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Steamed broccoli sprouts alleviate gut inflammation and retain gut microbiota against DSS-induced symptoms.

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Abstract: Inflammatory Bowel Diseases (IBD) are devastating conditions of the gastrointestinal tract with limited treatments, and dietary intervention may be effective, affordable, and safe for managing symptoms. Ongoing research has identified inactive compounds in broccoli sprouts, like glucoraphanin, and that mammalian gut microbiota play a role in metabolizing it to the anti-inflammatory sulforaphane. The biogeographic location of participating microbiota and how that affects anti-inflammatory benefits to the host is unknown. We fed specific pathogen free C57BL/6 mice either a control diet or a 10% steamed broccoli sprout diet, and gave a three-cycle regimen of 2.5% dextran sodium sulfate (DSS) in drinking water over a 34-day experiment to simulate chronic, relapsing ulcerative colitis. We monitored body weight, fecal characteristics, lipocalin, and bacterial communities from the contents and mucosa in the jejunum, cecum, and colon. Mice fed the broccoli sprout diet while receiving DSS performed better than mice fed the control diet while receiving DSS for all disease parameters, including significantly more weight gain, lower Disease Activity Index scores, lower serum lipocalin, and higher bacterial richness in all gut locations. Bacterial communities were assorted by gut location except in the mice receiving the control diet and DSS treatment. Importantly, our results suggested that broccoli sprout feeding effectively abrogated the effects of DSS on gut microbiota, as bacterial communities were similar between mice receiving broccoli sprouts with and without DSS. Collectively, these results strongly support the protective effect of steamed broccoli sprouts against gut microbiota dysbiosis and development of colitis induced by DSS.

Importance: Spatially resolved microbial communities provide greater insight than fecal microbial communities when investigating host-microbe interactions. Here, we show that a 10% broccoli sprout diet protects mice from the negative effects of dextran sodium sulfate induced

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colitis, that colitis erases biogeographical patterns of bacterial communities in the gut, and that the cecum is not likely to be a significant contributor to colonic bacteria of interest in the DSS mouse model of ulcerative colitis. Mice fed the broccoli sprout diet while receiving DSS performed better than mice fed the control diet while receiving DSS for all disease parameters, including significantly more weight gain, lower Disease Activity Index scores. Broccoli sprouts represent an affordable, accessible, and safe method of resolving DSS-induced inflammation and colitis. Information on how the gut microbiome mediates health benefits is critical to making effective medical recommendations.

Introduction

Inflammatory Bowel Diseases (IBD) are globally prevalent, chronic inflammatory diseases of multifactorial origin with an annual healthcare cost of billions of dollars in the United States alone (1, 2). IBD occurs in the gastrointestinal tract, and can be accompanied by autoimmune dysfunction, leading to microbial community changes in the gut. 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 (3–6). Treatments are currently limited to alleviating inflammatory symptoms, and returning patients to as close to homeostasis as possible. Diet can play an important role in the management of IBD as a source of anti-inflammatory metabolites, and as a tool for influencing the robustness of gut microbiomes. However, many guidelines for IBD patients recommend avoiding sulfur-rich foods which produce metabolites that might exacerbate symptoms in the gut (7), even as other sulfurous components reduce symptoms (8). Diet can be beneficial or detrimental to gut inflammation (9), but IBD patients choose to avoid all fiber based on a lost nuance that only some fiber sources and situations may induce negative

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side effects, e.g., (10). Thus, a better understanding of the interaction of diet, microorganisms, and disease is needed before dietary recommendations can be made.

Diets which are high in cruciferous vegetables such as broccoli have been associated with reduced inflammation and cancer risk (11–15) due to a group of specific plant secondary compounds, glucosinolates, which are used for defense against insect herbivory. Glucosinolates are very high in broccoli and can be converted into bioactive metabolites (16–18) such as sulfur-containing isothiocyanates, by the action of myrosinase, an enzyme present in these vegetables. In humans, isothiocyanates have been identified as bioactive candidates for reducing gut inflammation (19–21). Specifically, sulforaphane (SFN), a well-studied isothiocyanate (22), has been shown to inhibit the action of Nuclear Factor – Kappa B (NF-κB) and Signal Transducer and Activator Of Transcription 3 (STAT3) which are responsible for upregulation of inflammatory cytokines interleukins-6, -8, -12, -21, and -23 (19, 23–26). However, raw broccoli and broccoli sprouts contain small amounts of this anti-inflammatory, as broccoli-sourced enzymes preferentially metabolize the precursor of sulforaphane, glucoraphanin (GLR), to an inactive byproduct instead (22). Cooking the sprouts can alter the enzymatic activity of broccoli enzymes to prevent the creation of an inactive byproduct, leaving glucosinolates intact.

Mammals do not produce the enzymes for converting glucosinolates to isothiocyanates; however, gut bacteria with β-thioglucosidase activity can convert GLR to bioactive SFN in the mammalian GI tract (27–29). In human participants who were pretreated with oral antibiotics and bowel cleansing, urinary isothiocyanates excretion after consumption of cooked broccoli decreased significantly (17), supporting a role for gut microbiota. Several microorganisms isolated from the mammalian gut appear to have myrosinase-like enzymes that cleave the glycoside moiety from glucosinolates precursors, and there is evidence for GLR hydrolysis to SFN by colonic and

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cecal bacteria, *ex vivo* and *in vivo* in mono-colonized animal models (27, 30, 31). The bacterial populations that are capable of metabolizing glucosinolates precursors are not fully known, but *Lactobacillus*, *Bifidobacteria,* and *Bacteroides* strains have been implicated (20, 31, 32).

The taxonomic and functional structure of microbial communities along the gastrointestinal tract are highly dependent on the diet, health status, age, and microbial encounters of the host (33–35). Specific organs and sites within organs foster different environmental conditions that can create spatial niches for microbial taxa, an ecological pattern known as biogeography (36–38). For example, pH changes dramatically along the gastrointestinal tract and is linked to microbial diversity and density. IBD patients often have a less acidic gastrointestinal tract (39) due to altered diet or treatments, and this may result in alterations to gut biogeography (40), especially in mucosal-associated bacterial fractions (41–43). Mouse studies suggest biotransformation of GLR to SFN occurs in the colon (31, 44, 45), and we confirmed the majority of SFN accumulation to occur in the colon with greater resolution using multiple locations along the GI tract in our recently published study (18). Altering the structure and function of bacterial communities could alter if and where sulforaphane is created, which would improve or lessen the impact of having a locally produced anti-inflammatory on the destructive effects of colitis.

The dextran sodium sulfate (DSS) mouse model is widely used to reflect human Ulcerative Colitis (UC) in many ways (46, 47), and has been used for studying diet-sourced anti-inflammatories for the prevention of IBD in animals (46, 48). Administration of DSS in drinking water modifies the expression of tight junction proteins in intestinal epithelial cells, leading to a leaky epithelial barrier (49). This is followed by goblet cell depletion, erosion, ulceration, and infiltration of neutrophils into the lamina propria and submucosa (50), triggering the innate immune response (51, 52).

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There are significant knowledge gaps regarding the biogeography of the participating microbiota and how this may impact glucosinolate metabolism and health benefits to the host; and meanwhile, how the populations of microbiota are impacted by these bioactives remains unclear. To address these, we assessed the impact of steamed broccoli sprouts on the biogeographic pattern of gut microbiota and disease outcome in a mouse model of chronic, relapsing colitis. We fed specific pathogen free (SPF) C57BL/6 mice either a control diet or a 10% steamed broccoli sprout diet, starting at 6 weeks of age and continuing for another 40 days. The fresh broccoli sprouts were steamed to inactivate plant enzymes, and thus the metabolism of glucosinolates to isothiocyanates would rely on the gut microbiota. A three-cycle regimen of DSS in drinking water was given to stimulate chronic colitis in the mice, which is a well-established method (48, 53). We analyzed the bacterial communities from the luminal-associated (i.e., digesta contents) and mucosal-associated (i.e., scrapings) fractions in the jejunum, cecum, and colon using 16S rRNA gene sequencing, and compared this to metrics of disease activity including weight gain/stagnation, fecal characteristics, and lipocalin as a surrogate marker for inflammation in IBD (Figure 1).

