Early life exposure to broccoli sprouts confers stronger protection against enterocolitis development in an immunological mouse model of inflammatory bowel disease.

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Keywords: Crohn's Disease, cruciferous vegetables, sulforaphane, glucoraphanin, gut microbiota, dietary bioactives, 16S rDNA, interleukin-10 knockout

Abstract

Inflammatory Bowel Diseases (IBD) are chronic conditions characterized by inflammation of the gastrointestinal tract that heavily burden daily life, result in surgery or other complications, and disrupt the gut microbiome. How IBD influences gut microbial ecology, especially biogeographic patterns of microbial location, and how the gut microbiota can use diet components and microbial metabolites to mediate disease, are still poorly understood. Many studies on diet and IBD in mice use a chemically induced ulcerative colitis model, despite the availability of an immune-modulated Crohn's Disease model. Interleukin-10-knockout (IL-10-ko) mice on a

C57BL/6 background, beginning at age 4 or 7 weeks, were fed either a control diet or one containing 10% (w/w) raw broccoli sprouts which was high in the sprout-sourced anti-inflammatory sulforaphane. Diets began 7 days prior to inoculation with *Helicobacter hepaticus,* which triggers Crohn's-like symptoms in these immune-impaired mice, and ran for two additional weeks. Key findings of this study suggest that the broccoli sprout diet increases sulforaphane concentration in plasma; decreases weight stagnation, fecal blood, and diarrhea associated with enterocolitis; and increases microbiota richness in the gut, especially in younger mice. Sprout diets resulted in some anatomically specific bacterial communities in younger mice, and reduced the prevalence and abundance of potentially pathogenic or otherwise-commensal bacteria which trigger inflammation in the IL-10 deficient mouse, for example, *Escherichia coli* and *Helicobacter*. Overall, the IL-10-ko mouse model is responsive to a raw broccoli sprout diet and represents an opportunity for more diet-host-microbiome research.

Importance

A diet containing 10% raw broccoli sprouts increased the plasma concentration of the anti-inflammatory compound sulforaphane, and may be protective against negative disease characteristics of *Helicobacter*-induced enterocolitis in interleukin-10 knockout mice, including weight loss or stagnation, fecal blood, and diarrhea. Younger mice responded more strongly to the diet intervention, and resulted in increased gut bacterial community richness and bacterial community similarity by diet treatment and some anatomical locations in the gut, even in mice with adverse reactions to gut microbiota and a relatively short time in which they had been able to recruit them. To our knowledge, IL-10-ko mice have not previously been used to investigate the interactions of host, microbiota, and broccoli, broccoli sprouts, or broccoli bioactives in resolving symptoms of CD.

Introduction

Inflammatory Bowel Diseases (IBD) affect more than 6 million people globally, with more than 25% of cases reported in the United States (1). The varied combination of symptoms, the intensity of presentation, and their multifactorial origin make symptoms difficult to manage, and leads to significant detriment to quality of life. Crohn's Disease (CD) is one of the primary immune-disordered presentations of IBD which typically presents during late adolescence and early adulthood (2), has strongly associated genetic (3) and environmental (4, 5) factors, and involves a loss of function of the innate immune system which disrupts host-microbial interactions in the gastrointestinal tract (6). CD treatments attempt to alleviate inflammation as a method of reducing various symptoms, and to return patients to as close to homeostasis as possible, although single-strategy treatments can yield mixed results (7). Diet can play an important, economical, and accessible role in the prevention and/or management of IBD as a source of anti-inflammatory metabolites (8, 9), and broadly as a tool for influencing the robustness of gut microbiomes (10–12). How IBD affects gut microbial ecology (13, 14), how gut microbiota mediate disease (15) or response to diet (16, 17), and how dietary components and microbial metabolites improve symptoms are still poorly understood (18, 19). Moreover, the delicate health status in CD patients requires a thorough understanding of host-microbe-diet interactions before suggesting therapeutic strategies.

Diets high in cruciferous vegetables, such as broccoli and broccoli sprouts, have particularly been associated with reducing inflammation and cancer risk in the gut (9, 20), in part because of the concentration of sulfur-containing glucosinolates which can be metabolized to bioactive compounds by broccoli- or microbially-sourced enzymes. Isothiocyanates, derived from glucosinolates, have been identified as bioactive candidates for inflammation reduction, including in IBD (9). Specifically, sulforaphane (SFN), the most well-studied isothiocyanate, inhibits the immune factor NF-kB and downregulates multiple inflammatory signaling pathways (9). Broccoli sprouts, or the purified SFN or precursor compounds, protect against chemical-induced ulcerative

colitis in mice (20, 21), but the applicability of previous diet research to CD has been hampered by the use of mouse models which lack the complex immune-dysfunction component of CD (22, 23). Medical professionals will need a clear understanding of how the immune system in CD patients will react to the microbiota changes induced by diet (18, 24), and especially the inclusion of a sulfur-rich diet which complicates hot-microbe interactions in the gut (25), as CD patients are historically been advised to avoid cruciferous vegetables (24).

Inflammation in CD is relapsing, occurs throughout the gastrointestinal tract, and results in several breakdowns of the innate immune system to allow microbial translocation from gut to other tissues. This includes decreased expression of the MUC1 gene, leading to reduced coverage of mucin in the ileum (26), reduced efficacy of tight junction proteins (27), and a loss of absorption of bile salts in the ileum which causes damage to the colon. Acute, non-pathological inflammation is a normal component of the innate immune response. This response is reportedly weak in CD patients who have low neutrophil counts in response to infection (6), in whom uncleared infection and infiltration of fecal material through the mucosal lining of the gastrointestinal tract drives dysregulation of the adaptive immune response and results in chronic inflammation (6, 28). The adaptive immune response in CD primarily involves excessive recruitment of effector T-cells (Th1 and Th17) by inflammatory cytokines (interleukins -12, -18, and -23) which are upregulated in CD lesions (2, 29, 30). In addition to being chronic and debilitating, the duration of IBD and of inflammation, damage to intestinal walls, and other negative outcomes, are associated with an increased risk of developing gastrointestinal cancers, such as colorectal cancer (31).

The interleukin 10 (-/-) knockout mouse model is commonly used to study inflammation in CD (32). Interleukins (IL) are signaling proteins that aid in immune function by reducing inflammation and counterbalancing the immune response, thus mediating host-microbial tolerance (33). IL-10 also stimulates the growth and differentiation of numerous cell types, suppresses macrophage activation, inhibits inflammatory cytokine production, and displays

multiple mechanisms of control of Th1 cells (34). IL-10, in particular, influences the innate immune response to microorganisms, and a lack of it is involved in the breakdown of host-microbial relations in IBD (35). IL-10 deficient mice (IL-10-ko) are raised in germ-free conditions, and develop chronic enterocolitis upon exposure to microorganisms which act commensally in immune-competent mice, and this response resembles the transmural inflammation of CD, complete with the formation of granulomas, crypt abscess, mucosal hyperplasia, as well as aberrant immune cell response (36, 37). This non-chemical, genetic model is particularly well-suited to studying the immune factors and microbiota of CD (38).

To our knowledge, IL-10-ko mice have not previously been used to investigate the interactions of host, microbiota, and broccoli, broccoli sprouts, or broccoli bioactives in reducing inflammation, modifying the immune response, and supporting gastrointestinal tract microbial systems in CD patients, and the objective of this study was to evaluate IL-10-ko mice as a model for studying the broccoli sprout bioactives. Our initial hypothesis was that a medium-high concentration (10% w/w) of raw broccoli sprouts in the diet, which contains both the glucoraphanin (GLR) precursor and the anti-inflammatory byproduct sulforaphane (SFN), would protect mice from the effects of inflammation triggered by microbial conventionalization, even in the absence of a developed gut microbiota which would perform glucosinolate metabolism. We hypothesized that the sprout diet would alter the gut microbiota, and result in the recruitment of potentially beneficial taxa while reducing the abundance of putative pathogens. It is standard to conventionalize IL-10-ko at approximately 8 weeks of age, after mice are fully weaned (39), to mimic adolescence and typically timing for the onset of symptoms. In conducting two replications of a diet trial with mice beginning at 4-weeks and 7-weeks of age, we observed a previously unreported effect of young age on the effectiveness of the response to broccoli sprout bioactives in resolving the symptoms of enterocolitis.

Results

Raw broccoli sprouts alleviated disease characteristics of immune-modulated enterocolitis

The experimental design was structured as a prevention paradigm (Figure 1) in which homozygous interleukin-10 knockout (IL-10-ko) mice were given *ad libitum* either the 5LOD irradiated control diet or the treatment diet consisting of 10% (w/w) raw broccoli sprouts for 7 days to allow them to acclimate, then for a further 16 days during the induction of colitis via microbial colonization and symptom onset. Raw broccoli sprouts contain concentrated amounts of glucoraphanin, some of which is metabolized to sulforaphane by mastication, or in this case, diet preparation, when the broccoli enzyme myrosinase is released from tissues. The control diet contained no GLR or SFN, and the broccoli diet contained on average 4 µg of GLR and 85 µg of SFN.

