physical activity levels, as well as body weight and health conditions. The inter-individual variation will likely add extra complexity to the studies. Many human studies reported inter-individual variation in GSL metabolism. Notably, while there was less inter-individual variation in urinary ITC levels if ITCs were ingested, substantially different amounts of urinary ITCs were found if GSLs or cruciferous vegetables were given [108,116,124,145–148]. In a clinical trial, all subjects consumed 200 g steamed broccoli in one meal, resulting in highly varied levels of urinary ITC excretion, which was associated with varied levels of ex vivo GLR degradation by fecal bacterial culture from these subjects [124].

It is becoming clear from the immense collective body of research on IBD that dietary bioactives and modification of the gut microbiota have an important and promising role in disease modification for individuals with IBD going forward. Biogeography has long been an ecological concept used to study the distribution of animals and plants with respect to geography, as it
influences environmental conditions, food supply, dispersal, and more. Microbial ecologists began using the term in the early 2000s to describe environmentally-located microbial communities [149], and as our research technology has improved, we have been able to identify microbial biogeography - or spatial and temporal distribution - in host-associated communities. Microbial biogeography is exquisitely clear in the gut as an ecological principle [48,49,150], and is critical to understanding how microbially-mediated dietary bioactives can be used to reduce inflammation and other complex diseases in the gut. Future research must take microbial biogeography into account when assessing the action of diet in this and other host-microbial-diet interactions. Combination and sequential therapy will be in part informed by what is learned from the microbial biogeography of the GI tract.

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CHAPTER TWO

INTERLEUKIN-10 KNOCKOUT MICE AS A NOVEL MODEL FOR STUDYING BROCCOLI BIOACTIVES IN INFLAMMATORY BOWEL DISEASE

Abstract

Inflammatory Bowel Diseases (IBD) are devastating conditions with global burden and a strong cancer association, and treatments for IBD are limited. Broccoli and broccoli bioactives are a promising dietary intervention that have the potential to be accessible and safe, and ongoing research has identified specific compounds that can alleviate and prevent inflammation. Even so, previous research has been limited to established models that may not accurately reflect the microbial and genetic aspects of the Crohn’s Disease (CD) presentation of IBD, and very little work has been done to assess the biogeographic specificity of broccoli interventions in the treatment of IBD. We used a novel interleukin 10 knockout (IL-10-ko) mouse study design as an immunological model of Crohn’s Disease, to study dietary bioactives. IL-10-ko mice raised in clean housing were colonized by conventional mice microbiota to trigger inflammation and an immune response. Mice were fed either a purified AIN93G diet, or a purified diet +/- 10% steamed broccoli sprouts. Broccoli-fed mice did not exhibit weight stagnation (p<0.0001), and had much lower Disease Activity Index scores (DAI) than control diet fed mice (p<0.0001), implying a protective effect from diet. Histological results appear to confirm the protective effect. The control mice displayed more architectural changes of gut epithelial cells (p<0.0001), more polymorphonucelar immune cell infiltrate (p<0.0001), and more damage at the apical surface of epithelia where the cells encounter microbes (p<0.01) than broccoli diet fed mice. We further compared our novel IL-10-ko mouse model to an established DSS model of mouse colitis and observed more statistically significant affects in the IL-10-ko model (p<0.03 vs p<0.00001 for weight parameters). Our study has established a novel IL-10-ko mouse model for studying broccoli and broccoli bioactive mediated prevention of inflammation.
Keywords: inflammatory bowel disease, Crohn’s Disease, ulcerative colitis, broccoli, broccoli sprouts, sulforaphane, glucoraphanin, gut microbiota, dietary bioactives.

Statement of Significance: The IL-10-ko mouse model is an effective tool for studying the effect of diet-microbe-host health and SFN interactions in environments of Crohn’s Disease-like inflammation.

1 Introduction

Inflammatory Bowel Diseases (IBD) are globally prevalent, chronic inflammatory diseases of multifactorial origin with an annual healthcare cost of 7.2 billion dollars in the US alone [15]. IBD occurs in the gastrointestinal tract (GI) and can be accompanied by autoimmune dysfunction, and lead to microbial community changes in the gut. Crohn’s Disease (CD) is one of the primary autoimmune disorder presentations of IBD, and has a strongly associated genetic component [151]. Inflammation in CD is relapsing, occurs throughout the GI, and is characterized by decreased expression of the MUC1 gene leading to reduced coverage of mucin in the terminal ileum [152]. Additionally, CD is associated with the modification of tight junction proteins, resulting in a leaky epithelial barrier [153]. Inflammation likely begins with the innate immune response, however this response is reportedly weak in CD patients [154], although a weak adaptive immune response is thought to be the primary driver behind chronic inflammation in CD [155,156]. The adaptive immune response in CD primarily involves the imbalance of effector T cells such as Th1 and Th17, versus interleukin-10 secreting regulatory T cells [155].