Results

Broccoli sprouts alleviated disease characteristics of DSS-induced colitis

Mice were divided into four groups: control diet (Control), control diet with 2.5% DSS in drinking water (Control+DSS), 10% (w/w) steamed broccoli sprout diet (Broccoli, with the control diet as the base), and 10% broccoli sprout diet with 2.5% DSS in drinking water (Broccoli+DSS) (Figure 1). The DSS regimen was designed to induce chronic, relapsing colitis (54). The 6-week-

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149 old mice were acclimatized for 1 week. Treatment began in two phases: broccoli mice began their
150 treatment at 7 weeks old (Day 0), and the first DSS cycle began at 8 weeks old (Day 8).
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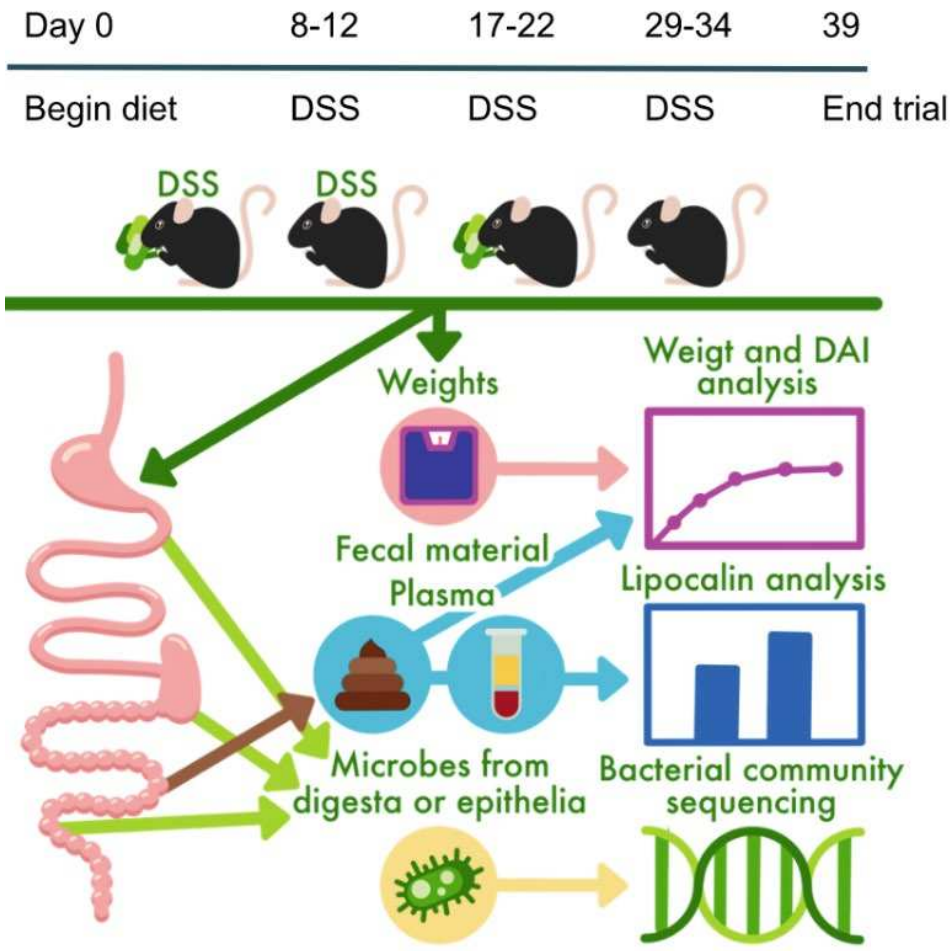

152
153
154 **Figure 1. Experimental design schematic for a chronic model of colitis induced by dextran**
155 **sodium sulfate (DSS) in 40 male mice (C57BL/6) beginning at 6-weeks of age.**
156

157 All mice gained weight over the study, as they are in a growth phase at this age (55). Mice
158 receiving the steamed broccoli sprout diet and DSS (Broccoli+DSS) gained significantly more
159 body weight over the trial than mice given the control diet and DSS (Control+DSS) (Figure 2a;
160 ANOVA, $p < 0.03$). There was no statistical difference observed in body weight among the three
161 groups, Broccoli+DSS, Broccoli, and Control (ANOVA, $p > 0.05$). Control and Broccoli mice

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occasionally exhibited slight weight loss as compared to body weight two days prior (Figure 2a), but these appeared to be random incidents which may be attributable to mice behavior and social dynamics. At the end of the first round of DSS treatment, Broccoli+DSS mice exhibited some weight loss and Control+DSS mice exhibited substantial weight loss (Figure 2a), but the second and third round of DSS only caused weight stagnation.

Mice in the Control+DSS group demonstrated elevated Disease Activity Index (DAI) scores as calculated by weight loss intensity score, fecal blood, and fecal consistency. Elevated DAI scores began at day 8 and lasted until day 34 (Figure 2b), and were significant on specific dates as compared to the mice in the Control group (Figure 2b, ANOVA, $p < 0.05$, comparisons by day, adjusted with TukeyHSD for multiple comparisons), as well as integrated over time (generalized additive model (GAM), $F = 308.32$, $p < 2e\text{-}16$).

Mice in the Broccoli+DSS group exhibited slightly elevated DAI scores during periods of DSS treatment, which returned to nearly 0 during rest periods, and which were lower as compared to Control+DSS mice (Figure 2b). Broccoli+DSS DAI scores were significantly elevated on specific dates (Figure 2b, ANOVA, $p < 0.05$, comparisons by treatment, adjusted with TukeyHSD for multiple comparisons), as well as integrated over time (GAM, $F = 17.47$, $p < 2e\text{-}16$). After the first round of DSS treatment, Broccoli+DSS and Control+DSS mice had similar elevated DAI scores (Figure 2b, ANOVA, $p > 0.05$). The diet control groups which were not treated with DSS, Control and Broccoli, did not have significantly different DAI scores from each other at any specific date (ANOVA, $p > 0.05$) or over time (GAM, $p > 0.05$). There was a lot of variability in the amount of fecal lipocalin between mice in treatment groups (Figure 3A), resulting in a lack of statistical significance between mice receiving DSS on the control diet and the steamed broccoli sprout diet except for day 19 on which steamed group had significantly (ANOVA, $p < 0.05$) higher

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lipocalin levels. Analysis of plasma samples showed DSS increased lipocalin levels in the plasma (Figure 3B). Feeding the mice with a 10% broccoli sprouts diet reduced the lipocalin levels in the group of mice treated with DSS comparable to the control group without DSS treatment (ANOVA, $p < 0.05$).

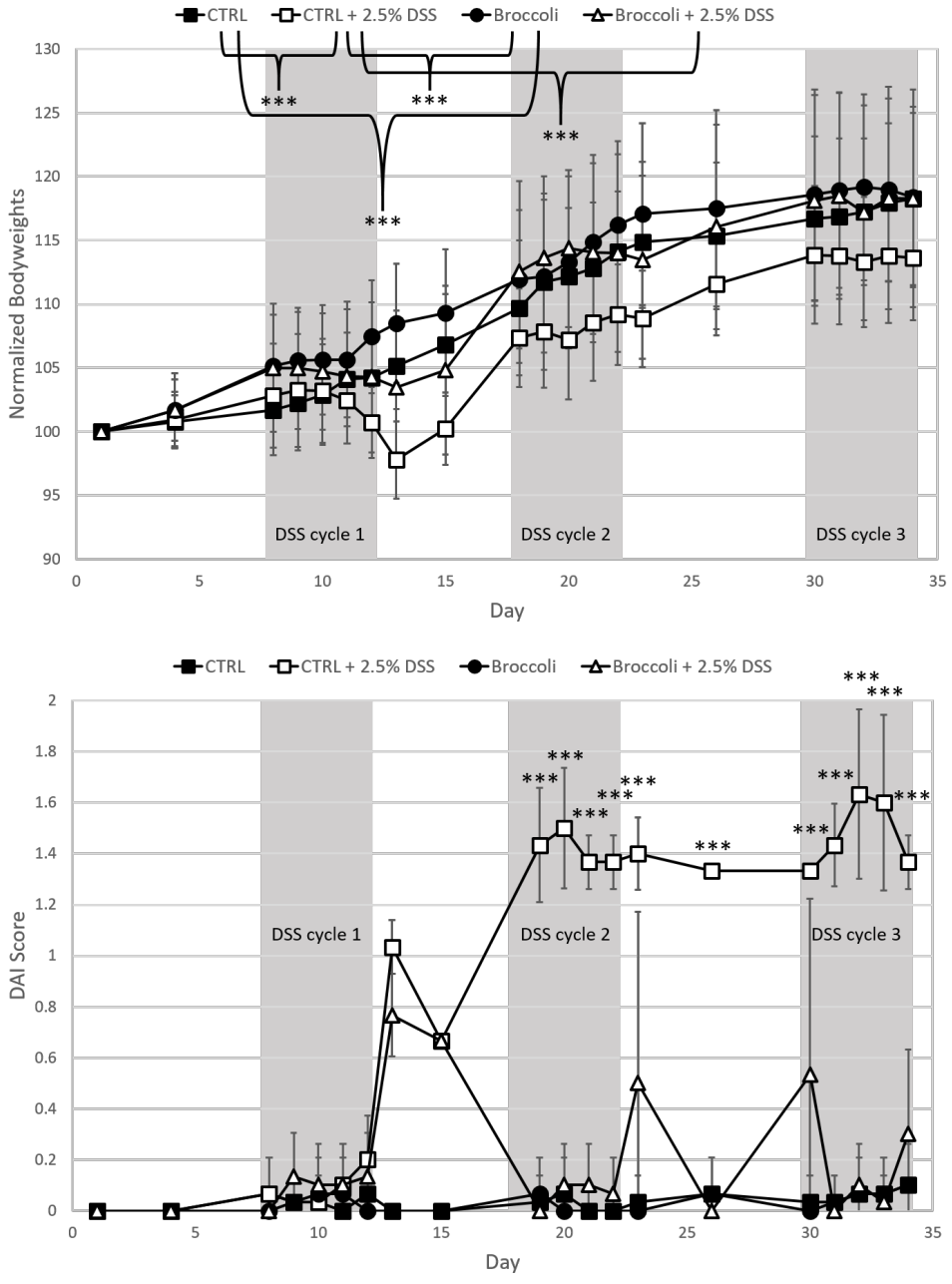

Figure 2. Metrics of disease status in a DSS-Induced model of chronic colitis, including (A) mouse body weights and (B) Disease Activity Index, by day of experiment. Body weights and