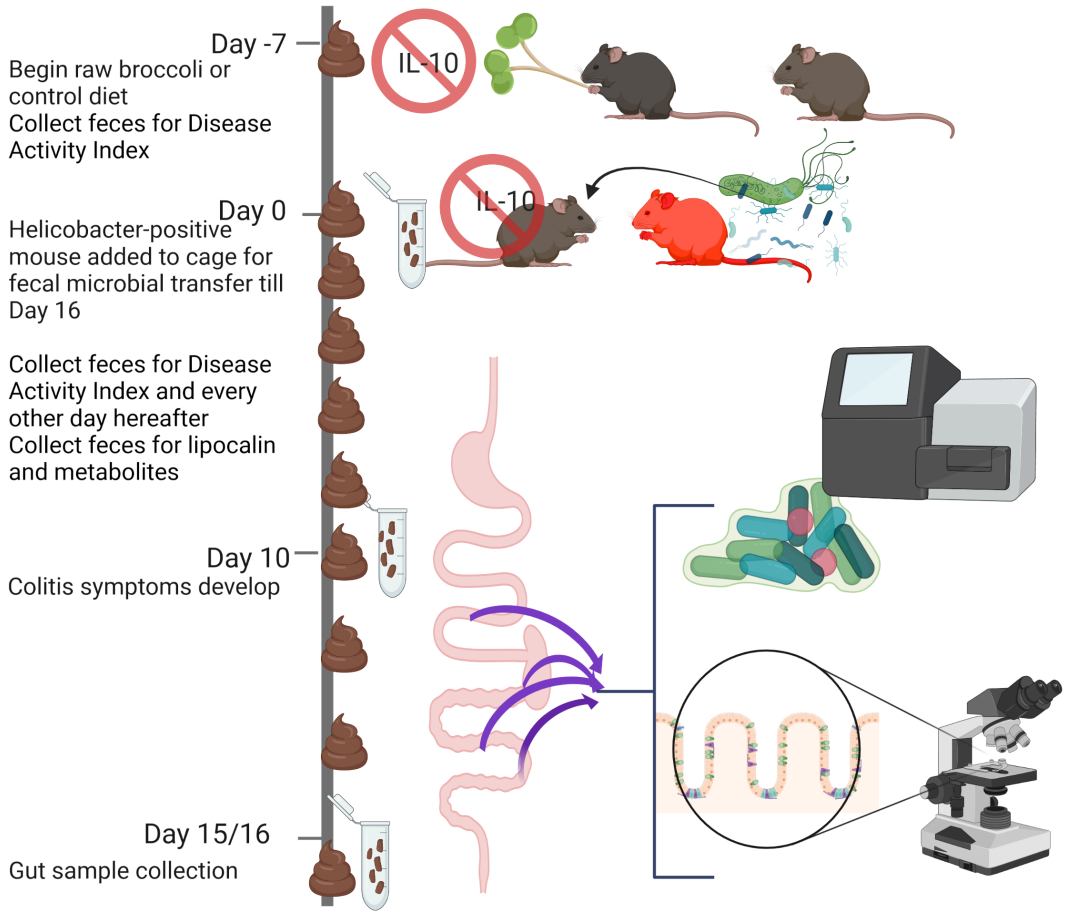

Figure 1. Experimental design.

Trial 1 included 9 mice (n = 5 in the treatment group and 4 in the control group) starting at 4 weeks of age, and Trial 2 included 10 mice (n = 5 in the treatment group and 6 in the control group) starting at 7 weeks of age, which is the standard age for IL-10 microbiota trials. The younger mice that were fed the broccoli diet continued to gain weight once enterocolitis was induced compared to their baseline weight, and the younger mice fed the control diet plateaued around 120% of body weight (Figure 2A, ANOVA $p < 0.05$). The older mice had a similar weight stagnation during enterocolitis regardless of diet (Figure 2B). Consumption of the broccoli diet significantly attenuated the development of inflammation in IL-10 KO mice regardless of age group, indicated by lower DAI scores, corresponding to firmer stool and the lack of blood in stool (Figure 2, ANOVA $p < 0.05$).

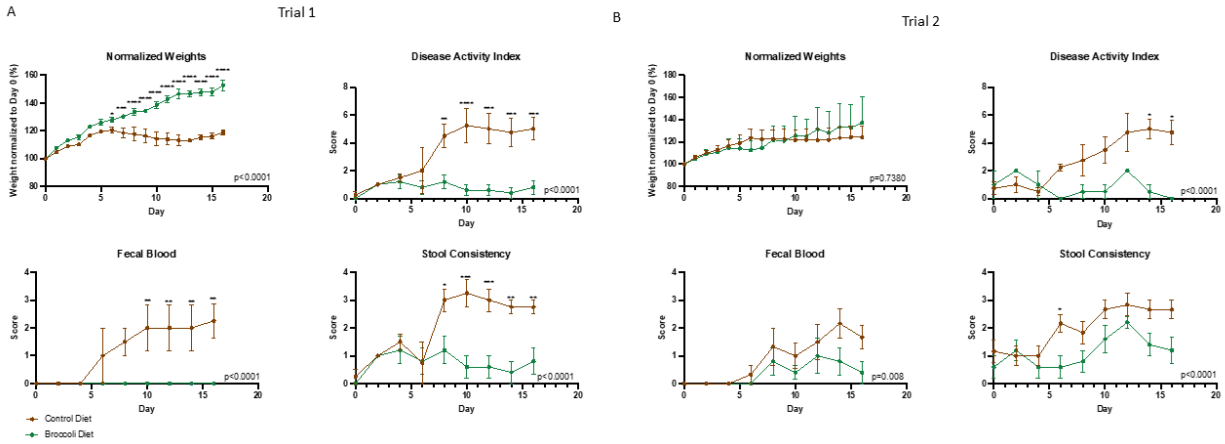

Figure 2. A one-week pretreatment, and continuation of a diet containing 10% broccoli sprouts significantly attenuated the development of colitis in IL-10-ko mice (broccoli, n=10; control, n=10). A) IL-10-ko mice used were 4 - 6 weeks old for the duration of the first trial, and B) 7 - 9 weeks old in the second trial. In the IL-10-ko model, animals with colitis tend to continue gaining weight, but more slowly. Thus, the animal's weights were normalized to their weight on the day of microbial exposure. Disease Activity Index scores are compiled from weight loss, fecal blood, and fecal consistency scores. Overall model significance is included on the chart, and

single timepoint comparisons significance was designated as $p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$ by two-way ANOVA, and post multiple comparisons by Sidak.

Sulforaphane (SFN) in the gut was absorbed by the mice consuming broccoli sprouts and was found in high concentrations in their plasma, while control mice exhibited no circulating SFN (Figure 3A, ANOVA $p < 0.05$). Lipocalin (LCN2), a neutrophil protein that binds bacterial siderophores, serves as a biomarker for intestinal inflammation (40). Analysis of fecal samples on the day of microbial exposure for conventionalization (D0), appearance of symptoms (D10), and end of the trial (D15/16) revealed a markedly lower LCN2 concentration in the group of mice fed the 10% raw broccoli sprouts diet compared to those fed the control diet at both Day 10 and Day 15/16 (Figure 3B, ANOVA $p < 0.05$). This was confirmed by lipocalin in serum on the last day of the trial, which showed a marked decrease in LCN2 concentration in the group of mice fed the 10% Raw Broccoli Sprouts Diet compared to those fed the control diet (Figure 3C, ANOVA $p < 0.05$).

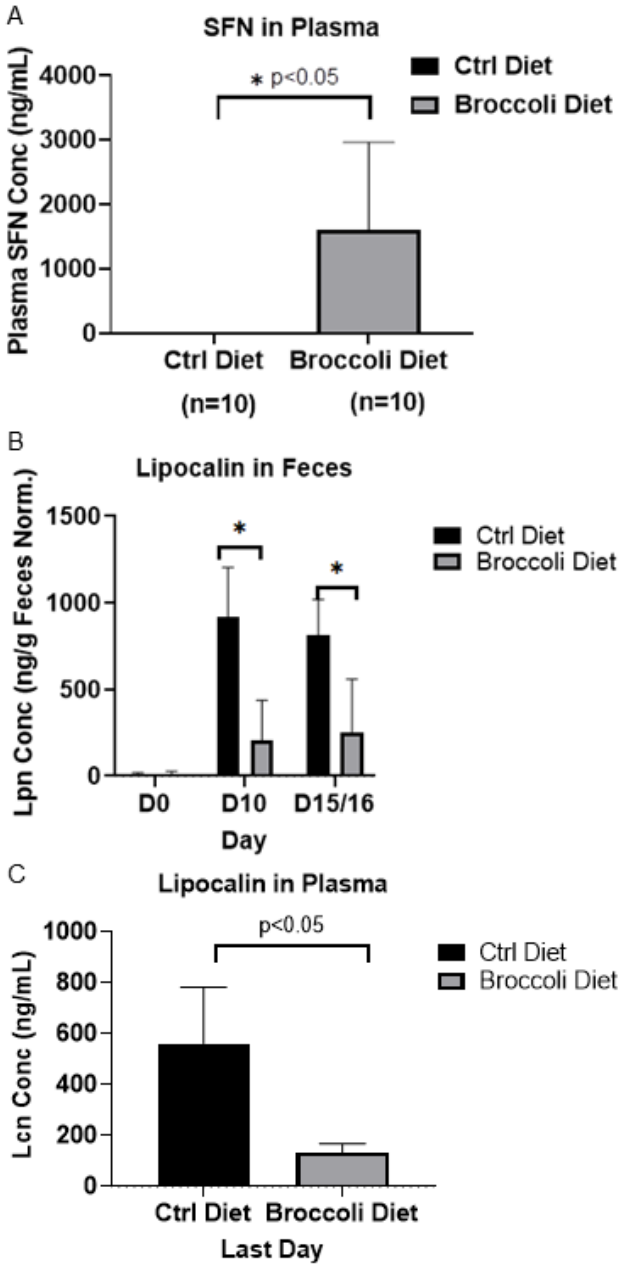

Figure 3. **Levels of (A) plasma sulforaphane and lipocalin in (B) feces and (C) serum measured by ELISA in conventionalized IL-10-ko mice during a diet trial featuring 10% (w/w) raw broccoli sprouts or control diets.** Stool samples were collected from mice (n=10/group) on Days 0 (conventionalization), 10 (onset of symptoms), and 15/16 of experiment for Lipocalin-2 concentration determination. Plasma was collected on Days 15/16 for sulforaphane concentration. Data were similar across both trials and combined. For fecal lipocalin, data was normalized by the weight of feces, and significance set as * $p<0.05$.

