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Steamed broccoli sprouts diet alleviates inflammatory bowel disease by increasing anti-inflammatory, antioxidant, and gut protective metabolites in an ulcerative colitis mouse model

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ABSTRACT

Background: Broccoli is a cruciferous vegetable rich in bioactive compounds that may be beneficial against inflammatory bowel disease. However, an in-depth annotation and understanding of the metabolites and microbial interactions associated with the broccoli sprouts diet is necessary.

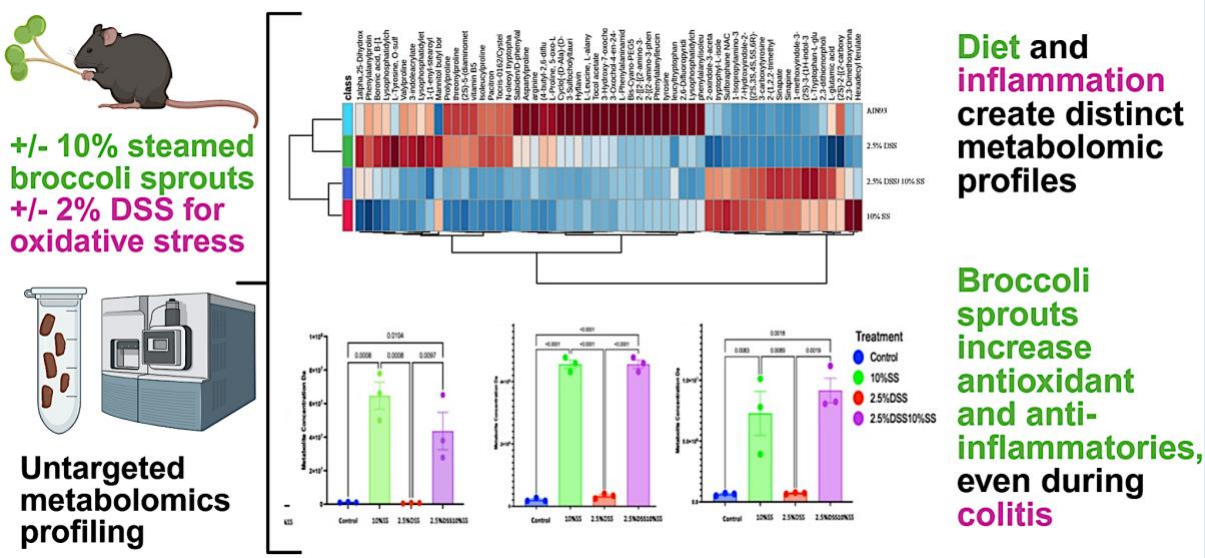
Methods: Specific pathogen-free C57BL/6 mice (male, 6 weeks old) were divided into four treatments: control diet; control diet with 2.5% DSS in water to induce symptoms of ulcerative colitis; 90% control diet plus 10% steamed broccoli sprouts; and 90% control diet/ 10% steamed broccoli sprouts plus 2.5% DSS in drinking water to induce symptoms. DSS treatment was cycled three times, with five days of DSS followed by a recovery period of five days. Mice continued their diets throughout the duration of the study. Fecal samples were collected on day 30 and frozen at -80°C until metabolomic analysis. Liquid chromatography, conducted using high-resolution mass spectrometry (LC-HRMS) was performed using a Waters Synapt G2, time-of-flight (TOF) XEVO. The LCMS metadata was processed in MassLynx, Progenesis QI, mzMine, and Sirius tools. This included the use of built-in search engines to obtain retention time, mass charge ratio, molecular weight, peak intensity, metabolites name, and formula using several libraries.

Results: Over 3,000 fecal metabolites were abundant in the feces of mice, with diet being a significant factor of differentiation (+/- steamed broccoli sprouts) and ulcerative colitis (+/- 2.5% DSS) treatment methods. The steamed broccoli sprouts intervention significantly increased the concentrations of beneficial metabolites, such as sulforaphane, short-chain fatty acids, tryptophan, indoles, glutamic acid, and polyphenolic metabolites. A positive correlation with commensal bacteria, *Bacteroides spp., Intestinimonas*, *Oscillibacter*, and Lachnospiraceae was found in the gut's colon, colon, cecal, and jejunum regions, and jejunum regions.