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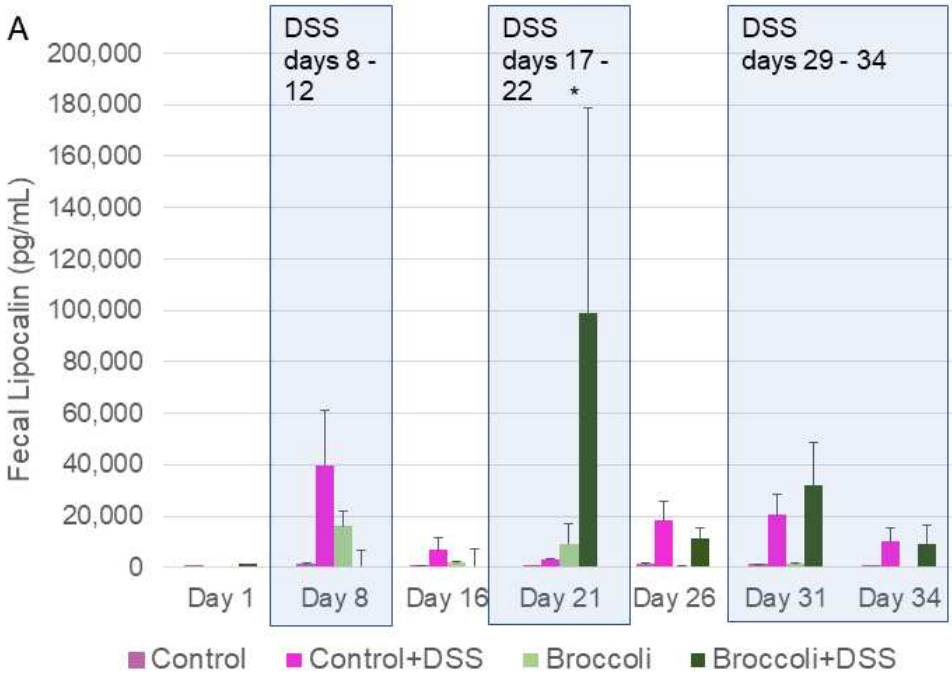
time scale were normalized to the day mice began the broccoli sprout diet. DAI scores are calculated by weight loss intensity score, fecal blood, and fecal consistency. Treatment comparisons at each day compared by ANOVA, and significance is designated as 0 - 0.001 '***'. Four treatment groups were used in a 34-day chronic, relapsing model of colitis: control diet, control diet with DSS added to drinking water, control diet adjusted with 10% by weight steamed broccoli sprouts, and 10% broccoli sprout diet with DSS added to drinking water.



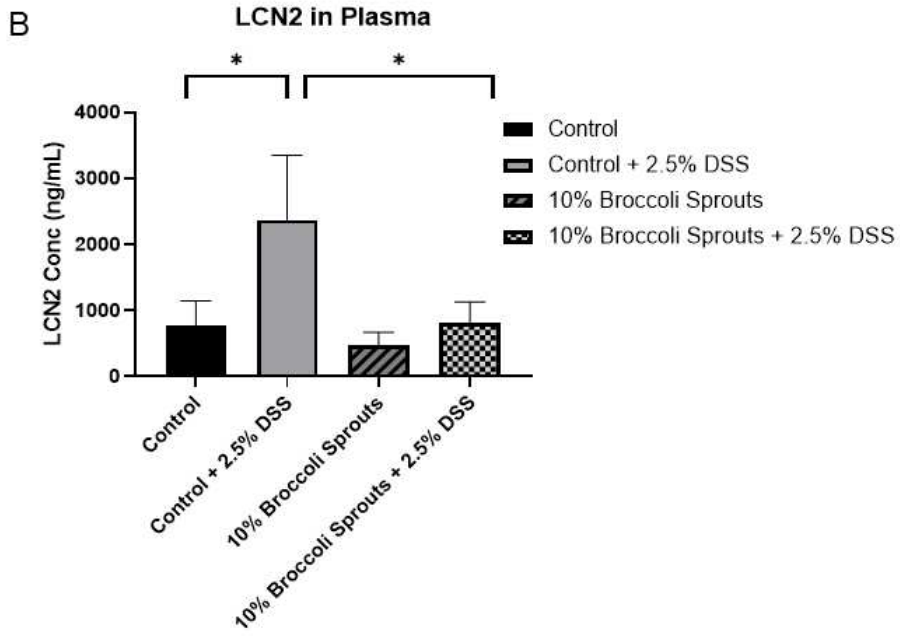


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Figure 3 Lipocalin in (A) feces at multiple timepoints and (B) serum on the last day of the trial from mice in a DSS-Induced model of chronic colitis. Significance was determined as $p < 0.05$, ANOVA.

Of the mice which had an elevated DAI score, some mice were positive for fecal blood, which can select for certain bacteria in the gut. In a subset of samples of mice which had an elevated DAI and were positive for fecal occult blood, there were different gut bacteria which were identified as important community features depending on whether they received the Control+DSS or the Broccoli+DSS diets (Figure 4, $p < 0.05$)). In Broccoli+DSS mice with positive fecal blood scores, these important taxa included a bacterial sequence variant (SV) identified in the Muribaculaceae family, and one in the *Clostridium* sensu stricto clade (Figure 4). For Control+DSS mice with a positive fecal blood score, this included different SVs identified in the *Clostridium* sensu stricto clade, *Cutibacterium*, and others (Figure 4).

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Figure 4. Gut bacterial taxa identified from mice receiving DSS which were associated with having a positive fecal occult blood score on the last day of the experiment, by treatment. Data were subset to mice with a positive score on the last day of the experiment, when the gut samples were collected. Important features (SVs) were identified through permutational random forest analysis, and only the features important to this group (>50 reads) are listed out of 143 significant ($p < 0.05$) features across all treatments. Model accuracy was 89.7%. Bacterial sequence variants (SV) are identified as the lowest level of taxonomic identity possible, with "NA" indicating which could not be identified to species, and the number indicating which specific SV it was. Four treatment groups were used in a 34-day chronic, relapsing model of colitis, those included here are a control diet with DSS added to drinking water, and 10% broccoli sprout diet with DSS added to drinking water.

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225

226 **Broccoli sprouts increased bacterial richness even during colitis**

227 Across all treatments and all mouse gastrointestinal samples, bacteria were primarily
228 identified as belonging to the phyla Bacillota (formerly Firmicutes), Bacteroidota (formerly
229 Bacteroidetes), Pseudomonadota (formerly Proteobacteria), Actinomycetota (formerly
230 Actinobacteria) which was higher in Control+DSS mice, and Verrucomicrobiota (formerly
231 Verrucomicrobia) which was higher in mice consuming broccoli sprouts (Figure S1).

232 Compared to the Control mice (receiving the control diet without DSS treatment), all other
233 treatment groups had more bacterial taxa present (measured as observed richness) in all locations
234 examined, with the exception of a loss of richness in the digesta/contents of the cecum and colon
235 in Control+DSS mice (Figure 5, Table 1, linear regression). Broccoli and Broccoli+DSS mice had
236 the highest bacterial richness in all gut locations, particularly in the colon contents and scraping,
237 but even the Control+DSS mice maintained high bacterial richness.

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Figure 5. Observed bacterial richness along the intestinal of mice on control diets with or without broccoli sprouts, and with or without DSS-induced chronic repeating colitis. Richness is calculated as the number of different bacterial sequence variants (SVs). Statistically significant comparisons are provided in Table 1. Four treatment groups were used in a 34-day chronic, relapsing model of colitis: control diet, control diet with DSS added to drinking water, control diet adjusted with 10% by weight steamed broccoli sprouts, and 10% broccoli sprout diet with DSS added to drinking water.

Table 1. Statistical comparison of observed richness along the intestinal of mice on control diets with or without broccoli sprouts, and with or without DSS-induced chronic repeating colitis. Comparisons were made using linear regression models comparing treatment, in subsets of the data by anatomical location. Only significant comparisons ($p < 0.05$) are listed. Bacterial richness is visualized in Figure 5.