Tissue from the ileum, proximal colon, and distal colon were collected for histological scoring (0 = no signs, and 2 = significant signs) of epithelial damage, architectural changes, infiltration of mononuclear cells into the lamina propria, infiltration of polymorphonuclear cells in the lamina propria or into the epithelium, as well as abscesses, ulcers, erosion, and branched crypts which were scored together (Figure S1). Treatment was not a significant factor in linear models for each of the scoring criteria, even when data were subset into trial 1 or 2, or subset into ileum, proximal colon, and distal colon. The exception to this was infiltration of mononuclear cells into the lamina propria, which was significantly lower (linear regression model (lm), $p = 0.02$) in the broccoli sprout mice compared to control mice in trial 1.

Age significantly influenced bacterial community responsiveness to raw broccoli sprouts

Of the three experimental factors analyzed in this study, we report that age is the most significant factor (relative to diet and anatomical location) in driving gut bacterial community richness, membership, and similarity between samples. In trial 1 group, mice that started consuming the raw broccoli sprouts at 4 weeks of age had greater observed bacterial richness by 6 weeks of age than their counterparts fed a control diet (Figure 4A, permANOVA $p < 0.05$). However, mice in trial 2, starting at 7 weeks, showed no significant difference in bacterial richness between the two treatment groups by age 9 weeks (Figure 4B, permANOVA $p > 0.05$). When comparing the effect of diet on bacterial communities within each age group and within each of the four anatomical locations studied, the broccoli sprout diet increased bacterial richness in the cecum and the proximal colon of the younger mice, compared to the younger controls (Figure 4B, Wilcox tests $p < 0.05$). There was no difference between bacterial richness in any gut location in the older mice consuming broccoli sprouts versus the control diet.

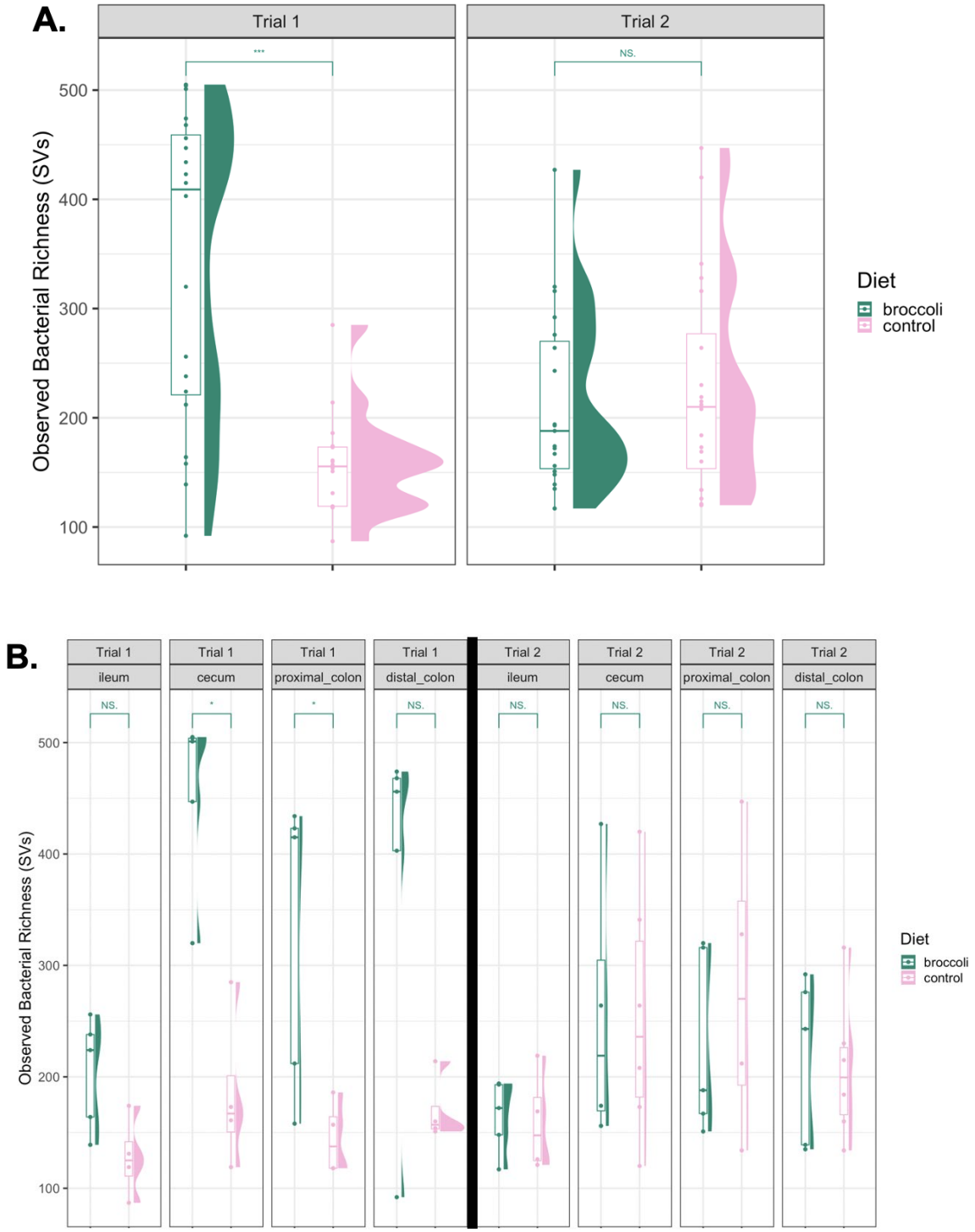

Figure 4. Observed bacterial richness within the gastrointestinal tracts IL-10-KO mice fed control diets or raw broccoli sprout diets from 4-6 weeks of age (Trial 1) and 7-9 weeks of age (Trial 2). Panel A shows comparison of broccoli-fed and control mice in Trial 1 and Trial 2 mice across the whole gastrointestinal tract (***p<0.001); Panel B shows comparisons of broccoli-fed and control mice in which were 4 - 6 weeks old for the duration of the first trial and 7 - 9 weeks old in the second trial, in each anatomical location scraping site (*p<0.05). Graphics made using

phyloseq and ggplot2 packages in R. Significance added by Wilcox tests from ggsignif package in R.

When comparing bacterial community similarity between samples, age was again the strongest explanatory factor in both unweighted (bacterial taxa presence/absence, Figures S2, S3) and weighted (bacterial taxa presence and abundance, Figure 5, 6) metrics. Within the trial 1 group, broccoli-fed samples clustered separately from control samples (Figure 5; Table 1, permANOVA $p < 0.001$), showing a significant difference in microbiota beta diversity between the two dietary treatment groups. This was in part due to significant taxonomic dissimilarity between the two diets in the cecum and from the distal colon between the two diet groups (Figure 6, Table 1), and differences in beta diversity within the proximal colon were nearly statistically significant.

Like the trial 1, dietary treatment was the most significant driver of bacterial community similarity in the older trial 2 mice, but to a lesser extent (Figure 5; Table 1, permANOVA: lower F values, $p < 0.03$). Overall, anatomical location was a significant factor, however; within each of the four anatomical locations studied, there was no significant diet effect shown within the older trial 2 group ($p > 0.05$). The bacterial taxa which drove the differences between age, diet, and anatomical treatment were primarily different SVs identified to the Muribaculaceae family (Figure S4), a taxon of common mouse commensals. SVs in the Muribaculaceae made up much of the core microbiota specific to each group, as well as several SVs identified to Lachnospiraceae, *Bacteroidetes*, *Helicobacter*, and others (Figure S5).