The GI tract has a commensal community of microorganisms, of which, bacteria are most prevalent and most well-studied, and are composed mainly of the bacterial phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, that may resist infection by pathogenic microbes and protect against disease [157]. It is debated whether or not macro-level ratios in these microbiota are truly indicators of disease; however, it is generally agreed that inflammatory
diseases such as CD are associated with changes in the microbial community structure of patients’ gut microbiomes [158,159]. Studies have shown microbial population clustering by host's health status and reduced diversity in Firmicutes and Bacteroidetes phyla in CD patients [160]. Additionally, CD has specifically been associated with a reduction in *Faecalibacterium prausnitzii*, and reintroduction of this microbe resulted in decreased inflammation [161].

In addition to being chronic and debilitating, a longer duration of IBD is associated with an increased risk of developing gastrointestinal cancers such as colorectal cancer (CRC) [6–9]. Treatments are currently restricted to alleviating inflammatory symptoms and returning patients to as close to homeostasis as possible. Diet can play an important role in the treatment of IBD, as a source of anti-inflammatory metabolites, and a tool for influencing the robustness of gut microbiomes. Diets high in cruciferous vegetables, such as broccoli, have particularly been associated with reduced inflammation and cancer risk [83,84]. Isothiocyanates, derived from broccoli compounds glucosinolates, have been identified as bioactive candidates for inflammation reduction in IBD [115,139]. Specifically, sulforaphane (SFN), perhaps the most well-studied ITC, has been shown to inhibit NF-kB, resulting in downregulation of multiple inflammatory signaling molecules [87,101,103,134,139].

Dextran sodium sulfate (DSS) is an established and widely used model for studying SFN reduction and prevention of IBD in animals [162,163]. Administration of DSS in drinking water results in a disease profile similar in progression and morphology to human Ulcerative Colitis (UC) [162,164]. DSS colitis begins by modifying the expression of tight junction proteins in intestinal epithelial cells, leading to a leaky epithelial barrier [165]. This is followed by goblet cell depletion, erosion, ulceration, and infiltration of neutrophils into the lamina propria and submucosa [166], triggering the innate immune response [167,168]. DSS stimulation in *in vivo* studies have shown a rapid development of colon tumors after treatment, making it an impressive tool in investigating IBD and CRC [162,166,168]. However, Bhattacharyya et al. reported that DSS treatment results in an excess of reactive oxygen species (ROS) which quickly dephosphorylate Hsp27 resulting in
direct IkBa dissociation, circumventing the canonical inflammatory pathway [41]. These results suggest that DSS may not be an accurate method for studying canonical IBD. Further, DSS treatment instigates colitis through chemical and physical damage, without a direct interaction with the immune system, thus it is inadequate for investigating the specific immunohistopathology present in CD.

The production of cytokine type interleukin 10 (IL-10) stimulates the growth and differentiation of numerous cell types in animals, in addition to suppressing macrophage activation, inhibiting inflammatory cytokine production, and displaying multiple control of Th1 cells [169]. IL-10-ko mice, a mouse model established by Kuhn et al, develops chronic enterocolitis which resembles the transmural inflammation of CD complete with granulomas, crypt abscess, mucosal hyperplasia, and aberrant immune cell response [170,171]. Germ-free IL-10 mice spontaneously develop chronic enterocolitis at a few weeks of age when exposed to a number of bacterial species which are commensal in conventional mice [172], and this non-chemical, genetic model may be particularly well-suited to studying the immune factors and microbiota of CD.

While the IL-10-ko mouse model has been used extensively to study the immune response of CD, there are few studies addressing dietary interventions for IBD in CD models, and none addressing broccoli, broccoli sprouts, or broccoli bioactive interventions. This pilot study, performed as a collaboration between the University of Vermont and the University of Maine Labs, established IL-10-ko mice as a working model for studying the role of dietary broccoli and broccoli bioactives in reducing inflammation, and modifying the immune response. We complemented this model with an established DSS model for studying broccoli in IBD, both to verify the efficacy of the IL-10-ko mouse model, and to compare the interactions of diet, gut microbes, and host in a more comprehensive experimental design.
2 Materials and Methods

2.1 Diet formulation

Multiple lots of Jonathan’s Sprouts™ (Rochester, Massachusetts, USA) broccoli sprouts were purchased from a nearby grocery store (Bangor, Maine, USA) and steamed in a double boiler for 10 minutes, after which they were immediately removed, spread into a thin layer, and placed in a -10°C freezer to halt cooking. After 30 minutes sprouts were moved to a -70°C freezer for storage until they could be freeze-dried at the University of Maine Pilot Plant (Orono, Maine, USA). We did not test the sprouts for SFN contents in this study, however we have previously assessed SFN content in raw, mild heat, and steamed sprouts, and found that SFN content is variable, and that it declines over time in the freezer, with freezer shelf life being approximately 1 year [190].