Treatment compared to control diet only group	Change in bacterial richness (SVs) ± Standard Error (rounded)	T-value	P-value

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Jejunum contents			
Control + 2.5% DSS	40 ± 11	3.790	0.000608 ***
10% Broccoli sprouts	23 ± 10	2.766	0.009207 **
10% Broccoli sprouts + 2.5% DSS	64 ± 10	6.141	5.05e-07 ***
Jejunum scraping			
Control + 2.5% DSS	52 ± 17	3.`04	0.00434 **
10% Broccoli sprouts	98 ± 16	6.230	1.16e-06 ***
10% Broccoli sprouts + 2.5% DSS	100 ± 15	6.708	3.35e-07 ***
Cecum contents			
Control + 2.5% DSS	-13 ± 15	-0.821	0.418
10% Broccoli sprouts	68 ± 13	5.120	1.54e-05 ***
10% Broccoli sprouts + 2.5% DSS	70 ± 13	5.225	1.54e-05 ***
Colon contents			
Control + 2.5% DSS	-12 ± 9	-1.316	0.197
10% Broccoli sprouts	43 ± 9	4.525	6.67e-05 ***
10% Broccoli sprouts + 2.5% DSS	90 ± 10	9.172	7.74e-11 ***
Colon scraping			
Control + 2.5% DSS	14 ± 12	1.225	0.129
10% Broccoli sprouts	60 ± 12	5.220	1.78e-05 ***

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10% Broccoli sprouts + 2.5% DSS	89 ± 12	7.749	2.81e-08 ***

252

253 **Broccoli sprouts protected against DSS-induced changes to bacterial communities**

254 Treatment was the most significant driver of bacterial community similarity in the
255 gastrointestinal tract of mice (Figure 6a,b), when considering which bacterial taxa were
256 present/absent (unweighted Jaccard Similarity, permANOVA: $F = 16.90$, $p < 0.001$) or when
257 considering presence as well as abundance (weighted Bray-Curtis Similarity, permANOVA: $F =$
258 29.97 $p < 0.001$). Mice fed a control diet clustered away from other treatments, as did mice on a
259 control diet with DSS-induced colitis, while mice on a broccoli sprout diet overlapped with mice
260 eating sprouts along with DSS-induced colitis (Figure 6a, b).

261 Using both metrics for calculating similarity, we evaluated the distance from centroids to
262 samples by treatment group (beta dispersion), as an indication of the strength of each treatment in
263 selecting bacterial communities. The Broccoli and Broccoli+DSS samples had the same amount
264 of dispersal between samples and the centroid for that treatment (beta dispersion, $p > 0.05$ for both
265 comparisons, adjusted with Tukey's Honestly Significant Difference correction for multiple
266 comparisons), while Control and Control+DSS groups had very different amounts of dispersal
267 between the samples and the centroids respective to those treatments (beta dispersion, $p < 0.05$ for
268 both comparisons, adjusted with Tukey's HSD).

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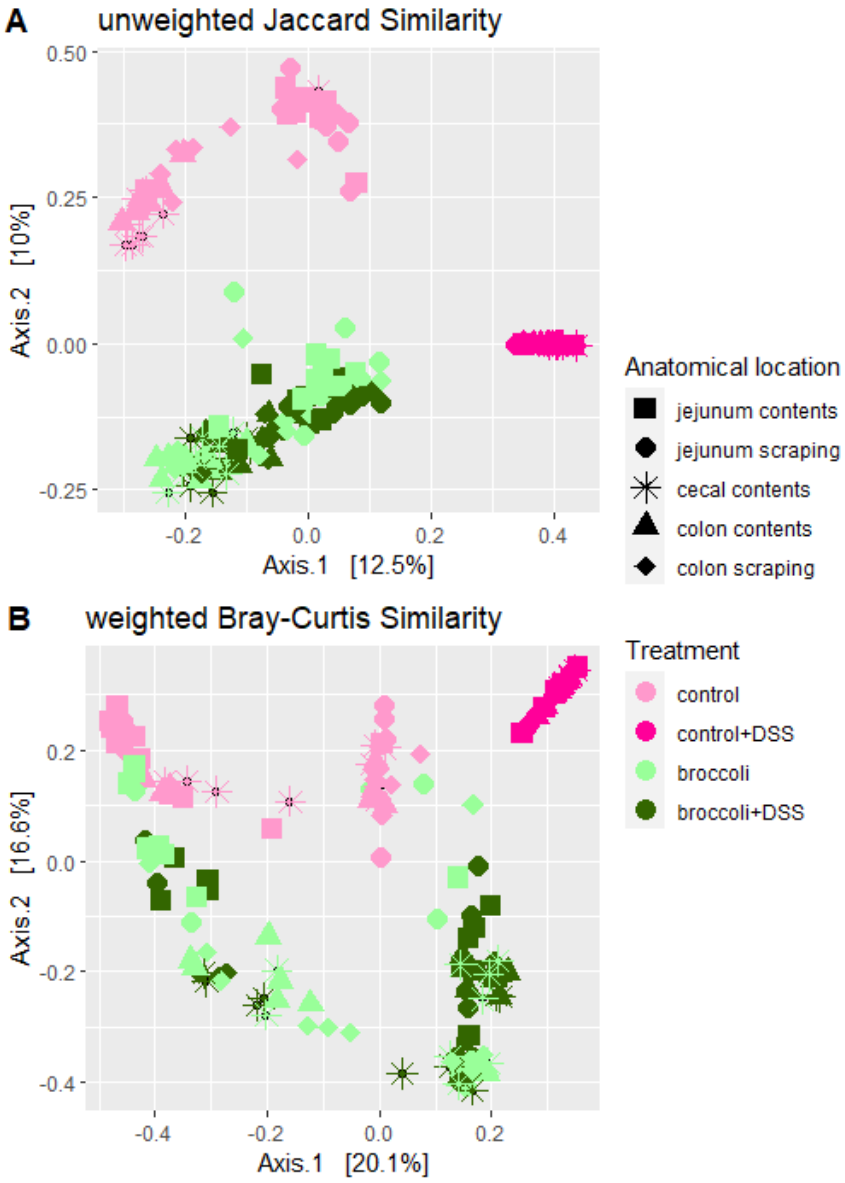

Figure 6. Principal coordinate analysis of bacterial community similarity along the intestines of mice on control diets with or without broccoli sprouts, and with or without DSS-induced chronic repeating colitis. Panel A was calculated with unweighted Jaccard Similarity to visualize differences in the taxonomic structure, and panel B with weighted Bray-Curtis to visualize structure and abundance. Four treatment groups were used in a 34-day chronic, relapsing model of colitis: control diet, control diet with DSS added to drinking water, control diet adjusted with 10% by weight steamed broccoli sprouts, and 10% broccoli sprout diet with DSS added to drinking water.

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Within the clusters of bacterial communities for each treatment, there was a secondary clustering effect of anatomical location, i.e., biogeography (Figure 7; permANOVA, uJS: $F = 3.42$, $p < 0.001$; wBC: $F = 4.36$, $p < 0.001$). This is easily visualized in Control, Broccoli, and Broccoli+DSS groups (Figure 6a, b), but was not observed in Control+DSS when compared with the other samples. When comparing all diet treatments, the variation between the Control+DSS group and the other treatments is so large that it obscures trends within the group (Figure 7a). Thus, we subset the Control+DSS samples (Figure 7b), and observed that the DSS alone diminishes the effect of biogeography such that there is no statistical difference (permANOVA, $p > 0.05$), except marginally between jejunum scraping vs. cecum ($p = 0.04$), between anatomical locations when considering the bacterial community structure (unweighted Jaccard Similarity; Figure 7b) or structure and abundance (weighted Bray-Curtis, data not shown).