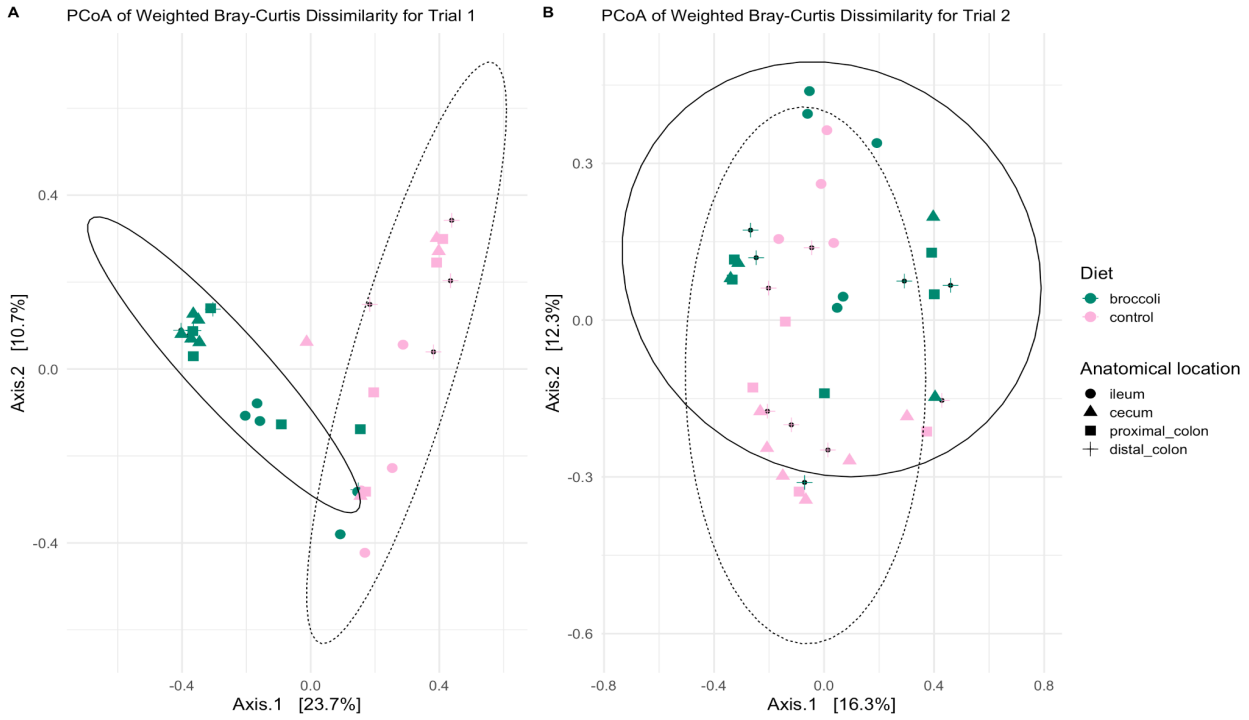

Figure 5. Principal coordinates analysis of bacterial community similarity within the gastrointestinal tracts of 4- or 7-week-old IL-10-ko mice fed control diets or broccoli sprout diets. Calculations using weighted Bray-Curtis dissimilarity show differences in the taxonomic structure of trial 1 mice in Panel A, and trial 2 mice in Panel B. Graphic made using phyloseq, and vegan packages in R.

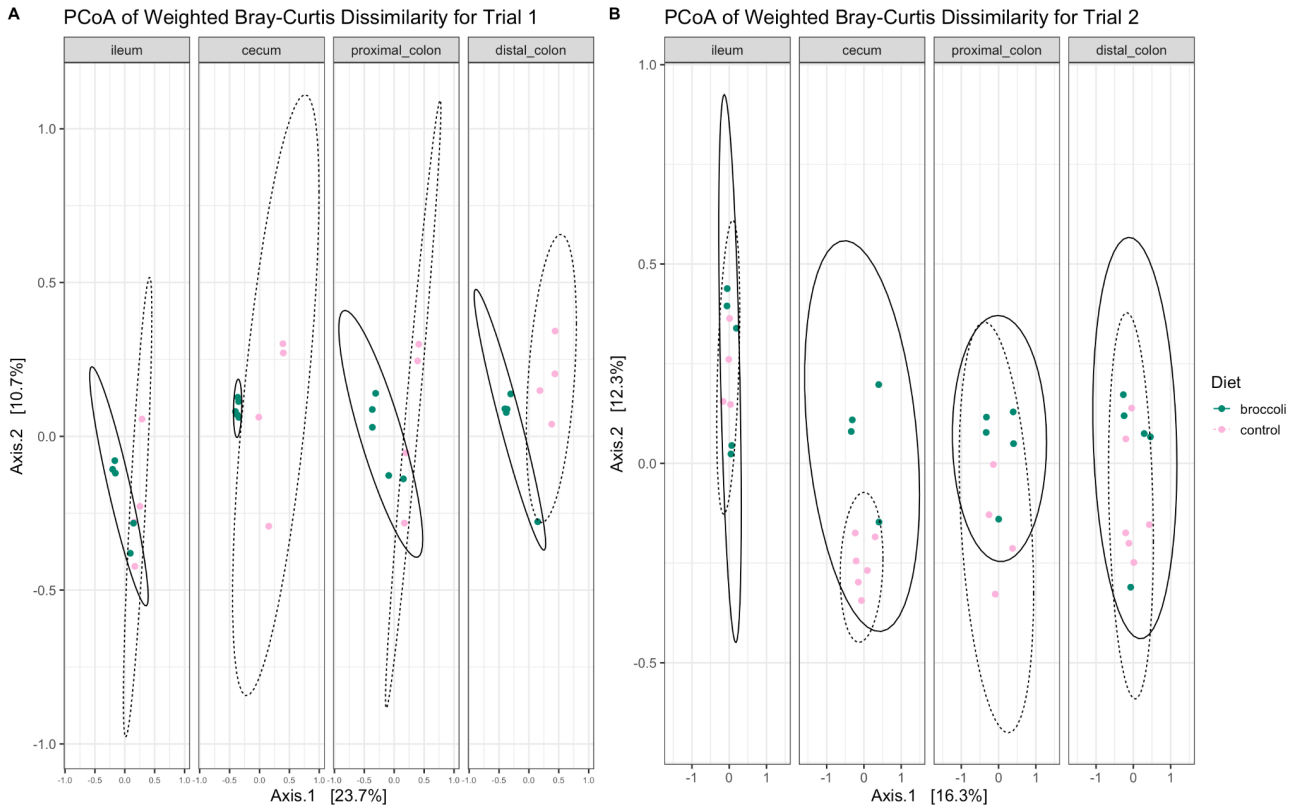

Figure 6. Bacterial community dissimilarity within a specific anatomical location along the gastrointestinal tract of IL-10-KO mice fed control diets or broccoli sprout diets beginning at 4 or 7 weeks of age. Calculations using weighted Bray-Curtis dissimilarity show differences in the taxonomic structure of (A) trial 1 mice sampled at age 6 weeks , and (B) trial 2 mice sampled at age 9 weeks, after each had been consuming diets for 3 weeks. Principal coordinates analysis graphic made using phyloseq, and vegan packages in R.

Table 1. Statistical comparison of principal coordinates analysis of bacterial community similarity within the gastrointestinal tracts of 4- or 7-week-old IL-10-KO mice fed control diets or broccoli sprout diets. Comparisons were done using dietary treatment and anatomical locations as factors, and subsetting by trial, as well as by anatomical location, as noted. Unweighted Jaccard Similarity and weighted Bray-Curtis metrics were used to calculate beta diversity. Number of permutations = 9999.

Permanova Tests of Dissimilarity	Df	Test type	Trial 1, aged 4-6 weeks		Trial 2, aged 7-9 weeks	
			F	P-value	F	P-value
Diet	1	Jac	2.7779	0.0001***	1.1898	0.0258*
		BC	7.5867	0.0001***	2.4498	0.0022**
Anatomical Location	3	Jac	1.2027	0.0291*	1.0955	0.0359*
		BC	1.5991	0.0205*	1.4958	0.0139*
Diet: Anatomical Location	3	Jac	1.0704	0.1780	0.9559	0.8477
		BC	1.0482	0.3504	0.8272	0.8300
Diet effect within ileum	1	Jac	1.1319	0.1242	1.0154	0.3133
		BC	1.4445	0.1220	1.3366	0.1699
Diet effect within cecum	1	Jac	1.9352	0.0074**	1.0393	0.2663
		BC	4.4036	0.0081**	1.6667	0.0818
Diet effect within proximal colon	1	Jac	1.3499	0.0554	0.9949	0.3998
		BC	2.0148	0.0247*	0.8776	0.6080
Diet effect within distal colon	1	Jac	1.7018	0.0261*	1.0058	0.4183
		BC	4.1923	0.0158*	0.9797	0.4679

Significance codes: $p \leq 0.001$ *** | $p \leq 0.01$ ** | $p \leq 0.05$ *
Jaccard dissimilarity = “Jac” | Bray-Curtis dissimilarity = “BC”

Raw broccoli sprout diet was bacteriostatic against putative pathogens

The raw broccoli sprouts had been ground and processed to prepare the diet, a process which had released broccoli myrosinase in plant tissues and metabolized the GLR to SFN prior to feeding the diet. Sulforaphane is bacteriostatic against common gut pathogens, including

Esherichia coli, *Klebsiella pneumonia*, *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis*, *Bacillus cereus,* and *Helicobacter* spp., which can be commensal in immune-competent mice (41, 42). Using that previous literature, we identified these pathogens in our mouse gut samples (Figure 7). The control groups contained more putative pathogenic genera, across more locations in the gut, and in higher abundance than the broccoli sprout groups. The younger broccoli-sprout fed mice contained less abundance of these genera than older mice.