The sprouts were then crushed by mortar and pestle into a fine powder and mixed with purified AIN93G rodent diet powder and water to a concentration of 10% by weight. Our labs have assessed the effects of different diet preparations and the percentage of broccoli sprouts, and found that 5-10% broccoli sprouts by weight reliably produces consistent anti-inflammatory results [190]. For this study we chose to use 10% steam broccoli sprouts both to assess the microbial conversion of GLR to SFN, and to ensure that the intervention would have a strong effect. Diet pellets were formed using a silicone mold to ensure consistent sizing, and allowed to dry at room temperature for up to 48 hours in a chemical safety hood to facilitate moisture evaporation, and after drying were stored in ziploc bags in a -10°C freezer until future use.
2.2 IL-10 mouse model design

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Vermont (Burlington, Vermont, USA; IACUC PROTO202000040), where the animals were housed and cared for during the trial by the Vermont team. Animals were euthanized by carbon dioxide and cervical dislocation at the end of the study for sample collection.

Eighty-four homozygous interleukin-10 knockout mice (IL-10-ko) on a C57BL/6 background (B6.129P2-Il10tm1Cgn/J, strain 2251; Jackson Laboratories, Bar Harbor, ME) were purchased, from the colony where they are bred and maintained in a barrier facility (Helicobacter (H.) hepaticus-free). These mice lack the gene to produce IL-10, an anti-inflammatory cytokine, and will develop colitis over the course of 10 days when exposed to Helicobacter bacteria which
are otherwise commensals in a conventional mouse. At 4 weeks of age, mice were transferred to the animal research facility at the University of Vermont, where they were promptly isolated in clean rooms to prevent microbial exposure and colonization (Figure 2). For the first seven days (days -7 to 0), mice were acclimated to the facility and to the control or experimental diet containing 10% broccoli sprouts (matched for gross nutrient intake to the control diet), which they would remain on for the duration of the experiment.

At 5 weeks of age, on Day 0, a baseline weight and Disease Activity Index (DAI) was evaluated, and fecal pellets were collected and frozen for later use in a lipocalin assay. Body weight was normalized to baseline for analysis using 2-way ANOVA generated with Prism9. The DAI is a cumulative score of stool consistency and presence of fecal blood, using Hemoccult Single Slide testing slides from Beckman Coulter (Brea, CA). Then, a *H. hepaticus*-positive C57BL/6 mouse was added to the cages of all IL-10-ko mice to induce colitis. For the rest of the trial, mice were weighed daily and a DAI was performed every other day until euthanasia on Day 16. After euthanasia, tissue from the ileum, proximal colon and distal colon of the IL-10-ko mice were collected and fixed in 4% paraformaldehyde overnight for histological evaluation.

**Figure 4. IL-10 KO mice experimental timeline.**
2.3 DSS mouse model design

Figure 5. DSS mice experimental design.

Forty male, 6 week old, aseptic mice (C57BL/6) were purchased from the Jackson Laboratory (Bar Harbor, Maine, U.S.) and transferred to the animal facility at the University of Maine (Orono, Maine, USA) where they were housed for the duration of the experiment (IACUC protocol A2020-01-04), and cared for by myself and the University of Maine team. The mice were acclimated to their new caged environment from -7-0 days, during which they received *ad libitum* autoclaved tap water and the AIN-93G purified rodent diet (control diet). After acclimation, the mice were divided randomly into 4 experimental groups: control diet without DSS treatment, 10% steamed broccoli sprout diet without DSS treatment, control diet with DSS treatment, and 10% steamed broccoli sprout diet with DSS treatment. All experimental groups were given another 7 days of acclimation to their respective diets (control or 10% steamed broccoli sprout), after which
DSS was added to the autoclaved tap water of the DSS treatment groups to a final concentration of 2.5%. Mice were given DSS for a period of 5 days, and then subsequently given a recovery period of water containing no DSS for 5 days. This was repeated for a total of 3 DSS on/off cycles, and mice were sacrificed and tissue collected after the third round of DSS on day 39. Feces for lipocalin analysis, bodyweight, and disease activity index (DAI) scores were taken every 2 - 3 days throughout the trial, and every day during the DSS cycles. Bodyweights were analyzed using 2-way ANOVA generated with R. After euthanasia, digesta (lumen contents) and epithelial-associated (tissue scrapings) microbial community samples were collected from the jejunum, cecum (contents only), and colon for DNA extraction, described below.

2.4 IL-10-ko tissue sample preparation for histology

Tissue samples (1 cm in length) from the ileum, proximal colon and distal colon of the IL-10-ko deficient mice were collected by the University of Vermont and rinsed with phosphate buffer solution (PBS), placed in 4% paraformaldehyde/0.2% picric acid as a preservative, and stored at 4°C until transport to the University of Maine Electron Microscopy Laboratory (Orono, Maine) for processing.

Hematoxylin and Eosin (H&E) staining was performed according to the methods laid out by Rolls and Sampius in 2019 [192]. All chemicals were acquired from Fisher Scientific. Upon arrival tissue samples were washed 4 times with 1X PBS over a period of 24 hours. After the final PBS wash, the samples were transferred to embedding baskets which were placed in a 1000 ml beaker containing a 50% ethanol (EtOH) solution and stored at 4°C for 2 hours, after which they were transferred to 70% EtOH for 2 hours. This process was repeated for 80% EtOH and 96% EtOH solutions. The samples remained immersed in 96% EtOH overnight at 4°C. Three washes of fresh 100% EtOH were performed at 25°C (20 min, 20 min, and 60 min) to ensure no PBS remained. The samples were then placed in 100% acetone for 17 minutes to serve as a transition.
solution from ethanol to xylene, after which the samples were given two rounds of xylene for an hour each.