Novelty: This study used innovative metabolomic methods to identify dietary and microbial anti-inflammatory, antioxidant, prebiotic, and gut-protective metabolites associated with diets including steamed broccoli sprouts. The research concluded that the diet was effective against ulcerative colitis in mice.

Conclusions: These data suggest that broccoli sprouts may positively affect metabolites and microbial interactions. These benefits include their antioxidant, anti-inflammatory, and gut-protective properties that help with inflammatory bowel disease symptoms.

Keywords: cruciferous vegetables, sulforaphane, glucoraphanin, gut microbiota, dietary bioactives, metabolomics, ulcerative colitis model



Graphic Abstract Title: Diet and inflammation create distinct metabolomic profiles, and broccoli sprouts increase antioxidant and anti-inflammatories, even during colitis. Figure made with BioRender under license.

INTRODUCTION

Broccoli (Brassica oleracea var. italica) is a cruciferous vegetable in the Brassicaceae family. Mature broccoli, immature broccoli sprouts, fermented broccoli, and extracts of bioactive components are effective dietary interventions because of their multi-faceted benefits [1–3]. These benefits are primarily displayed via anti-inflammatory and antioxidant activities [4] which reduce

oxidative stress [5,6]. Broccoli sprouts have been reported to possess more beneficial phytochemicals than mature broccoli heads [7–9]. Diets rich in broccoli sprouts may contribute to overall well-being in healthy and diseased individuals [10–14]. This study found that the active ingredients in broccoli sprouts have anti-inflammatory and antioxidative properties [15-16]. Various preparations of broccoli sprouts reduce the inflammatory oxidative stress in the gut while promoting gut microbial diversity and intestinal structures [1,17–19].

Diets rich in broccoli also increase oxygen radical absorption while scavenging for other compounds' hydroxyl radicals in vitro [20]. Broccoli diets increase the production of Interleukin-10 (IL-10), an anti-inflammatory cytokine, while maintaining the cell-signaling membrane of glycoprotein CD36, which can reduce antigen responses. This effect decreases pro-inflammatory components produced by macrophages and monocytes: cytokine IL1β, the inducible nitric oxide synthase (iNOS) enzyme, the Cyclooxygenase-2 (COX-2) enzyme, and several classes of chemokine ligands (CCLs and CXCCLs) in vitro in colitis mouse models [20–22]. Similarly, steamed and raw broccoli sprouts protect the colon against damage through their antioxidant properties, increased expression of genes for nuclear factor erythroid 2-related factors 2 (NRF2), and the protein peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1α). The glutathione peroxidase 2 (GPX2) and glutathione S-transferase (GSTM1) enzymes increased the expression of tight junction protein ZO-1. Occludin and claudin-2 decreased proinflammatory cytokines IL6, IL1β, and tumor necrosis factor alpha (TNFα), while increasing microbial richness in mouse models with ulcerative colitis (dextran sodium sulfate (DSS) induced) or Crohn's disease (microbially induced in interleukin 10 knockouts (IL10 KO) [18-19].

These benefits may be associated with the hydrolysis effect of some gut bacteria on dietary metabolites from broccoli, such as glucosinolates (GSL), amines, polyphenols, and fibers. Gut microbes can hydrolyze these plant metabolites into isothiocyanates (ITC), which are conjugates of flavonoids, phenolic acids, short-chain fatty acids (SCFA), and vitamins [1,8,20]. For instance, consuming cooked or raw broccoli for four or more days could increase the abundance of myrosinase-like microbial hydrolases in Escherichia coli, Lactobacillus spp., Bifidobacterium spp., and Bacteroides spp. These metabolize GSLs, such as glucoraphanin (GLR), glucoerucin, and sinigrin, into ITCs such as nitriles (inactive versions), erucin, and indole conjugates. These microbial metabolites can upregulate genes to produce antioxidant and anti-inflammatory factors such as glutathione (GSH), NRF2, quinone oxidoreductase 1 (NQO1), and IL10. This improves the gut mucosal barrier, alleviating IBD pathologies [23–26]. Similarly, flavonoids and polyphenols such as curcumin, ferulic acids, and quercetin can be metabolized by Clostridium, Bacteroides, Ruminococcus spp., and Lactobacillus spp. through their ability to decrease proinflammatory cells, increase Treg cells, promote antioxidants (SOD), and enhance barrier protection such as ZO-1 and occludin [27–29]. The gut microbiome contains phenolic-cleaving bacteria such as Aldercreutzia equolifaciens and Flavonifractor plautii. Enzymes in the gut include: beta-glucuronidase, sulfotransferases (SULT), and catechol-O-methyltransferases (COMT) [30,31]. Microbiota such as Bacteroides, Lactobacillus, and Eubacterium can hydrolyze dietary fibers into SCFAs such as butyrate, propionate, and acetate, which stimulate anti-inflammatory responses in Treg cells that release IL-10 and protect epithelial tight junctions from damage [32-33]. Broccoli may change the intestinal microbial population for tryptophan hydrolysis by Clostridium sporogenes and Peptostreptococcus spp., which increases the indole metabolites that provide anti-inflammatory effects through the inhibition of COX2 and stimulation of NRF2 pathways [34–36]. Phenylalanine, a