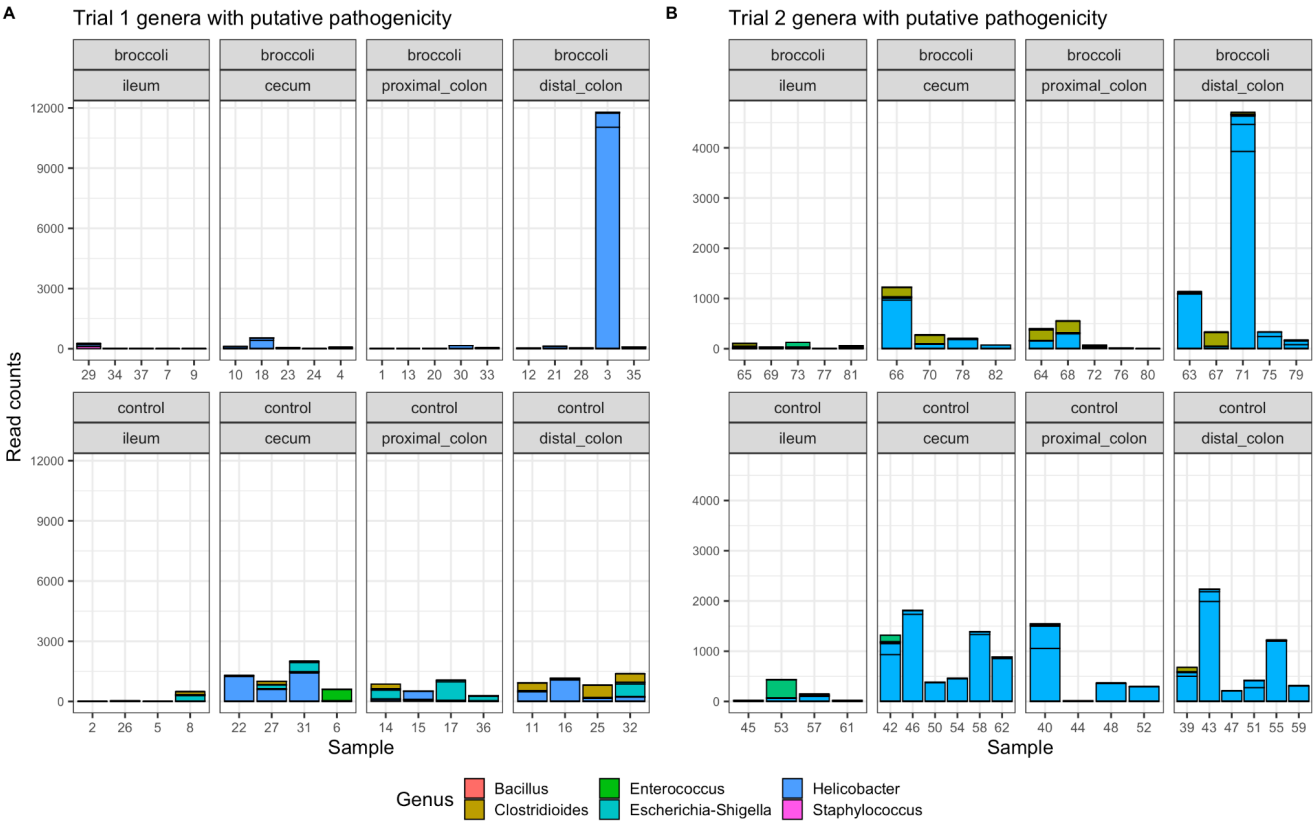

Figure 7. Bacterial sequence variants (SVs) of genera which have putative pathogenicity. Trial 1: 36 samples and 107 SVs; Trial 2: 39 samples and 103 SVs. Graphic made using phyloseq and ggplot2 packages in R.

<u>*Bacteroides* and *Lactobacillus* are present in disease models</u>

Several bacterial taxa have the capability to perform myrosinase-like enzymatic activity and metabolize glucoraphanin into sulforaphane. After consulting the current available literature, we identified 19 taxa to analyze in detail (38, 85, 86). From our samples, we identified 562 SVs belonging to the following genera: *Bacillus, Bacteroides, Enterococcus, Lactobacillus, Lactococcus, Pseudomonas,* and *Staphylococcus* (Figure 8). Though, we did not find *Bifidobacterium, Listeria, Pediococcus, Streptomyces, Aerobacter, Citrobacter, Enterobacter, Escherichia, Salmonella, Paracolobactrum, Proteus,* or *Faecalibacterium.* There were few putative GSL-converting taxa found in the younger trial 1 mice fed the broccoli sprout diet, however, there were high numbers of read counts for *Bacteroides* and *Lactobacillus* in the trial 1 controls. Both dietary treatment groups of the older trial 2 mice exhibited high levels of *Bacillus* and *Enterococcus* SVs (Figure 8).

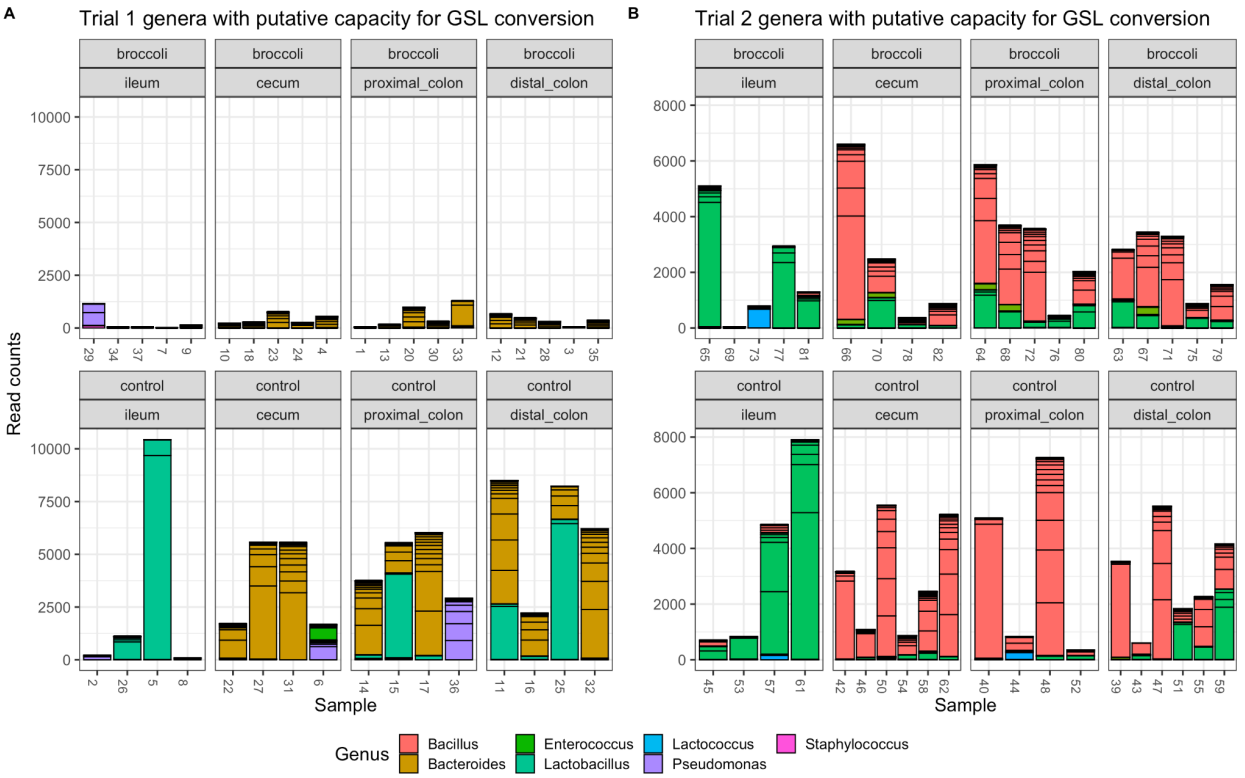

Figure 8. Bacterial sequence variants (SVs) of genera which have putative capacity to convert glucoraphanin to sulforaphane (GSL). Strains of bacteria in these genera have been demonstrated to perform myrosinase-like activity in the digestive tract, as reviewed in (43). Trial

1: 36 samples and 241 SVs; Trial 2: 39 samples and 353 SVs. Graphic made using phyloseq and ggplot2 packages in R.

There were few species from putative GSL-converting genera found in the younger trial 1 mice fed the raw broccoli sprout diet (Fig. 9). The cecum and distal colon had a significant presence of *B. sartorii* and *B. acidifaciens.* The proximal colon included *B. caecimuris*, *B. sartorii*, and *B. acidifaciens.* Most of the species in the ileum could not be identified. By contrast, the trial 1 control group had significant presence in four of the identified GSL-converting species (*Lactobacillus gasseri* in the ileum, proximal and distal colon; *B. sartorii*, *B. caecimuris*, and *B. acidifaciens* in the cecum, proximal and distal colon).

In the older trial 2 mice, the overall presence of GSL-converting taxa was similar to the trial 1 control, but with five of the identified species having significant presence (Fig. 9). In the trial 2, broccoli-fed mice, the highest presence was *Lactobacillus intestinalis* in the ileum with less but still significant presence in the cecum, proximal and distal colon. These mice also had a significant presence of *B. caecimuris* and *B. acidifaciens* in the cecum, and proximal and distal colon, with *B. sartorii* in the proximal and distal colon. As in the trial 1 control, the trial 2 control had a high presence of *L. gasseri* in the ileum, but there was much less in the proximal and distal colon. *B. acidifaciens* was dominant in the cecum, proximal and distal colon. *L. intestinalis* had a significant presence in the ileum, cecum, and distal colon as did *B. sartorii* in cecum, proximal and distal colon. There was a minor presence of *E. casseliflavus*, *E. faecalis, B. thetaiotaomicron, E. cecorum*, and *S. aureus*.