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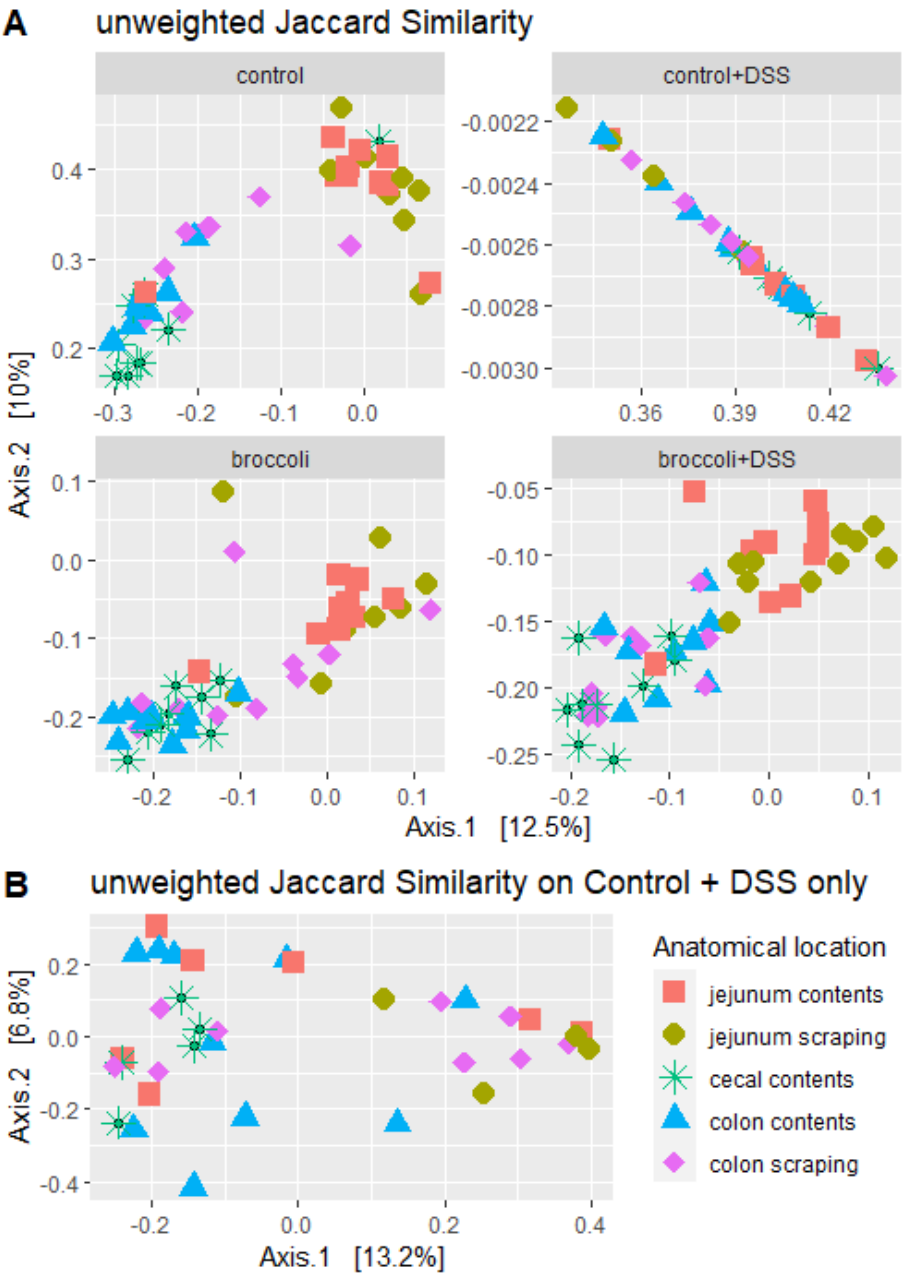

290

291 **Figure 7. Principal coordinate analysis of bacterial communities from locations along the**
292 **intestines of mice, across all treatments (A) and in DSS-induced colitis alone (B).** Bacterial
293 communities in the jejunum, cecum, and colon were statistically different (permANOVA, $p < 0.05$)
294 from each other in the treatment groups (A) Control, Broccoli, and Broccoli+DSS, were not
295 statistically different from each other after multiple bouts of colitis in the (B) Control+DSS group.
296

297

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When identifying significant taxa which defined the samples in different treatments, there were 188 significant ($p < 0.05$) features across all treatments with a permutational random forest model accuracy of 98%. The Control mice contained a high abundance of mouse commensals, such as *Dubosiella* spp. and strains from the Muribaculaceae and Lachnospiraceae families (Figure S2). The Control+DSS mice contained much lower abundance of those known mouse commensal taxa and higher abundance of the *Clostridium sensu stricto* clade (Figure S3). The Broccoli sprout group was high in *Dubosiella* spp., *Parasutterella excrementihominis*, and *Bacteroides* spp. (Figure S4). The Broccoli+DSS group had many of the same important taxa as the group consuming Broccoli sprouts without colitis (Figure S5), implying a strong selective pressure of the broccoli sprouts on the bacterial community structure such that many SVs were found in high abundance across at least 70% of the samples in those mice (Figure S6). There were no bacterial SVs which were shared across at least 70% of samples in the Broccoli+DSS and the Control+DSS group, indicating there was not a specific community which was enriched or associated with the DSS.

Genera with putative GLR metabolism capacity present with broccoli sprout diet even during colitis

There are several bacterial taxa which are known or reputed to express myrosinase-like enzymatic activity and transform glucoraphanin into sulforaphane, and we selected taxa from previous literature to examine in greater detail (31, 56, 57). There were 309 bacterial SVs identified to the genera *Bifidobacterium, Bacteroides, Enterococcus, Lactobacillus, Lactococcus, Pseudomonas, Staphylococcus,* and *Streptomyces.* We found no *Faecalibacterium*, *Bacillus, Listeria, Pediococcus, Aerobacter, Citrobacter, Enterobacter, Escherichia, Salmonella,*

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Paracolobactrum, or *Proteus* in our samples (Figure 8). Only a few of these were found in Control mice, with some of these genera found in the jejunum of Control+DSS mice (Figure 8), although without species-level resolution it cannot be determined if these are beneficial strains.

Importantly, most of these reads belonged to *Bacteroides* (1,394,934 frequency across all samples compared to a total frequency of 6,791 for all other sequences) and were found along the gastrointestinal tract of mice consuming the broccoli sprout diet, with or without DSS (Figure 8), suggesting improved GLR metabolism capacity upon the exposure to broccoli sprout diet. However, for the broccoli and DSS treatment, only the cecum contents and colon scrapings retained a significant proportion of *Bacteroides*. The *Bacteroides* content of the colon contents appears to have been adversely affected by the addition of DSS to the broccoli diet.

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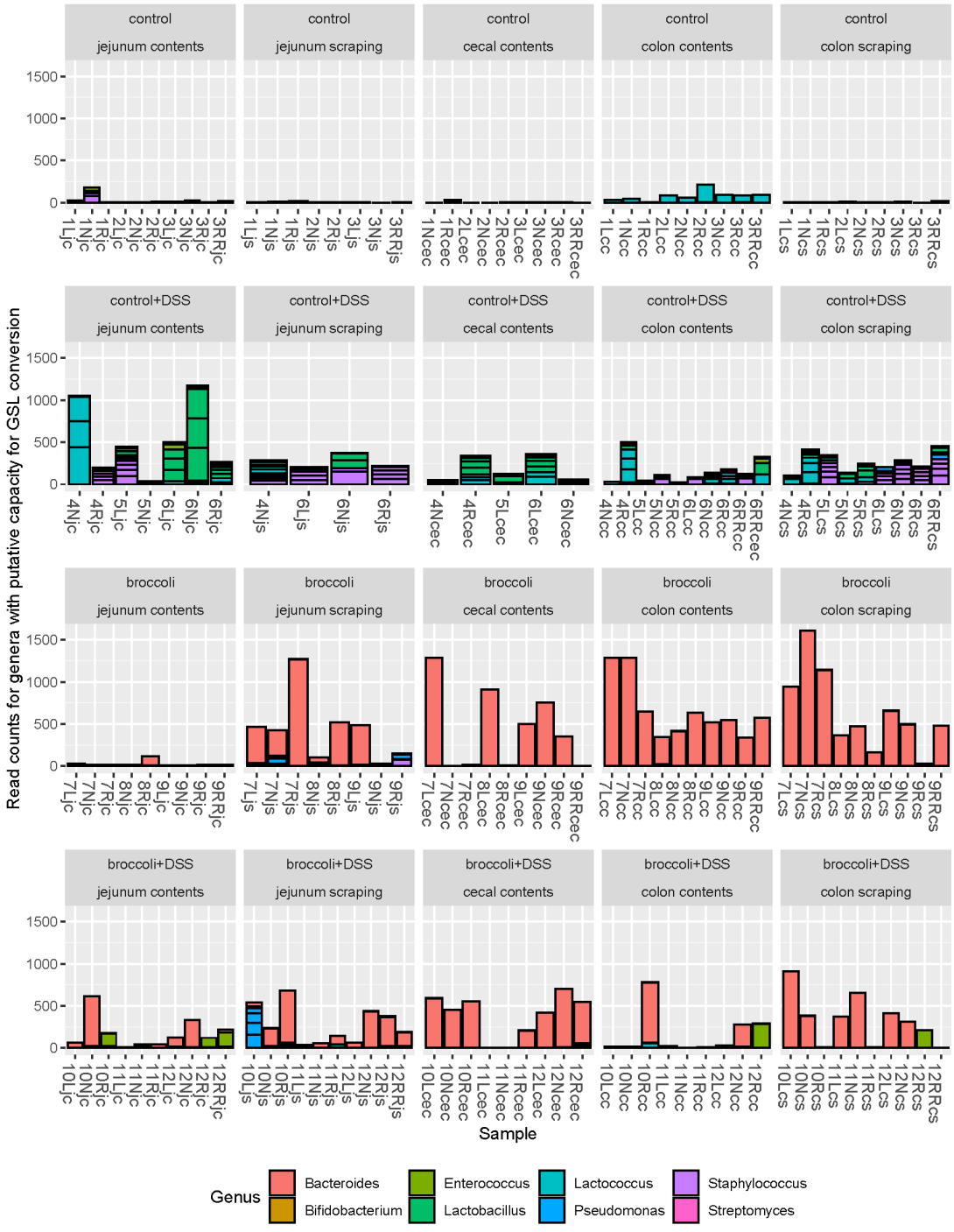

Figure 8. Bacterial sequence variants (SVs) belonging to the genera which have putative capacity to convert glucoraphanin to sulforaphane. Strains of bacteria in these genera have been demonstrated to perform myrosinase-like activity in the digestive tract, as reviewed in (56). Four treatment groups were used in a 34-day chronic, relapsing model of colitis: control diet, control diet with DSS added to drinking water, control diet adjusted with 10% by weight steamed broccoli sprouts, and 10% broccoli sprout diet with DSS added to drinking water.