dietary amino acid in broccoli, can be metabolized by Enterococcus and Lactobacillus spp. to increase anti-inflammatory and neuroprotective metabolites, which decrease cytokines such as interleukins [6,37–39].

Inflammatory bowel disease (IBD) is a major intestinal pathological condition that has a rising global concern [40]. The disease is difficult to treat due to the complex interplay between multiple pathological pathways, such as inflammation, oxidative damage, dysbiosis, and gut damage [1,41]. Symptom management is often conducted through diet [1,42]. Despite the reports that steamed broccoli sprouts could improve IBD pathologies in mouse models with decreased inflammatory markers (IL6, TNFα, and IL1β) and maintained microbial richness within the gut [17-18], information regarding the role of microbiota-derived metabolites modulating IBD pathways to create healthy conditions is limited. Therefore, it is essential to analyze the metabolomic intensity of compounds with antioxidant, anti-inflammatory, and gut-protective properties during IBD management with broccoli sprouts.

In this study, we hypothesized that steamed broccoli sprouts would increase the concentration of anti-inflammatory, antioxidant, prebiotic, and gut-protective compounds against IBD. We treated mice with DSS and/or steamed broccoli sprouts and conducted untargeted metabolomics with fecal samples. To further explore broccoli's nutraceutical effect against IBD, we compared the fecal metabolites in mice fed steamed broccoli diets alone with steamed broccoli diets in induced IBD conditions.

METHODS

Diet preparation and mouse study design: Broccoli sprouts were purchased from a local grocery store (Jonathan's Sprouts, Rochester, Massachusetts, USA), steamed for 10 minutes over boiling water, freeze-dried, and ground into fine powder. The powder was mixed with purified AIN93G rodent chow base (Inotiv, Indianapolis, IN, USA) to make a 10% (w/w) steamed broccoli diet according to previous studies, which reported adequate conversion of GLS to ITC [17,18]. Specific pathogen-free C57BL/6 mice were purchased from Jackson Laboratory at six weeks of age, acclimated for seven days, then distributed into cages based on four treatment groups as reported in the study [18]: 1) Control mice fed with AIN93G diet; 2) 2.5% DSS mice fed with DSS in water to induce IBD; 3) 10% SS mice fed with 10% steamed broccoli sprouts diet; 4) 2.5% DSS + 10% SS mice fed with both DSS and steamed broccoli sprouts diets. After the first seven days of the diet intervention, mice were placed on 2.5% DSS treatment in drinking water for five days, followed by a recovery period of five days. Mice remained on their diets for the duration of the study. After two more DSS treatment and recovery cycles, fecal samples were collected on day 30 and frozen at -80℃ until metabolomic analysis.