Wax molds were pre-folded out of paper. The prepped tissue in their embedding baskets went through 3 cycles of Paraplast X-tra paraffin wax warmers to rinse off the xylene before molding. The samples spent one hour in each of four successive wax warmer trays. The tissues were then taken from their embedding baskets and oriented vertically in the paper molds, to which wax from the last warmer tray had been added. Once the wax developed a “skin”, the mold was placed in a cool water bath and allowed to solidify overnight.

Each wax block, containing the dissected specimen, was sliced using a Rotary Microtome (Spencers World, [https://www.spencersworld.com/default.htm](https://www.spencersworld.com/default.htm)) and placed on Epredia™ Shandon™ Polysine Slides (Thermo Scientific, Waltham MA). The slides were heated on a Fisher Scientific 77 Microscope Slide Warmer (Fisher Scientific, Waltham MA) at 35°C for 15 minutes to melt the sample onto the slide. The tissue was then stained through a time-specified solvent series in Coplin staining jars and transferred using tweezers. The staining process began with 3 treatments of 100% xylene for two minutes to melt the wax. The slides were then given 3 treatments of 100 % EtOH for 2 minutes, and then transferred to 95% EtOH for 2 minutes, and then 70% EtOH for another 2 minutes. After the EtOH series, the slides were gently rinsed with running tap water (ensuring not to rinse off the sample). The slides were then stained with Hematoxylin for 30 minutes and an acid rinse of 1% HCl in distilled water for 8 minutes. After the acid rinse slides were dipped in a bluing agent containing 1% ammonium in H₂O. The slides were transferred into Eosin y for 2 minutes to provide the background stain, and then given 2 treatments of 95% EtOH for 2 minutes. Final steps include 2 treatments of 100% EtOH for 2 minutes and 3 treatments of 100% xylene for 2 minutes. Once dried, one drop of DPX Mountant for Histology (Sigma Aldrich, St. Louis MO) was placed on each cross-section using a wooden applicator. Corning® glass coverslips (Corning Inc, Corning NY) were placed over the cross-sections.
2.5 IL-10-ko Histological Scoring

Six criteria were used to assess inflammation in the ileum, and the proximal and distal colon tissues. Epithelial damage and architectural changes were scored from 0 (no damage) to 2 (extensive damage). Similarly, infiltration of mononuclear cells in the lamina propria was scored on a scale of 0 (no infiltration) to 2 (extensive infiltration). Infiltration of polymorphonuclear cells in both the lamina propria and the epithelium were scored on a scale of 0-2, with 0 indicating no infiltration, 1 indicating sighting of $\geq 1$ cell in a 40x magnification viewing field, and 2 equating to $>3$ cells in a given viewing field. Abscesses, ulcers, erosion, and branched crypts were scored together with 0 being absence of these damage indicators and 1 being presence. Linear mixed model plots of histology scores were made using the ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2022) packages in the R statistical program (R Core Team, 2022), and a 2-way ANOVA was used to compare means with the vegan package.

2.6 Microbial Isolation from DSS Mice

At the conclusion of the DSS project, digesta (lumen contents) and epithelial-associated microbial community samples were collected from the jejunum, cecum (contents only), and colon by removing the GI, and identifying and separating anatomical locations. GI contents were flushed by running closed tweezers down the length of the section into a 2 mL microcentrifuge tube. Scrapings were then obtained by slicing the tissue open and scraping along the interior with a scalpel. Tissues and scraping were collected for the purpose of bacterial isolation using selective media.

2.6.1 Preparing Bacto Tryptone Yeast Broth and Agar Plates

Bacto tryptone yeast agar (BTYA) was prepared using the recipe outlined in Whitaker et al 2017 [173]. All chemicals were obtained from Fisher Scientific or Sigma Aldrich. Briefly, 5.0 grams of Bacto Tryptone™, 2.5 g of yeast extract, 1.0 g of glucose, 10.0 g of agar powder, 500 µL of 0.8% CaCl, 500.0 µL of 0.4 mg/mL FeSO4, 500.0 µL of 1mg/mL K3 solution, 50.00 mL of
KPO4 buffer solution, 20.00 mL salt solution, and 2.5 mL of cysteine were measured into a 1L bottle. Distilled water was added to make a total of 1L of agar broth. The broth was then autoclaved for 15 minutes at 121 °C. After autoclaving the agar, 2.5 mL of hematin-histidine solution was prepared in a clean BSL2 hood with distilled-deionized water and was then added to the agar and inverted to evenly mix. Agar was poured into 100 mL petri dishes, allowed to sit for 30 minutes in a BSL2 hood to solidify, and stored inverted in a 4°C refrigerator. To make Bacto Tryptone Yeast Broth (BTYB), the above method was used without agar powder, autoclaved, and stored in 1L bottles in a 4°C refrigerator.