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Several *Bacteroides* species were identified (*acidifaciens*, *massiliensis*, *sartorii*), but the dominant sequence variant was not identified to species-level by the Silva Database taxonomy assignment (Figure 9, 10). This unidentified *Bacteroides* dominant SV (*B.*-dominant) may be associated with multiple species using the NCBI Database (BLASTN): *Bacteroides thetaiotaomicron (B. theta)*, *Bacteroides faecis* and *Bacteroides zhangwenhongiim* (all with 100% Query Cover and Percent Identity). *B. theta* has been linked to myrosinase-like enzyme activity and a broccoli diet, but *Bacteroides faecis* and *Bacteroides zhangwenhongii* have not.

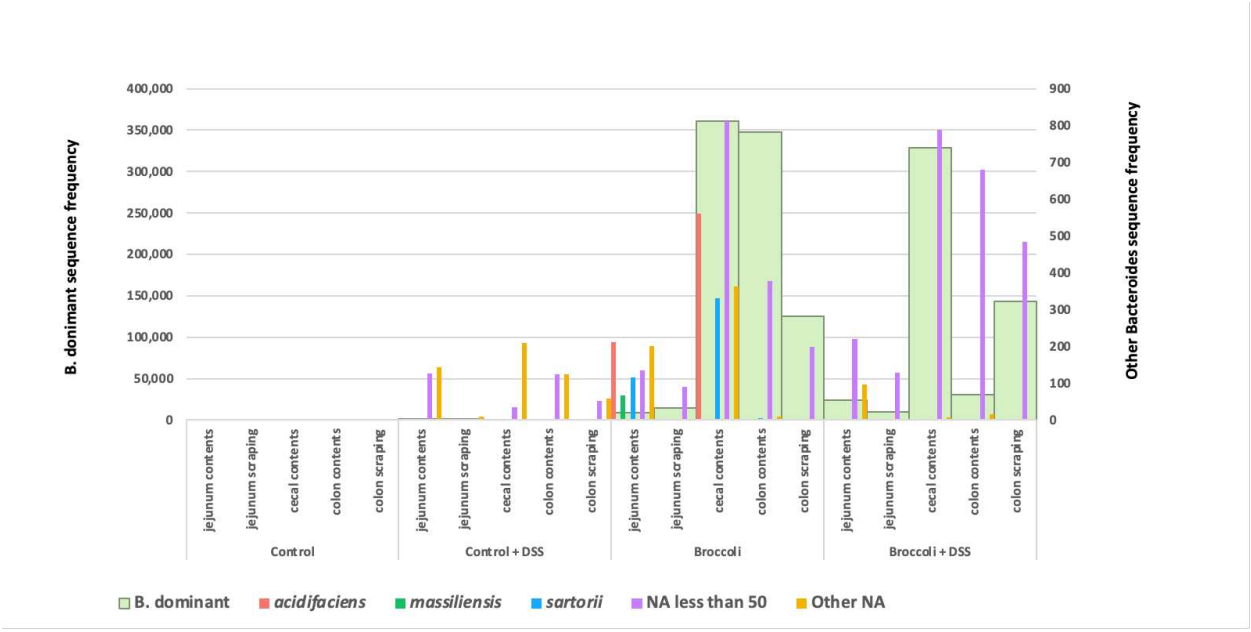

Figure 9. Bacteroides species by diet treatment and anatomical location in the gastrointestinal tract of mice. The Silva Database identified *Bacteroides* species *acidifaciens*, *massiliensis*, and *sartorii*. Of the BLASTN identified species for *B.*-dominant SVs, *B. thetaiotaomicron* was the only species only found in broccoli-sprout-fed mice.

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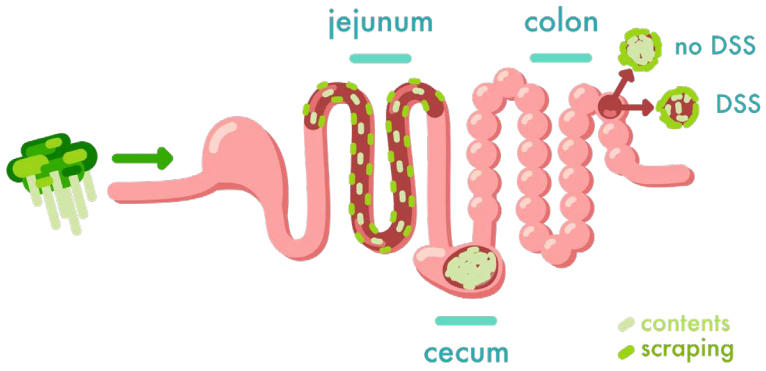

Figure 10. Biogeography of the dominant Bacillus SV, which was found exclusively in broccoli-sprout-fed mice. *B.*-dominant was present in the jejunum contents and scrapings and abundant in the cecum contents, colon contents, and colon scrapings. DSS reduced *B.*-dominant colon content density.

Discussion

Broccoli sprouts protected mice against DSS-induced colitis

The anti-inflammatory effects of broccoli and broccoli sprout bioactives, in particular sulforaphane (SFN), have been well-established in various cell, animal, and human trials of IBD (22). However, studies have not elucidated the role of the gut microbiota and the effect of biogeography in metabolizing the glucoraphanin (GLR) precursor to SFN, and the subsequent prevention and alleviation of inflammation in the gut. The present study sought to address these investigatory gaps by using a DSS-induced mouse model of chronic, relapsing colitis and comparing treatment group performance across multiple measures, including bacterial richness and abundance across treatment groups and anatomical locations, body weight, disease activity, and lipocalin analysis. We fed a 10% steamed broccoli sprout diet to SPF C57BL/6 mice beginning

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374 at 6 weeks old and throughout a three-cycle regimen of DSS. Here, we showed that a diet featuring
375 10% steamed broccoli sprouts reduced the Disease Activity Index and prevented DSS-induced
376 shift in bacterial community structures along the intestines. This included preventing a shift in the
377 microbial community caused by fecal blood, which can increase the abundance of pathobionts and
378 reduce commensals (58, 59).

379 We began broccoli sprout feeding prior to introducing DSS, in part to acclimate mice to
380 the diet, but primarily to investigate the use of broccoli sprouts as a prevention strategy before the
381 initiation of colitis, as its usefulness in alleviating symptoms during active disease states has been
382 previously demonstrated (18). We chose 10% to demonstrate proof of concept and used male mice
383 as they are known to suffer more histopathological damage from DSS-induced colitis (60–62).
384 Regular access to fiber is critical to recruiting and retaining beneficial microbiota in the gut (63),
385 and regular exposure to glucosinolates is needed to stimulate gut microbial conversion (30). In
386 people sporadically consuming broccoli or broccoli sprouts, or consuming over short periods of
387 time, cooking preparation and the activity/inactivity of plant myrosinase were often the
388 determining factors in how much SFN was produced, if any, as reviewed in (30). However,
389 providing SFN directly in the diet is not feasible because it is unstable (64), and also would
390 preclude other benefits, and the enjoyability, of consuming a phenol and fiber-rich food.

391 We acknowledge that a major limitation in this study was only using male mice, as it is
392 known that hormones mediate both the gut microbiome and the type and intensity of DSS-induced
393 colitis. Thus, any future research on the application of this diet in people will require diversity in
394 the gender of participants.

395

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Bacterial communities are highly responsive to DSS and broccoli sprouts

Mice fed broccoli sprout diets showed increased bacterial richness with and without DSS compared to mice fed a control diet. We previously showed that the concentration of SFN is highest in the colon after feeding C57BL/6 mice steamed broccoli sprout diets with reduced myrosinase concentrations, suggesting that primary hydrolysis of SFN to GLR by microbial communities occurs in the colon [32]. The current study found effects on microbial richness from the broccoli sprout diet manifested most strongly in the colon, supporting our previous findings. Given the location-specific dynamics of gut anatomy and physiology, digestion and the availability of different nutrients, and microbial communities, a smaller change in jejunum tissues could indicate that the biochemical effects of the diet are not available to the host until it reaches the colon.

When identifying significant taxa which defined the samples in different treatments, the Control mice contained a high abundance of known mouse commensal taxa, including *Dubosiella* spp. and strains from the Muribaculaceae and Lachnospiraceae families. The Control+DSS mice contained much lower abundance of known mouse commensal taxa and higher abundance of the *Clostridium sensu stricto* clade (65), which contains known pathogens such as *Clostridium perfringens* (66), and well-known butyrate-producing symbionts, such as *Clostridium butyricum* (67).