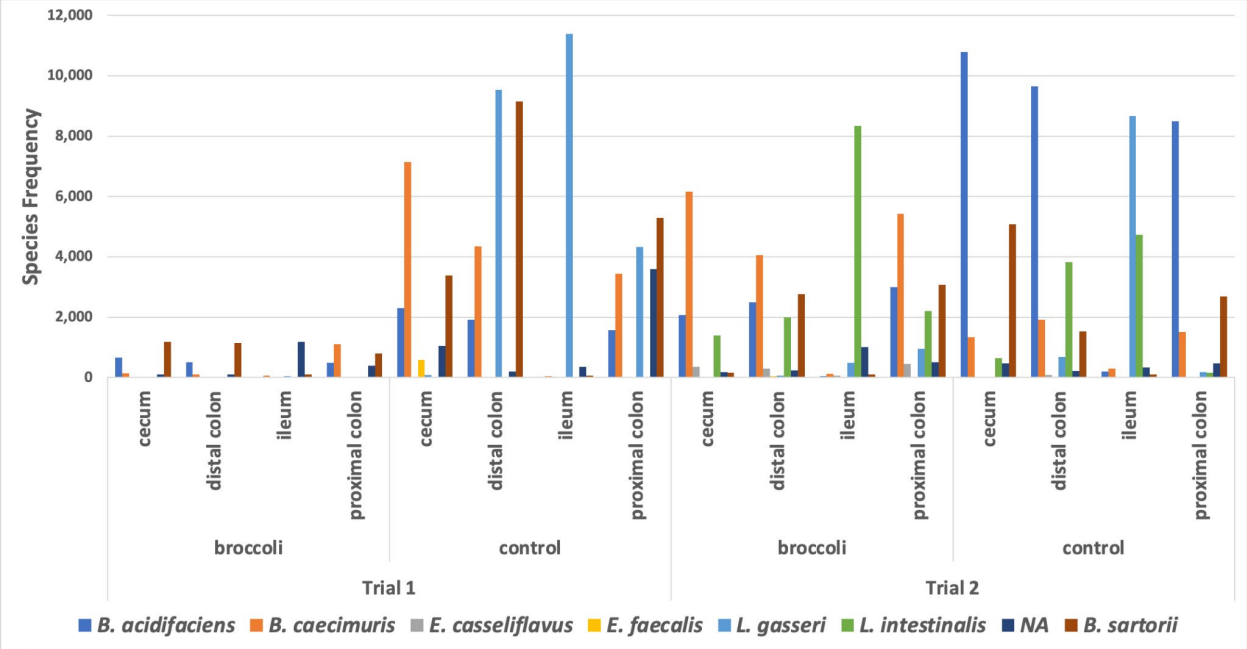

Figure 9. Biogeographic frequency of species from putative GSL-converting genera in the treatment groups. The Silva Database identified *Bacteroides* species *acidifaciens*, *caecimuris*, and *sartorii*, *Enterococcus cecorum*, *Staphylococcus aureus*, and *Lactobacillus intestinalis*. NCBI BLASTN identified additional *B. acidifaciens*, *B. caecimuris*, and *B. sartorii.* BLASTN uniquely identified *Enterococcus* species *casseliflavus* and *faecalis*, and *Lactobacillus gasseri*. *Bacteroides thetaiotaomicron* was linked to two (2) SVs based on genome mapping. *E. cecorum* (frequency 12), *S. aureus* (2), and *B. thetaiotaomicron* (5) were not included in the figure as their frequencies were minimal.

Discussion

The objective of this pilot study was to evaluate IL-10 deficient mice as a model for studying the role of dietary broccoli and broccoli bioactives in reducing inflammation, modifying the immune response, and supporting gastrointestinal tract microbial systems. Diet is well-recognized to drive gut microbial diversity, and in particular, high-fiber diets can increase microbial diversity, recover beneficial symbiotic species in the gut, and reduce inflammation by providing antioxidants against reactive oxygen species or antimicrobials against microbial competitors. Our initial hypothesis was that a medium-high concentration of raw broccoli sprouts in the diet of mice would induce changes to the gut microbiota, increase SFN present in the plasma, and collectively

reduce the symptoms of enterocolitis. In conducting two replications of a diet trial, we observed a previously unreported effect of age in early life – a three week difference – on the response to broccoli sprout bioactives.

Dextran sodium sulfate (DSS) is an established and widely used model for studying SFN reduction and prevention of IBD in animals (22, 23). Treatment of DSS in drinking water results in a disease profile similar in progression and morphology to human Ulcerative Colitis (22, 44). DSS colitis begins by modifying the expression of tight junction proteins in intestinal epithelial cells, leading to a leaky epithelial barrier (45). This is followed by goblet cell depletion, erosion, ulceration, and infiltration of neutrophils into the lamina propria and submucosa (46), triggering the innate immune response (47, 48). However, Bhattacharyya et al reported DSS treatment results in an excess of ROS species which quickly dephosphorylate Hsp27 resulting in direct IkBa dissociation, circumventing the canonical inflammatory pathway (49). Further, DSS treatment instigates colitis through chemical and physical damage, without a direct interaction on the immune system, thus it is inadequate for investigating the specific immunohistopathology present in Crohn's Disease. While a few studies have used IL-10-ko mice to study the effects of certain mineral additives (50, 51), isomaltodextrin supplementation (52), or high-fat diets on colitis (53, 54), the use of this model for diet studies is still nascent.

Younger IL-10-KO mice were more responsive to diet compared to adolescent counterparts

A key finding from the bacterial community analyses performed in this study was that bacterial richness and community similarity were greater in younger mice (4 - 6 weeks old during trial 1) than in adolescent mice (7 - 9 weeks old during trial 2) after three weeks on either the broccoli or control diet, and that the difference between raw broccoli sprouts and control diets was significant in the younger mice but not in older ones. We hypothesized that the responsiveness of young mice to our dietary treatment was driven by the instability of the developing gut microbiota and its amenability to selective pressures. Mice weaned at approximately 20-22 days

of life (3 weeks) and this time period is critical for the development of the neonate's immune system and gut microbiota, a community succession which is coordinated by immune factors in milk, growth of the gastrointestinal tract, and improvement of epithelial barrier function (55). By 28 - 35 days of life (4 - 5 weeks), mice are just beginning sexual development but are still juveniles, and still have a changing gut microbiome (56). By days 35 - 42 (5 - 6 weeks) they are beginning adolescence (57), and during this period their gut microbiome stabilizes and will remain through adulthood unless it is perturbed (56). Thus, our trial 1 mice were still in a transitional state of life and trial 2 mice were just beginning a period of life marked by stability of the immune system and gut microbiome. Our findings are consistent with previous human studies that have suggested that gut microbiota of infants and children are more plastic than that of adults (11). In humans, starting around age 3, gut microbiota fall into long-term patterns driven by consistency in diet and lifestyle (11, 58–60).

In mouse models, events in early life like stress, e.g., (61), antibiotics, e.g., (62), or other disruptions like colitis, e.g., (63), are shown to negatively impact microbial communities well into adulthood. IL-10-ko mice will acquire a microbiota as they age if they are placed in non-sterile rearing conditions (64), but even when they acquire microbiota they appear to never fully develop diverse, commensal gut microbiota and this appears to be attributable to the spontaneous generation of enterocolitis at the 4 - 6 weeks of age (65). In humans, IBD is partly linked to certain disruptions in early life, including exposure to antibiotics (5) or cigarette smoke (4), and being fed formula instead of breastmilk (4).

In their review article, Derrien et al. highlight the importance of diet in microbiota development in early life (11). Consumption of foods rich in fiber, such as fruits, vegetables, and grains, has been associated with microbiota stabilization (60), because the wealth of microbial byproducts is demonstrated to recruit and maintain a diverse microbial community (66), reduce inflammation, improve nutrition, and stimulate gut motility: aspects which are limited in IBD (67–69). Furthermore, in adults, undergoing dietary change is one of the few external factors that can

“destabilize,” or subject the adult gut microbiota to change (11, 59), which may be useful in removing gut microbial communities which are not providing functional benefits and recruiting a different community instead.

Raw broccoli sprouts increase microbiota richness and diversity while possibly reducing proinflammatory bacteria

A functional gastrointestinal tract has a stable community of gut symbionts, composed mainly of bacteria, which provides support for digestion, immune function, and which can resist infiltration and infection by pathogenic microorganisms and protect against metabolic disease (70). Inflammatory diseases such as CD are associated with changes in the presence, abundance, and functionality of a patient's microbiomes (68, 71, 72). Studies in CD have shown population clustering and reduced bacterial taxonomic diversity within the Firmicutes and Bacteroidetes phyla (73), both of which are dominant in the gut and are indicators of broad-changes to the gut community. Additionally, CD has specifically been associated with a reduction in *Faecalibacterium prausnitzii*, a putative glucosinolate metabolizer, and reintroduction of this bacteria resulted in decreased inflammation (74). However, not all studies on the gut microbiota in IBD patients point to specific patterns of change across all disease states and people, and many gut microbiota are only useful to the host in specific metabolic conditions (75, 76), which can obfuscate our understanding of which microbiota may be useful to try and maintain in order to preserve beneficial interactions with the host.

After subsetting our data by trial (age), we found that dietary treatment was the next most significant driver of bacterial community structure (Table 1). We observed that mice fed broccoli sprouts had greater bacterial community richness when compared to the control group. Also, compared to the controls, the younger mice fed a diet containing broccoli sprouts had more bacterial genera meet the core genera standard of prevalence and abundance (Figure S5). This

finding is of interest because it may suggest that young age and/or a broccoli diet promote a larger, more diverse core community of bacteria within the diseased gut of our mouse models. We also found that the broccoli-fed mice of trial 2 had fewer genera that met the core standard, which could suggest that the core gut microbial community diminishes with age.