2.6.2 Culturing Spread Plates with Mice Gut Samples

All work for culturing spread plates was done in a Coy Type A anaerobic chamber accessed using an airlock system, maintained at ~90% nitrogen gas, 5% hydrogen gas, and 5% carbon dioxide gas atmosphere located in the Ishaq Microbiology Lab at the University of Maine. Oxygen was removed using a palladium catalyst and dehydrators to remove the resulting moisture. A Coy Forced Air Incubator (Coy Laboratory Products Inc, Grass Lake Michigan) was maintained at 35°C inside the anaerobic chamber.

A total of 400.0 µL of BTYB was added to sterile 1.5 mL Eppendorf microcentrifuge tubes containing 100 uL of experimental group tissue samples in PBS. The microcentrifuge tubes were closed and inverted to evenly disperse the contents. 100.0 µL of the tissue sample solution was then pipetted onto a media plate. A sterile spreader was used to evenly distribute microbes across the plate, and the plates were incubated in the anaerobic chamber for 48 hours at 35°C. After incubation individual colonies were streaked onto BYTA plates. The isolation plates were incubated for 48 hours at 37°C in the anaerobic chamber. 10 mL of broth was pipetted into glass culture tubes inside the anaerobic chamber to ensure minimal dissolved oxygen. Individual colonies were taken from the streak plates and inserted into the culture tubes containing broth and then incubated at 37°C for 48 hours. After incubation, 1000 µL glycerol was administered into
2 mL cryotubes. The colony-broth test tubes were inverted to evenly distribute the microbes, and 1000 µL of the samples were pipetted into the 2.0 mL cryotubes. Cryotubes were then stored in the -20°C freezer until needed for DNA extraction.

Bacterial isolation, identification, and myrosinase assays are still in progress, and are not the focus of this thesis, however, some results are presented in APPENDIX B.

2.7 Bacterial community sequencing

Intestinal tissue sections (2 cm in length) from the ileum, cecum, proximal colon, and distal colon of IL-10-ko mice were placed in RNAlater (Zymo Research, Irvine CA) preservative and transported overnight on ice from the University of Vermont to the University of Maine for DNA extraction. From the DSS mice, digesta (lumen contents) and epithelial-associated (tissue scrapings) microbial community samples were collected from the jejunum, cecum (contents only), and colon. All tissues containing their resident gut microbiota were gently homogenized with vortexing, then treated with propidium monoazide (PMA; BioTium) following kit protocols at a final concentration of 25 µM. PMA covalently binds to relic/free DNA and DNA inside compromised/dead cell membranes, and prevents amplification in downstream protocols to preclude dead DNA from the sequence data.

Following PMA treatment, bulk DNA was extracted from tissue-associated bacterial communities (n = 80 samples), or no-template (water) control samples (n = 4, one for each extraction batch) using commercially available kits optimized for water and tissue-based microbial communities (Quick-DNA Fecak/Soil Kit, Zymo Research). DNA extract was roughly quantified and purity-checked with a Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham MA). Samples underwent DNA amplicon sequencing of the 16S rRNA gene V3-V4 region, using primers 341F [174] and 806R [175] and protocols consistent with The Earth Microbiome Project [176], and sequenced on an Illumina MiSeq platform using the 2 x 300-nt V3 kit (Molecular Research Labs, Clearwater, TX). Raw
sequence data (fastq files and metadata) will be made publicly available from the NCBI Sequence Read Archive (SRA) prior to publication.

Amplicon sequence data was processed using previously curated workflows, for example; (Ishaq et al., 2019, 2020; Yeoman et al., 2018), which used the DADA2 pipeline ver. 1.26 (DADA2 Pipeline Tutorial (1.4), 2016.) in the R software environment ver. 4.1.1 [177]. The dataset started with 28,128,268 raw reads. Trimming parameters were designated based on visual assessment of the aggregated quality scores at each base from all samples (plotQualityProfile in DADA2): the first and last 10 bases were trimmed, and sequences were discarded if they had ambiguous bases, more than two errors, or matching the phi X genome (used as sequencing positive-control). After filtering, 21,759,650 paired non-unique reads remained.

The DADA algorithm was used to estimate the error rates for the sequencing run, dereplicate the reads, pick sequence variants (SVs) which represent ‘microbial individuals’, and remove chimeric artifacts from the sequence table. Taxonomy was assigned using the Silva taxonomic training data version 138.1 [178] and reads matching chloroplasts and mitochondria taxa were removed using the dplyr package [179]. No-template control samples were used to remove contaminating sequences from the samples by extraction batch [180]. The sequence table, taxonomy, and metadata were combined for each experiment using the phyloseq package [181].

Normality was checked using a Shapiro-Wilkes test on alpha diversity metrics generated from rarefied data, including observed richness, evenness, and Shannon diversity. Linear models were run for comparisons of alpha diversity metrics using linear models to compare by sample type, (lme4 package [182]), in which anatomical location and diet treatment were used as fixed effects. Jaccard unweighted similarity was used to calculate sample similarity based on community membership (species presence/absence), visualized with non-parametric multidimensional scaling, and tested with permutational analysis of variance (permANOVA) by using the vegan package [183]. Random forest feature prediction with permutation was used to
identify differentially abundant SVs based on factorial conditions [184]. Plots were made using the ggplot2 [185], ggpubr [186], and phyloseq packages. Sequencing data analysis is still in progress, and is not the focus of this thesis, however, some results are presented in APPENDIX B.