The Broccoli sprout group was also high in commensal taxa such as *Dubosiella* spp., and had enrichment of *Bacteroides* spp., a genus known to contain glucosinolate-metabolizing bacteria and modified by cruciferous vegetable consumption (68). The broccoli sprout diet also enriched for *Parasutterella excrementihominis*, which is commonly found in human murine gut communities and is associated with carbohydrate intake (69). It is possible that other breakdown

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products of glucosinolates, such as aromatic carbohydrate compounds in broccoli sprouts (70), caused this enrichment. *P. excrementihominis* has also been associated with inflammation in the gut in observation-based studies; however in metabolic pathway-based studies it appears to be an important contributor to nitrate reduction in the gut and in reducing stress-related inflammation (71). Nitrates are low in raw broccoli and other cruciferous vegetables, but this can be increased by freezing (72). Thus, our diet preparation – which included freezing and freeze-drying steps – may have selected for nitrate-reducers.

Importantly, the Broccoli+DSS group had many of the same important taxa as the group consuming Broccoli sprouts without colitis, implying that the selective pressure of the broccoli sprouts on the bacterial community structure may be stronger than that of the DSS. The two treatments were so similar that many SVs were found in high abundance across at least 70% of the samples in those mice. There were no bacterial SVs shared across at least 70% of the Broccoli+DSS and the Control+DSS group, indicating there was not a specific community which was enriched or associated with the DSS.

The inclusion of DSS in drinking water demonstrably affects mice and gut bacterial communities, but it does not necessarily reduce bacterial richness. In part, this is because certain bacteria, like *Proteus vulgaris,* can make use of DSS (73), while other novel commensals appear to offer protective effects (74). We did not identify any bacterial SVs belonging to *Proteus*, although we found significantly higher amounts of *Bacteroides* sp. in broccoli sprout-fed mice. *Bacteroides thetaiotaomicron* has been demonstrated to metabolize glucoraphanin to sulforaphane and offer protection against colitis in mice (31). In this experiment, for the broccoli and niche microbiomes, *B.*-dominant flourished in the cecum and colon. *B.*-dominant was present under broccoli treatment (no DSS) in the jejunum contents and scrapings, but was abundant in the cecum

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442 contents and the colon contents and scrapings. However, for the broccoli and DSS treatment, DSS
443 presence in the colon contents did not provide as favorable an environment for *B.*-dominant.

444 Across multiple mouse lineages, individualized bacterial communities responded
445 differently to the inclusion of DSS, and the presence of *Duncaniella muricolitica* and *Alistipes*
446 *okayasuensis* were implicated in better mouse health outcomes (75). Modeling of the gut
447 microbiome indicates that certain taxa may act as keystone species – critical to building the rest of
448 the community and particularly in circumstances in which the microbial community has been
449 destabilized (76). However, no singular important bacteria has been identified in meta analyses of
450 IBD in humans (77, 78), and some studies have pointed to the switch from bacterial commensals
451 to pathobionts as important (79).

452 There is considerable evidence that microbes provide metabolism of biologically inert
453 glucosinolates to biologically active isothiocyanates, and several cultured bacterial strains from
454 fermented foods and the digestive tract have been shown to perform this conversion (31, 56, 57).
455 Additionally, a single bacterial equivalent of the plant myrosinase enzyme has not been identified,
456 and there is the possibility that several enzymes in bacteria may work in concert to achieve
457 metabolism of GSLs. Further, research is lacking to determine if the effect on the gut microbiome
458 is metabolite (isothiocyanates)-mediated or precursor (glucosinolates)-mediated. The scope of
459 research in this field should be expanded by investigating the concepts of biogeographic specificity
460 of both bioactive production and absorption and microbial community dynamics.
461

462 **Biogeography reveals location-specific trends in the bacterial community**

463 It has been well-demonstrated that distinct bacterial communities exist in different
464 locations along the gastrointestinal tract in mammals, related to the local anatomical,

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environmental, nutritional, and host immunological conditions in different organs (36, 80, 81). Further, the effect of foods on the gut microbiome can be specific to an individual's communities (34). Given the complexity of microbial community function, as well as the spatially explicit biochemical digestion activities of the host, it follows that the location of glucoraphanin metabolism may influence how well the host can absorb SFN, whether it may be distributed systematically, and where it will be effective at preventing or treating symptoms. It has previously been demonstrated in mouse trials using 2,4,6 trinitrobenzene sulfonic acid to chemically induce colitis that the mucosal-associated bacterial population is more affected by colitis than the lumen-associated (digesta) community (43).

Here, we showed that DSS-induced colitis effectively erased the biogeographical specificity of communities in the mouse gastrointestinal tract (Figure 4, Table 1), with the exception of communities in jejunum scrapings remaining distinct from those in cecal contents. Given the highly specific function of the cecum in separating fibers by size and furthering microbial fermentation, it is not surprising that it would be distinct. The DSS may be a stronger selective force than anatomical location in driving bacterial communities in the gut (74). For example, its destruction of the epithelial cell surface may change the microarchitecture and alter microbial attachment in the gut (82), and DSS increases the hydrophobicity of bile acids (83), which may affect microbial survival and ability to attach to host epithelial cells, thus increasing microbial washout through the gut. Any of these could possibly explain our findings that DSS eliminated biogeographical specificity of communities in the mouse gastrointestinal tract. However, more research would be needed to determine causation.

Mouse studies suggest biotransformation of glucoraphanin to SFN occurs in the colon (31, 44, 45), and we previously confirmed the majority of SFN accumulation takes place in the colon,

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with greater resolution using multiple locations along the GI tract (18). Critically, the cecum had low SFN, indicating that it is not responsible for hosting bacterial biotransformation of these bioactives (18). Here, we also showed that only a few bacterial taxa were estimated to be sourced in the cecum and creating sink populations in the colon (Figure S7), and of these, none were identified to be putatively responsible for metabolism of glucoraphanin. Collectively, this confirms that this is a valid model for generalization to the human gut.

Considerations for the application of this work

Access to fresh or frozen broccoli and broccoli sprouts, the cooking preparation, and the ability to regularly consume these vegetables will have implications for the feasibility and success of a dietary intervention for preventing or reducing inflammation in the gut. For example, sprouts from various plants have been implicated in food-borne illness because of their proximity to soil, and people may be wary of or discouraged from consuming raw or slightly cooked sprouts. However, more recent research has shown that sprouts can be consumed safely, especially with improvements in hygiene and agricultural regulations, as well as in food processing (84). Further, broccoli sprouts can be grown at home in windowsill seed-bed germinators without requiring soil, gardening tools, or specialized gardening knowledge, which could ease the financial burden of purchasing healthy foods (85). This may prove to be particularly important in areas without access to healthcare or affordable prescriptions, or in areas without close proximity to fresh, healthy fruits and vegetables (7, 86, 87) as this can preclude being able to make the dietary recommendations set forth by medical professionals (88). Including broccoli sprouts as 10% of the diet could be potentially too high for IBD patients to comply with, and future studies on the application of this

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diet will require a deeper understanding of the biological, microbiological, immunological, as well as social and logistical factors involved in dietary interventions in people.

Materials and Methods

Diet preparation

Jonathan's Sprouts™ (Rochester, Massachusetts, USA) broccoli sprouts were purchased from a grocery store (Bangor, Maine, USA) and steamed in a double boiler for 10 minutes and immediately cooled down. They were stored in a -80°C freezer until freeze-drying (University of Maine Pilot Plant, Orono, Maine, USA). The freeze-dried broccoli sprouts were ground into a fine powder and mixed with purified AIN93G rodent base diet to a concentration of 10% by weight. Our previous work 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 in mice (18). For this study, we chose to use 10% steamed 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 sealable plastic bags in a -20°C freezer.

DSS colitis model

Forty male, 6-week-old, specific pathogen free (SPF) C57BL/6 mice (*Mus musculus*) were purchased from the Jackson Laboratory (Bar Harbor, Maine, U.S.) and transferred to the animal facility at the University of Maine (Orono, Maine, U.S.). The animal protocol (IACUC protocol

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A2020-01-04) was approved by the University Committee on the Use and Care of Animals, and all biosafety work was approved by the Institutional Biosafety Committee (protocol SI-092220). The mice were acclimated to the facility for 7 days (day -7 to 0), during which they received *ad libitum* autoclaved tap water and the AIN-93G purified rodent diet (control diet). After initial acclimation, the mice were randomly assigned to one of 4 experimental groups beginning on experimental day 0: control diet without DSS treatment (Control), 10% steamed broccoli sprout diet without DSS treatment (Broccoli), control diet with DSS treatment (Control+DSS), and 10% steamed broccoli sprout diet with DSS treatment (Broccoli+DSS). All experimental groups were on 7 days of their respective diets (control or 10% steamed broccoli sprout), after which DSS (Alfa Aesar, molecular weight ~40 kD (53)) was added to the drinking water of the DSS treatment groups to a final concentration of 2.5%. Mice were given DSS for 5 days, followed by a recovery period for 5 - 7 days. This was repeated for a total of 3 cycles to induce chronic colitis (48, 89). Mice were sacrificed and tissue collected after the third round of DSS, on day 35 of the experiment.