Interestingly, our results suggest that consumption of broccoli sprouts may prevent the accumulation of putatively harmful, pro-inflammatory bacteria. When compared to the controls, the broccoli-fed mice exhibited less prevalence and abundance of *Helicobacter*, which maybe act is a non-harmful commensal in conventionally raised and immune competent mice, but acts as a pro-inflammatory bacterial genus in IL-10-ko mice with little ability to moderate their own response to the presence of microbiota (35). The specific species of *Helicobacter* which colonies IL-10-ko mice can also affect the host's immune response and severity of symptoms (77). Sulforaphane is bacteriostatic against several bacteria, including *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis*, *Bacillus cereus,* and *Helicobacter* spp. (41, 42, 78).

Sulforaphane is also bacteriostatic against bacterial taxa which may provide some benefit to mice, including *Bacteroides (79)*, a genera containing known glucosinolate metabolizers (80). Across many of our experimental groups, we found several bacterial genera, including *Bacteroides* (but not *Bacteroides thetaiotaomicron*), *Enterococcus casseliflavus*, *Lactobacillus gasseri*, and others in very low abundance, that may perform myrosinase-like activity in the digestive tract (Figure 8), as reviewed in (43). *Enterococcus casseliflavus* are gram-positive, motile facultative anaerobes sourced from vegetables, with high glucosinolate degradation efficiency (81). One (1) SV was matched to *E. casseliflavus* based on BLASTN results in the trial 2 mice, and favored the broccoli mice in trial 2 (1164 count) compared to the control (113). Although GLR levels were low in the broccoli group (4 µg on average), *E. casseliflavus* may have been responsible for some of the conversion to SFN.

Lactobacillus gasseri was the most abundant species with putative myrosinase-like enzymatic activity. A probiotic strain with implications for fighting pathogens, *L. gasseri* has hydrolyzed GLR to SFN *in situ* in the cecum of rats (82, 83). Four (4) SVs were mapped to *L. gasseri* using BLASTN. These sequences were most abundant in the control for both trials (trial 1: 25342 vs. 97; trial 2: 9567 vs. 1532), with the highest counts occurring in the younger trial 1 control mice.

Contrary to expectations, with the exception of *Bacteroides caecimuris*, *Enterococcus casseliflavus*, and *Lactobacillus intestinalis*, the species identified from GSL converting genera tended to favor the control treatments for both trial 1 and 2. As a whole, the presence of GSL genera negatively correlated with the broccoli treatments. The high concentration of SFN in the diet, and the relatively low amount of the GLR precursor, may have precluded the need to select for metabolizing-competent bacteria, and it may be that the taxa present in younger mice were sensitive to the presence of SFN while the community present in older mice was not. However, in these mice without the ability to recruit commensal bacteria, commensalism cannot be assumed for any taxa. For example, IL-10-ko mice infected with Helicobacter also exhibited higher abundance of *Bacteroides* and *Lactobacillus* species (84).

Biogeographical patterns emergent in younger mice consuming broccoli sprouts

Anatomical location within the gut, including different organs and solid-associated, liquid-associated, and mucosal-associated locations, select for different bacterial communities due to environmental conditions and anatomical features specific to each location, i.e. biogeography (85–87), even within the first few days of life, e.g. (88). In IL-10-ko mice, which are raised in 'germ-free' conditions and do not acquire gut microbiota, there is no biogeographical signal in the gut, and the damage to the epithelium caused by inflammation or the lack of IL-10 may preclude the ability of a typical biogeographical signal to form. The addition of a conventionally raised mouse to the IL-10-ko mouse cages, and placement in a conventional mouse room, is enough to

stimulate microbial transmission (89) and for IL-10-ko mice to acquire a gut microbiota even as they react negatively to it.

The third experimental factor of this study, and the one that showed to be the least significant driver of microbial community dissimilarity, was the anatomical location of sample derivation. We found that the number of bacterial taxa (SVs) in the cecum and proximal colon of the younger trial 1 mice fed broccoli sprouts was significantly elevated, as compared to the control trial 1 mice (Figure 4B), and significant community dissimilarity between the broccoli sprout diet group the control group in both the cecum and the distal colon. There were no significant dietary treatment effects on richness or bacterial community similarity in any anatomical location studied in the older trial 2 mice (Figure 4B). The cecum, a muscular and oblong pouch emerging from the junction of the ileum and proximal colon, houses a diverse community of fibrolytic bacteria, and specialized peristaltic movements allow for fiber in the ileocecal junction to be pulled into the cecum, as well as separate large and small fibers to allow for retention of particles to induce more microbial fermentation. Mice have a large cecum, even in IL-10-ko mice this organ acquires a diverse microbiota given enough microbial exposure and time (64). Studies on inflammation for IBD models report significant inflammation and changes to cecal microbiota in mice (21, 90, 91). It is likely that given the short duration of this experimental period, the cecum and parts of the colon that also house diverse microbiota were the only organs which were suited to recruit differential bacterial communities fast enough to be statistically significant. While humans have a very small cecum relative to our size, and do suffer inflammation there in IBD patients, the effect on IBD of the microbiota there is unknown.

Limitations and Future Directions

In our study, raw broccoli sprouts in the diet provided during the critical period of microbiome stabilization and enterocolitis development in these IL-10-ko mice resulted in an increase in microbial diversity. Due to the short duration of the experiment, we were unable to

discern if this increase in diversity persists into adulthood, and if this would preclude the development of more intense symptoms and damage to the intestinal epithelium. Further, we lacked sufficient power to determine sex specific differences in response to diet (52, 92), immune function (93), and microbial acquisition (94).

Future research will also be required to examine the effect of cooking preparation on the concentrations of precursors and bioactives which are contained in the diet and available in the gut. The myrosinase enzyme present in broccoli and sprouts can metabolize the glucoraphanin precursor to bioactive SFN when the plant tissues are cut or chewed, but most of the precursor is converted to biologically inactive SFN nitrile by the epithiospecifier protein, also present in broccoli (9). SFN was high in our raw sprout diet, which was an effect of the preparation process stimulating release of myrosinase. However, SFN is an unstable molecule and will not persist in processed raw sprouts unless kept frozen. These enzymes can be inactivated by cooking, preserving the stable precursor in the diet and allowing gut microbiota to perform the conversation in the intestines (20).

Future research will also be needed into the feasibility and adoptability of this intervention. The difficulty in managing CD symptoms is costly: patients spend an average of $2,000 USD/year on out-of-pocket costs (95), and collectively create billions in U.S. healthcare costs (96, 97). Further, many IBD patients may be told to avoid fiber for fear of exacerbating symptoms or aggravating tender intestines (18, 24). Given the importance of diet in supporting host health and microbiota, we underscore the need for understanding dietary preferences, and for incorporating nutritional intervention as part of a holistic treatment for gastrointestinal inflammation.

Materials and Methods

Diet

Multiple lots of Jonathan's Sprouts™ (Rochester, MA, U.S.) broccoli sprouts were purchased from a nearby grocery store (Bangor, ME, U.S.) and stored in a -70℃ freezer for storage until they could be freeze-dried at the University of Maine Pilot Plant (Orono, ME, U.S.). The sprouts were then crushed by mortar and pestle into a fine powder and mixed with purified 5LOD 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 (98). 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℃ freezer until future use. Samples from the control and the sprout diet were tested for glucoraphanin and sulforaphane concentrations using LC/MS (20), none was found in the control and the sprout diet contained an average 4 µg of GLR and 85 µg of SFN (induced when raw diets are crushed and myrosinase is released from broccoli tissue vesicles).

IL-10 Mouse Model

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Vermont (PROTO202000040), in Burlington, Vermont, U.S. and all biosafety work at the University of Maine was approved by the Institutional Biosafety Committee (protocol SI-092220). Two replicate trials were conducted using male and female IL-10 knockout mice (*Mus musculus*) on a C57BL/6 background (B6.129P2-Il10tm1Cgn/J, strain 2251; Jackson Laboratories, Bar Harbor, Maine, U.S.). IL-10-ko mice are a well-demonstrated model for Crohn's research and the C57BL/6 genetic line shows some genetic-based resistance to developing symptoms (32), which allowed us to focus on environmental triggers of disease.

Trial 1 included 9 mice starting at 3 weeks of age. Trial 2 included 10 mice starting at 3-4 weeks of age. At 3-4 weeks of age the mice were moved from the barrier facility to the conventional mouse room and placed on their respective diets.

The experimental design was structured as a prevention paradigm (Figure 1). A colony of homozygous interleukin-10 knockout mice was maintained in a barrier facility which is *Helicobacter (H.) hepaticus* free. From weaning and for the duration of the trial, they were given either the 5LOD control diet or the treatment diet consisting of 10% by volume raw broccoli sprouts and 90% control diet. Mice had access to tap water *ad libitum*. Mice consumed the respective diets for 7 days prior to the induction of colitis, and remained on their respective diets for the remaining 16 days of the experiment. Induction of colitis was achieved by adding an H. species positive mouse in the cage for the remainder of the experiment. The H positive mouse transfers commensal bacteria via feces and induces colitis over the course of 10 days (32, 39). Day 0 of each trial was set as the day the IL-10 mice were exposed to the H. positive mouse, such that experimental Day 10 is when symptoms appear (Figure 1). Animals were euthanized by carbon dioxide and cervical dislocation on Day 16.