3 Results

3.1 Weight and fecal characteristics of IL-10-ko mice

Figure 7. Daily mouse body weights (n=84). A. Daily mouse body weights in an IL-10-ko mouse model. B. Daily DAI scores. p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 by two-way ANOVA.

A one-week pretreatment, and continuation of a diet containing 10% broccoli sprouts significantly attenuated the development of colitis in IL-10 KO mice (broccoli, n=10; control, n=10). IL-10 KO mice used were 5-7 weeks old for the duration of the experiment, thus were still in a growth phase. In the IL-10 model, animals with colitis tend to continue gaining weight, but more slowly. Thus, the animal’s weights were normalized to their original baseline weight. The mice fed the broccoli diet continued to gain weight compared to their baseline, while the mice fed the control diet plateaued around 120% (p<0.0001). Consumption of the broccoli diet significantly attenuated
the development of inflammation in IL-10 KO mice, indicated by lower DAI scores, corresponding to firmer stool and the absence of blood in stool.

3.2 Weight and characteristics of DSS mice

Figure 8. Mouse body weights in a DSS-Induced model of chronic colitis (n=40).

Mice fed a diet of 10% steamed broccoli diet and treated with 2.5% DSS in the drinking water gained significantly more weight than mice given a control diet and treated with DSS (p<0.03). Mice fed a broccoli diet and treated with DSS, mice fed a broccoli diet and no DSS treatment, and mice fed a control diet and no DSS treatment were not statistically dissimilar.
3.3 Histology

Figure 9. Linear mixed model fit plots for 6 histology scoring criteria. p<0.001 ‘***’, 0.001 ‘**’, 0.01 ‘*’, 0.05 ‘.’. Stars along y axis title indicate intercept significance. Stars above “treatment” indicate significance of overall treatment vs control.

Broccoli diet fed mice scored better than control mice for polymorphonuclear cells in LP (p<0.0001), presence of erosion or ulcers (p<0.0001), architectural changes (p<0.0001), and
polymorphonuclear cells in the epithelium (p<0.001). Epithelial damage was consistently worse in the proximal and distal colons of the control diet fed mice (p<0.05 and p<0.01 respectively).

4 Discussion

The anti-inflammatory effects of dietary broccoli and broccoli metabolite SFN in IBD have been well established in the literature as noted in Chapter 1. However, studies often focus on a single model of IBD which is similar in disease presentation to UC, and which may not be an accurate reflection of the genetic factors or microbial reactions to inflammation and dietary changes of CD. The present study sought to address these investigatory gaps by using complimentary mouse models of IBD, introducing an IL-10-ko mouse model, and using standard IBD assessment techniques to compare model performance across multiple measures; weight, fecal blood, fecal consistency, and intestinal inflammation scoring.

We fed a 10% steamed broccoli sprout diet to 5 week old IL-10-ko mice prior to exposing them to an *H. hepaticus*-positive C57BL/6 mouse, and took daily body weights over the course of the study. Young mice are expected to gain weight over time, however inflammation causes weight stagnation and sometimes weight loss. Mice fed a broccoli sprout diet gained significantly more weight than mice fed a control diet, and DAI scores were significantly better for broccoli sprout diet mice than control diet mice. In addition to the histology scores, this indicates a protective effect of the broccoli sprout diet against developing symptoms of CD. Further, the appearance of classic CD symptoms in the control mice and the positive response of the broccoli-fed mice indicates that our use of the IL-10-ko mouse for dietary bioactive trials was an accurate one.

We also fed a 10% steamed broccoli sprout diet to 6 week old C57BL/6 mice prior to introducing 2.5% DSS into their drinking water, which is an established protocol for researching UC in mice as a model for humans. Similar to the IL-10 mice, the broccoli diet mitigated the effects
of DSS on mice weight stagnation when compared to control diet mice, confirming that our model was successful. Interestingly, we observed a stronger effect in the IL-10-ko mouse study compared to the DSS mouse study, as it pertains to the mitigation of weight stagnation. This may reflect that IL-10-ko mice are younger than the DSS model mice and still in an active growth phase, because triggering inflammation in IL-10 mice is timed to a specific age. Mice in DSS studies are typically 6-8 weeks of age at the start of treatment for the purpose of observing DSS effects on weight gain during a growth phase. IL-10-ko mice are usually around 6-8 weeks old as well, however for this study they happened to be available at 5 weeks of age. Younger animals have a more variable gut microbial community and immune system than older animals [187–189], thus an intervention at a younger age during a growth phase might have a more pronounced effect on the health of the animal. Future research should focus on temporal variation of mouse ages when treatment commences to understand the nuance of each model and the effects of dietary treatment at younger ages. Further, if mouse age is an important factor in the strength of dietary broccoli sprout treatment affects, implications should be considered for human research.