Bodyweight, fecal blood, and fecal consistency were used to calculate Disease Activity Index (DAI) scores (52). Samples were taken every 2 - 3 days throughout the trial, and daily during the DSS cycles. Body weights and DAI were analyzed using 2-way ANOVA generated with R to compare differences between treatments for each day. A generalized additive model (GAM) was used in R to compare DAI differences (R-sq.(adj) = 0.861, Deviance explained = 86.4%, GCV = 0.036031) by treatment across the entire study, using Mouse ID to account for repeated measures. Lipocalin-2 concentration in the plasma and fecal samples were determined by a mouse Lipocalin-2/NGAL DuoSet ELISA kit (R & D Biosystems, USA) following the manufacturer's instructions. Lipocalin is a neutrophil protein that binds bacterial siderophores and serves as a surrogate marker for intestinal inflammation in IBD (90). The readings at wavelengths of 540 nm and 450 nm were

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556 measured by a Thermo Scientific Varioskan LUX Multimode Microplate Reader. The readings at
557 540 nm were subtracted from the readings at 450 nm to correct for the discoloration of the solution.
558 After euthanasia, lumen-associated (digesta contents) and mucosal-associated (epithelial
559 scrapings) microbial community samples were collected from the jejunum, cecum (contents only),
560 and colon for DNA extraction and community sequencing as described below.
561
562 **Bacterial community sequencing and analysis**
563 From the DSS mice, digesta (lumen contents) and epithelial associated (tissue scrapings)
564 microbial community samples were collected from the jejunum, cecum (contents only), and colon.
565 All tissues containing their resident gut microbiota were gently homogenized with vortexing, then
566 treated with propidium monoazide (PMA; BioTium) following kit protocols at a final
567 concentration of 25 µm. PMA covalently binds to relic/free DNA and DNA inside
568 compromised/dead cell membranes, and prevents amplification in downstream protocols to
569 preclude dead DNA from the sequence data (91).
570 Following PMA treatment, bulk DNA was extracted from tissue-associated bacterial
571 communities (n = 200 samples), or no-template (water) control samples (n = 10, one for each
572 extraction batch) using commercially available kits optimized for water and tissue-based microbial
573 communities (Quick-DNA Fecal/Soil Kit, Zymo Research). DNA extract was roughly quantified
574 and purity-checked with a Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis
575 Spectrophotometer (Thermo Scientific, Waltham, MA, U.S.). Samples underwent DNA amplicon
576 sequencing of the 16S rRNA gene V3-V4 region, using primers 341F [174] and 806R [175] and
577 protocols consistent with The Earth Microbiome Project [176], and sequenced on an Illumina
578 MiSeq platform using the 2 x 300-nt V3 kit (Molecular Research Labs, Clearwater, TX, U.S.).

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Raw sequence data (fastq files and metadata) is available from NCBI through BioProject Accession number PRJNA911821.

Amplicon sequence data was processed using previously curated workflows in the Ishaq Lab (R code supplied as Supplemental Material), 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 46,581,832 paired raw reads, and based on initial quality assessment only the forward reads were processed. Trimming parameters were designated based on visual assessment of the aggregated quality scores at each base from all samples (plotQualityProfile in DADA2): the first 10 bases were trimmed, sequences were trimmed to 225 bases in length, and were discarded if they had ambiguous bases, more than two errors, or matched the PhiX version 3 positive control (Illumina; FC-110-3001). After filtering, 34,009,802 non-unique forward/read 1 sequences 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] down to species where possible, 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], which was also used for basic visualization and statistical analysis in combination with other packages. Samples from one mouse (4L, in the Control+DSS group) were dropped from further analysis as they were outliers on all visualizations and may have been contaminated during DNA extraction.

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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 to compare by sample type, (lme4 package [182]), in which anatomical location and diet treatment were used as fixed effects, and mouse ID used to control for repeated sampling as needed. Generalized additive models were used to assess trends in alpha diversity using time as a smoother (92). 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 (93). Random forest feature prediction with permutation was used to identify differentially abundant SVs based on factorial conditions (94). Plots were made using the ggplot2 (95), ggpubr (96), and phyloseq packages.

Source Tracker algorithms which had been modified for the R platform (97, 98) 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 142 SVs were identified as possibly sourced from the cecum, and 95 SVs were estimated to make up >1% of the proportion of sources (Figure S7). The putative GLR converting bacteria in the Broccoli and Broccoli+DSS mouse gut samples were not among those taxa identified as sourced in the cecum.

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Author Contributions

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

Figure Legends

Figure 1. Experimental design schematic for a chronic model of colitis induced by dextran sodium sulfate (DSS) in 40 male mice (C57BL/6) beginning at 6-weeks of age.

Figure 2. Metrics of disease status in a DSS-Induced model of chronic colitis, including (A) mouse body weights and (B) Disease Activity Index, by day of experiment. Body weights and time scale were normalized to the day mice began the broccoli sprout diet. DAI scores are calculated by weight loss intensity score, fecal blood, and fecal consistency. Treatment comparisons at each day compared by ANOVA, and significance is designated as 0 - 0.001 '***'. Four treatment groups were used in a 34-day chronic, relapsing model of colitis: control diet, control diet with DSS added to drinking water, control diet adjusted with 10% by weight steamed broccoli sprouts, and 10% broccoli sprout diet with DSS added to drinking water.

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Figure 3 Lipocalin in (A) feces at multiple timepoints and (B) serum on the last day of the trial from mice in a DSS-Induced model of chronic colitis. Significance was determined as $p < 0.05$, ANOVA.

Figure 4. Gut bacterial taxa identified from mice receiving DSS which were associated with having a positive fecal occult blood score on the last day of the experiment, by treatment. Data were subset to mice with a positive score on the last day of the experiment, when the gut samples were collected. Important features (SVs) were identified through permutational random forest analysis, and only the features important to this group (>50 reads) are listed out of 143 significant ($p < 0.05$) features across all treatments. Model accuracy was 89.7%. Bacterial sequence variants (SV) are identified as the lowest level of taxonomic identity possible, with "NA" indicating which could not be identified to species, and the number indicating which specific SV it was. Four treatment groups were used in a 34-day chronic, relapsing model of colitis, those included here are a control diet with DSS added to drinking water, and 10% broccoli sprout diet with DSS added to drinking water.

Figure 5. Observed bacterial richness along the intestinal of mice on control diets with or without broccoli sprouts, and with or without DSS-induced chronic repeating colitis. Richness is calculated as the number of different bacterial sequence variants (SVs). Statistically significant comparisons are provided in Table 1. Four treatment groups were used in a 34-day chronic, relapsing model of colitis: control diet, control diet with DSS added to drinking water, control diet adjusted with 10% by weight steamed broccoli sprouts, and 10% broccoli sprout diet with DSS added to drinking water.

Figure 6. Principal coordinate analysis of bacterial community similarity along the intestines of mice on control diets with or without broccoli sprouts, and with or without DSS-induced chronic repeating colitis. Panel A was calculated with unweighted Jaccard Similarity to visualize differences in the taxonomic structure, and panel B with weighted Bray-Curtis to visualize structure and abundance. Four treatment groups were used in a 34-day chronic, relapsing model of colitis: control diet, control diet with DSS added to drinking water, control diet adjusted with 10% by weight steamed broccoli sprouts, and 10% broccoli sprout diet with DSS added to drinking water.

Figure 7. Principal coordinate analysis of bacterial communities from locations along the intestines of mice, across all treatments (A) and in DSS-induced colitis alone (B). Bacterial communities in the jejunum, cecum, and colon were statistically different (permANOVA, $p < 0.05$) from each other in the treatment groups (A) Control, Broccoli, and Broccoli+DSS, were not statistically different from each other after multiple bouts of colitis in the (B) Control+DSS group.

Figure 8. Bacterial sequence variants (SVs) belonging to the genera which have putative capacity to convert glucoraphanin to sulforaphane. Strains of bacteria in these genera have been demonstrated to perform myrosinase-like activity in the digestive tract, as reviewed in (56).

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699 Four treatment groups were used in a 34-day chronic, relapsing model of colitis: control diet,
700 control diet with DSS added to drinking water, control diet adjusted with 10% by weight steamed
701 broccoli sprouts, and 10% broccoli sprout diet with DSS added to drinking water.
702
703 **Figure 9. Bacteroides species by diet treatment and anatomical location in the**
704 **gastrointestinal tract of mice.** The Silva Database identified *Bacteroides* species *acidifaciens*,
705 *massiliensis*, and *sartorii*. Of the BLASTN identified species for *B.*-dominant SVs, *B.*
706 *thetaiotaomicron* was the only species only found in broccoli-sprout-fed mice.
707
708
709 **Figure 10. Biogeography of the dominant Bacillus SV, which was found exclusively in**
710 **broccoli-sprout-fed mice.** *B.*-dominant was present in the jejunum contents and scrapings and
711 abundant in the cecum contents, colon contents, and colon scrapings. DSS reduced *B.*-dominant
712 colon content density.
713

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