Assessment of Disease Activity

Disease severity was assessed every other day beginning on Day 0 when mice were combined with the *H.* positive mouse, using a disease activity index (DAI). This included a score that combined fecal blood presence and severity, and stool consistency (99). The animals' weights were normalized to their baseline weight on Day 0 of the trial, as at this age, mice are still in a growth phase and likely to gain weight even when under duress; this is also the rationale for excluding weight loss as part of the DAI criteria. The presence of fecal blood was assessed using Hemoccult Single Slide testing slides (Beckman Coulter, Brea, CA, U.S.).

Assessment of Lipocalin

Serum samples from the last day of the trial immediately after euthanasia, as well as fecal samples at various time points were frozen for evaluation of fecal lipocalin (LCN2); a neutrophil protein that binds bacterial siderophores and serves as a biomarker for intestinal inflammation (40). Frozen fecal samples were weighed up to 20 mg and reconstituted in phosphate buffered solution (PBS) with 0.2 mL of 0.1% Tween 20. Samples were then thawed and vortexed to create a homogenous fecal suspension. The samples were centrifuged for 10 minutes at 12,000 rpm at 4 °C. Clear supernatant was collected and stored at -20 °C until analysis. Lipocalin-2 concentration, a neutrophil protein that binds bacterial siderophores and serves as a biomarker for intestinal inflammation (40), was measured in the samples by a mouse Lipocalin-2/NGAL DuoSet ELISA kit (R & D Biosystems, USA) following the manufacturer's instructions. The readings at wavelengths of 540 nm and 450 nm were measured by a Thermo Scientific Varioskan LUX Multimode Microplate Reader. The readings at 540 nm were subtracted from the readings at 450 nm to correct for the discoloration of the solution in serum. Serum was also measured for sulforaphane concentration using LC/MS (20).

Histological Analysis of Tissues

After euthanasia, tissue segments (1 cm, collected in duplicate) from the ileum, proximal colon and distal colon were collected and fixed in 4% paraformaldehyde overnight for histological evaluation. Tissues were immediately rinsed with phosphate buffer solution (PBS), placed in 2% paraformaldehyde/0.2% picric acid as a preservative, and stored at 4℃ until transport to the University of Maine Electron Microscopy Laboratory (Orono, Maine) for processing. All processing protocols took place in a biosafety cabinet using aseptic techniques to reduce contamination. A pipette was used to gently remove the previous PBS that the tissue was submerged in without disturbing tissue while leaving a little bit of PBS behind to prevent the tissue drying out. The sample tubes were refilled with fresh PBS, and the wash step was repeated four times throughout the day every three hours with samples stored at 4℃ between washes.

After the final wash step, the tissue samples were transferred into embedding baskets. The samples were dehydrated by immersing the samples in a graded series of ethanol at 4℃ (50%, 70%, 80%, 95%, 100% ethanol), cleared in xylene, and then infiltrated and finally embedded in Paraplast X-tra. During embedding, care was taken to orient the intestine samples to give a cross section during sectioning. Slides were sectioned at 5μm and stained in Hematoxylin and Eosin (Cardiff et al. 2014). Images were taken using an Olympus BX41 microscope with a Zeiss ERc 5s digital camera.

The tissues were scored with 7 criteria (Figure S1) used to assess inflammation in the ileum, and the proximal and distal colon tissues, performed according to parameters provided by the Mawe Lab at the University of Vermont (100). 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 equating to sighting of ≥1 cell in a given 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. Finally, presence of granulomas was scored with 0 indicating no granulomas and 1 equating to ≥1 granulomas. Scores by treatment, anatomical location, and trial were compared with analysis of variance.

DNA extraction and 16S rRNA bacterial sequencing library preparation

On Day 16, following euthanasia, intestinal tissue segments (2 cm in length) were collected from the ileum, cecum, proximal colon, and distal colon, and placed in RNAlater preservative (Invitrogen, Waltham, MA, U.S.) and transported overnight on ice to the University of Maine for DNA extraction. All DNA extraction processing steps took place in a biosafety cabinet using aseptic techniques to reduce contamination. 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 µmol. 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 fecal-based microbial communities (Zymo fecal/soil kit), and some aliquots archived. DNA extract was roughly quantified and purity-checked with a Nanodrop spectrophotometer. Samples underwent DNA amplicon sequencing of the 16S rRNA gene V3-V4 region, using primers 341F (101) and 806R (102) and protocols consistent with The Earth Microbiome Project (103), and sequenced on an Illumina MiSeq platform using the 2 x 300-nt V3 kit (Molecular Research Labs, Clearwater, TX). Nine samples failed the first sequencing run, and were extracted again for repeated sequencing. Raw sequence data (fastq files and metadata) from both runs are publicly available from the NCBI Sequence Read Archive (SRA) under BioProject Accession PRJNA909836.

16S rRNA bacterial community sequencing analysis

Amplicon sequence data was processed using previously curated workflows in the Ishaq Lab (Supplemental Material) which used the DADA2 pipeline ver. 1.22 (104) in the R software environment ver. 4.1.1 (105). The dataset started with 38,287,212 million raw reads from 80 samples and 4 negative controls. All samples were sequenced in an initial batch together, but 5 samples failed to meet all quality control standards and were resequenced. The two sequencing batches were put through quality control steps separately in DADA2 and combined before rarefaction. 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 PhiX version 3 positive control (Illumina; FC-110-3001). After filtering, 43,382,928 paired non-unique reads and 289 samples 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 (106) and reads matching chloroplasts and mitochondria taxa were removed using the dplyr package (107). No-template control samples were used to remove contaminating sequences from the samples by extraction batch (108). The sequence table, taxonomy, and metadata were combined using the phyloseq package (109) to facilitate statistical analysis and visualization, representing 80 samples and 22,805 taxa. Due to the large variability in sequences per sample which passed quality assurance parameters (range 12,060 - 157,040 sequences/sample), and the knowledge that some sample types would contain much lower microbial diversity than others, the data were rarefied (110, 111) to 12,060 sequences/sample.

Normality was checked using a Shapiro-Wilkes test on alpha diversity metrics generated from rarefied data; observed richness (W = 0.86662, p-value = 1.186e-06) and evenness (W = 0.89141, p-value = 9.775e-06) were not normally distributed. Shannon diversity was also not normally distributed (W = 0.95012, p-value = 0.005061). Linear models were run for comparisons of alpha diversity metrics using linear models to compare by sample type, (lme4 package (112)), in which anatomical location, diet treatment, and trial (either 1 or 2) were used as factors.

Jaccard unweighted similarity was used to calculate sample similarity based on community membership (species presence/absence) and non-parametric multidimensional scaling (Trial 1 run 20 stress = 0.1940131; Trial 2 run 20 stress = 0.1748438) and tested with permutational analysis of variance (permANOVA) by using the vegan package (113). Random forest feature prediction with permutation was used to identify differentially abundant SVs based on factorial conditions (114). Plots were made using the ggplot2 (115), ggpubr (116), and

phyloseq packages.

Source Tracker algorithms which had been modified for the R platform (117, 118) were used to identify source: sink effects based on anatomical location. This was used to determine if the cecum could be the source for population sinks in the colon, as a proxy for the model's applicability to the human gut anatomical features and microbial communities. A total of 38 SVs were identified as possibly sourced from the cecum (Figure S5). Common mouse commensals, but not the putative GSL converting bacteria in the Broccoli mouse gut samples, were among those taxa identified as sourced in the cecum and sinking in the proximal or distal colon.

Acknowledgements

All authors have read and approved the final manuscript. This project was supported by the USDA National Institute of Food and Agriculture through the Maine Agricultural & Forest Experiment Station: Hatch Project Numbers ME022102 and ME022329 (Ishaq) and ME022303 (Li); the USDA-NIFA-AFRI Foundational Program [Li and Chen; USDA/NIFA 2018-67017-27520/2018-67017-36797]; and the National Institute of Health [Li and Ishaq; NIH/NIDDK 1R15DK133826-01]. Johanna Holman was supported by ME0-22303 (Li), and Lola Holcomb was supported by US National Science Foundation One Health and the Environment (OG&E): Convergence of Social and Biological Sciences NRT program grant DGE-1922560, and the UMaine Graduate School of Biomedical Science and Engineering.

Author Contributions

Conceptualization, S.L.I., Y.L., T.Z., G.M., P.M., G.C.; Methodology, S.L.I., Y.L., T.Z., G.M., P.M., J.H., L.H., M.H., B.L., EP; Software, S.L.I.; Formal Analysis, J.H., L.H., S.L.I.; M.H., A.S., T.H., B.H.; Investigation, M.H., B.L.; J.H., L.H., L.C.; A.S.; Resources, G.M., S.L.I., Y.L.; Data Curation, S.L.I., J.H., L.H.; Writing - Original Draft, J.H., L.H., S.L.I., Y.L.; Writing - Review and Editing; S.L.I., Y.L., T.Z., G.M., P.M., J.H., L.H., M.H., B.L., G.C.,T.H., B.H., E.P.; Visualization, J.H., L.H., S.L.I., A.S.,T.H., B.H.; Supervision, S.L.I., Y.L., T.Z.; G.M.; Project Administration, S.L.I., Y.L., G.M.; Funding Acquisition, S.L.I., Y.L., G.M. G.C.

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