Inflammation in CD is relapsing, occurs throughout the GI, and is characterized by decreased expression of the MUC1 gene leading to reduced coverage of mucin in the terminal ileum [152]. Thus, the ileum is a site of interest for intervention studies, along with the colon where much of the inflammation occurs in IBD patients. There was no statistical difference between IL-10-ko treatment groups in the ileum for any histological scoring criteria. Effects from the broccoli diet appear most strongly in the colon, which is consistent with our previous findings that microbial conversion of GLR to SFN and resulting absorption of SFN by the GI occurs mainly in the colon [190]. Given the location-specific dynamics of gut anatomy and physiology, digestion and the availability of different nutrients, and microbial communities, a lack of change in ileum tissues could indicate that the biochemical effects of the diet are not available to the host until the colon.

The IL-10 deficient mouse model may be an excellent environment for studying broccoli diet and SFN interactions with gut microbes, and we have already begun work to address this
potential. We have collected a considerable number of samples from our IL-10 deficient mice GIs for 16S rRNA sequence data analysis of the bacterial communities present, and we hope to culture specific gut-obtained strains and assess them for myrosinase activity in the future.

While our work verified IL-10 deficient mice as a research tool for studying broccoli diet driven prevention of inflammation, as an animal study it is mostly inapplicable to dietary recommendations in humans. Our study used a 10% steamed broccoli diet by weight, which would be impractical in human studies: broccoli and broccoli sprouts are dietarily controversial vegetables and people have strong reactions to both the flavor and side effects, such as bloating, which can be attributed to its sulfur content. It is therefore necessary to test broccoli interventions in human subjects, to gain insight into dose efficacy, and best preparation methods.

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CHAPTER THREE

CONCLUSION

1 Summary of Findings

Inflammatory Bowel Diseases have complicated presentations with genetic, environmental, dietary, immune, microbial, and stress components. To be an accurate reflection of IBD, research studies should expand their scope, for example by addressing the concepts of biogeographic specificity of both nutrient absorption and microbial community dynamics, or by using multiple research tools to better mimic the multiple presentations of IBD. Too often, research focuses on just one aspect of IBD or uses one model with a narrow scope, that may result in unanticipated microbial changes, or that are not representative of genetic factors. This is reflected in the absence of genetic models in biochemical-centric research focused on the role of broccoli-metabolite SFN in preventing and treating IBD. To date, no previous SFN or broccoli diet studies have used the IL-10-ko mouse model. With our study, we sought to cover this research gap by, first, proving broccoli dietary measures in IL-10-ko mice that have a Crohn's disease-like presentation of inflammation. Does this model work? Will we see a change in the inflammatory response of mice fed a broccoli diet versus mice fed a control diet? Will these inflammatory response changes be comparable to a well-established model for studying broccoli diet in IBD?

We fed IL-10-ko mice either a broccoli diet or a control diet, initiated inflammation, and assessed that inflammation using bodyweight gain, a disease activity index score, and immunohistology. All three of the parameters measured showed a consistent and marked reduction of inflammation in mice that were fed a broccoli diet. To assess the performance of this study, we also compared the bodyweight results of our novel IL-10-ko model to the results of an established DSS model of IBD. As expected, the broccoli diet prevented inflammation in the DSS model when compared to control diet fed mice. Excitingly, the IL-10 model had a much more
pronounced effect on bodyweight gain (p<0.0001 for the IL-10-ko model vs p<0.03 for the DSS model). These results serve to establish IL-10-ko mice as a functional model for studying the role of broccoli diet in IBD.

2 Reflection on Research Undertaken

Over the past two years I have participated in still-ongoing research on broccoli, broccoli bioactives, and their roles in preventing inflammation in multiple disease models of IBD. We worked collaboratively with IBD and broccoli experts at a number of institutions outside the University of Maine on this project. I added to my background library of laboratory skills a variety of new techniques, including anaerobic microbial culture and isolation, and histology tissue preparation and staining, and gained familiarity in biochemical function testing of bacteria, DNA extraction, and bacterial community sequencing analysis. I participated in 2 mouse trials, one a well established model and one a novel model. I trained a number of undergraduates in laboratory safety, research tools and equipment use, and research techniques, as well as communications skills such as writing and creating oral presentations of research outcomes. Collectively, this project provided me with professional development in research, science communications, and the management of laboratory, project, and personnel. We did all of this and more under the working conditions of a global pandemic that limited access to laboratory equipment and materials. Between the pandemic and the time limit of two years, undertaking this project was difficult and frustrating at times, and we did not complete all of the work we hoped to complete. We have, still, more than 800 bacterial isolates from the GI of the DSS mice to identify and test for myrosinase activity. We also have numerous samples from the GI of both the DSS and IL-10-ko mice that are waiting for 1Ss rRNA data analysis to indentify the bacterial communities present and trends based on biogeography, diet treatment, sex, and time. Despite these limitations, our work added
to the body of research dedicated to broccoli, SFN, and IBD, by establishing new research tools that may help resolve genetic and microbial factors which have confounded previous research.

3 Future Directions and Limitations

At this point in GI inflammatory research, it is generally well-accepted that microbial communities are important drivers behind homeostasis and disease severity. However, the specific role of microbes in SFN and broccoli diet IBD interventions remains to be elucidated. There is considerable evidence that microbes provide metabolism of biologically inert glucosinolates to biologically active isothiocyanates, and although cultured bacterial strains have been shown to perform this conversion, specific gut strains have not yet been identified. Additionally, a bacterial myrosinase enzyme has not been pinned down. There is also evidence that broccoli diets and SFN impact the abundance and diversity of gut microbiomes; however, studies addressing this interaction are limited, and better methods must be employed to understand the complex and delicate gut microbiome system. The IL-10-ko mouse model may be an excellent environment for studying broccoli diet and SFN interactions with gut microbes, and we have already begun work to address this potential. We have collected a considerable number of samples from our IL-10-ko mice GIs for 16S rRNA sequence data analysis of the bacterial communities present, and we hope to culture specific gut-obtained strains and assess them for myrosinase activity in the future.

While our work verified IL-10-ko mice as a research tool for studying broccoli diet driven prevention of inflammation, as an animal study it is mostly inapplicable to dietary recommendations in humans. Humans are unlikely to adjust their diets towards 10% broccoli sprouts by weight, and although research suggests consumption of broccoli reduces the risk of developing IBDs and CRC [191], it is unclear what amount of dietary broccoli or broccoli sprouts
would result in the specific effects witnessed in our mouse trials. Future research should therefore focus on broccoli interventions in human subjects, and address dose efficacy and best preparation methods.


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A total of 806 bacterial strains were isolated from the digesta (lumen contents) and epithelial-associated (tissue scrapings) microbial community samples were collected from the jejunum, cecum (contents only), and colon of DSS mice. These stocks have been archived in glycerol at -80°C for future research on bacterial utilization of glucoraphanin, the related compound sinigrin, and sulforaphane in biochemical assays.

**Figure 10. Photos of bacteria isolated from the GI tract of mice** during the DSS trial on selective media culture plates being incubated inside an anaerobic chamber with a 90% nitrogen, 5% carbon dioxide, and 5% hydrogen gas mix to mimic conditions in the digestive tract of mammals.
Figure 11. Observed bacterial richness (number of Sequence Variants) in microbial communities from the GI tract of IL-10-ko mice on a broccoli sprout or control diet. Asterisks represent significant comparisons of means using linear modeling.
Figure 12. Non-metric multidimensional Scaling (NMDS) ordination plot visualizing the bacterial community similarity in gut samples from mice in the IL-10-ko study. Unweighted Jaccard Index was used to calculate community similarities based on the presence/absence of bacteria in each sample. Each dot represents one bacterial community from one mouse sample. Shapes indicate the anatomical location of the bacterial community in the GI of mice, and color indicates diet treatment. The size of the dot indicates the two experimental trials. There is a division between trial 1 and trial 2. In trial 1, the broccoli samples are packed tighter together, suggesting the broccoli sprout diet pressured mice to acquire a similar bacterial community regardless of the GI location. However in trial 2, the broccoli samples are spread out and overlap with the controls, and there is less of a treatment effect.
Figure 13. Non-metric multidimensional Scaling (NMDS) ordination plot visualizing the bacterial community similarity in gut samples from mice in the IL-10-ko study by anatomical location and trial. Unweighted Jaccard Index was used to calculate community similarities based on the presence/absence of bacteria in each sample. Each dot represents one bacterial community from one mouse sample. Color indicates diet treatment. Panels are used to better focus on the differences between diet within each anatomical location and experimental trials. There are separate clusters for broccoli vs. control, and for anatomical location vs. anatomical location. There are not separate clustering for diet x location, however there are tighter clusters in some areas. There are some differences between trial 1 and trial 2 mice, possibly due to age, trial 1 mice were 5 weeks (just weaned) at starting, and trial 2 mice were 7 weeks at starting.
BIOGRAPHY OF THE AUTHOR

Johanna Holman was born in Syracuse, New York in 1994, and was homeschooled by her mother until her graduation in 2012. She attended the University of Maine as an art student from 2012-2014, before finally transferring to Husson University to begin her career as a Biology student. Her very first experience with research was as an undergraduate at Husson University, where she participated in a Research Experience for Undergraduates at the Single Cell Genomics Center at Bigelow Laboratories in 2016. After graduating with her B.S. in Biology at Husson in 2017, she turned to practical laboratory experience and began working with Drs. Tao Zhang and Yanyan Li at Husson University as a research assistant on their project involving broccoli metabolite sulforaphane and its role in the treatment and prevention of inflammatory bowel disease. It was with their encouragement that Johanna finally applied and was accepted into the M.S degree under the Food Science and Human Nutrition department at the University of Maine in 2020. After receiving her degree, Johanna will continue her education at the University of Maine as a Ph.D. student in the Food Science program. She is a candidate for the degree of Master of Science Food Science and Human Nutrition from the University of Maine in August 2022.