Untargeted metabolomics data acquisition and statistical analyses: Frozen fecal samples (50 mg) were sonicated in 200 µl of water and homogenized with 800 µl of cold solvent (1:1 of MeCN:MeOH), vortexed for 30 seconds, then centrifuged at 14,000 g for 5 mins at 4°C. The supernatant (900 µl) was collected into a fresh tube and centrifuged, followed by the collection of the supernatant (800 µl) into another tube, which was dried in a centrifugal vacuum at room temperature. Metabolites were reconstituted in a cold 400 µl buffer (1:1 of water + 0.1 % formic acid: MeCN) for metabolomic analysis. Liquid chromatography was performed with a high-resolution mass spectrometer (LC-HRMS). Data acquisition was performed using a Waters Synapt G2, time-of-flight (TOF) XEVO machine at the mass spectrometry facility of the University of California, Irvine, using the well-established method previously reported [43]. 250 µl of each sample was transferred to the LC vials for use in the column: 10 µl injection (C18 column, 7 x 40 mm) at mobile phase conditions as shown in Table 3.1 for metabolite analysis in an Acquity iClass

UPLC and Synapt G2 system (Waters Corp., Manchester, UK) [44]. The LC-MS metadata was processed in MassLynx, Progenesis QI, mzMine, and Sirius tools, along with built-in search engines to obtain the retention time, mass charge ratio, molecular weight, peak intensity, metabolite name, and formula using several libraries [45-46].

Table 1: Liquid Chromatography conditions for metabolomics.

Time (min)	A(%)	B(%)
0.00	98.0	2.0
1.00	98.0	2.0
15.00	2.0	98.0
17.00	2.0	98.0
18.10	98.0	2.0
20.00	98.0	2.0

A: 95:5 water: acetonitrile with 0.1% formic acid

B: acetonitrile with 0.1% formic acid. Flow rate of 3 µl/min.

The metadata was pre-processed in R to remove duplicate metabolite calls and correct peak intensity errors before importing the data into MetaboAnalyst 5.0 (http://www.metaboanalyst.ca) [47]. The data was normalized using a log transformation, a univariate One-way ANOVA analysis at $p< 0.05$ in a scatter plot done in MetaboAnalyst and Prism with Fisher's post-hoc test. The metabolites were evaluated for statistical significance across the experimental groups using bar plots. This was performed unsupervised, with supervised discriminant analysis for sample and group variation. The unsupervised principal component analysis (PCA) created a 2-way comparison of variability and sample cluster. Hierarchical clusters were conducted with heatmaps to observe the correlation between the experimental groups and metabolite peak intensity.

Correlations to microbial communities: To explore correlations to gut microbiota, sequence tables were taken from sequencing data in the 16S rRNA gene V3-V4 region to assess live bacterial communities from jejunum contents. Jejunum scrapings were obtained to evaluate mucosal-associated communities, cecal contents, colon contents, and colon scrapings [18]. Bacterial community data were generated with an Illumina MiSeq and processed with the DADA2 pipeline ver. 1.26 [48] in R software ver. 4.1.1 [49]. Raw sequence data (fastq files and metadata) is available from NCBI through BioProject Accession number PRJNA911821, along with a detailed workflow and code [18]. Previously generated read count data from bacterial genes that convert GSLs to ITCs identified in Bacteroides thetaiotaomicron VPI-5482 [50]. Quantitative polymerase chain reaction (qPCR) was performed on extracted DNA from mouse gut samples [18]. A Spearman correlation was performed to analyze p-values less than 0.05 between the metabolites of interest and previously published microbiota richness, taxa diversity, and B. thetaiotaomicron genes (BT2160-2156 operon) [18].

RESULTS

Diet and ulcerative colitis treatments induced differential metabolite profiles in feces: There were substantial differences in the metabolite profiles driven by diet (+/- 10% SS) and ulcerative colitis (+/- 2.5% DSS) treatments, visualized as distinct clusters (Figure 1a-c). A supervised statistical model partial least squares-discriminant analysis (PLS-DA) (Figure 1a), and an unsupervised K-means clustering approach to highlight sample similarity (Figure 1b) both resulted in similar

clustering by treatment group, while indicating similar amounts of data variation explained by the first (13.8 and 21.9%) and second (35.1% and 38.7%) axes. The K-means clustering of the PCA plot identified inter-cluster similarity between the 10% SS and 2.5% DSS + 10% SS groups (Figure 1b). However, the hierarchical clustering of samples confirmed the separation of samples into their treatment groups (Figure 1c). A total of 12,313 metabolites were observed based on metabolomics analysis. Yet, about 7,000 were filtered out due to quality control analysis done in MetaboAnalyst. 3,103 were found to have significantly increased or decreased concentrations across the four treatment groups (one-way ANOVA at $p < 0.05$; Figure 1d), which drove the cluster patterns.

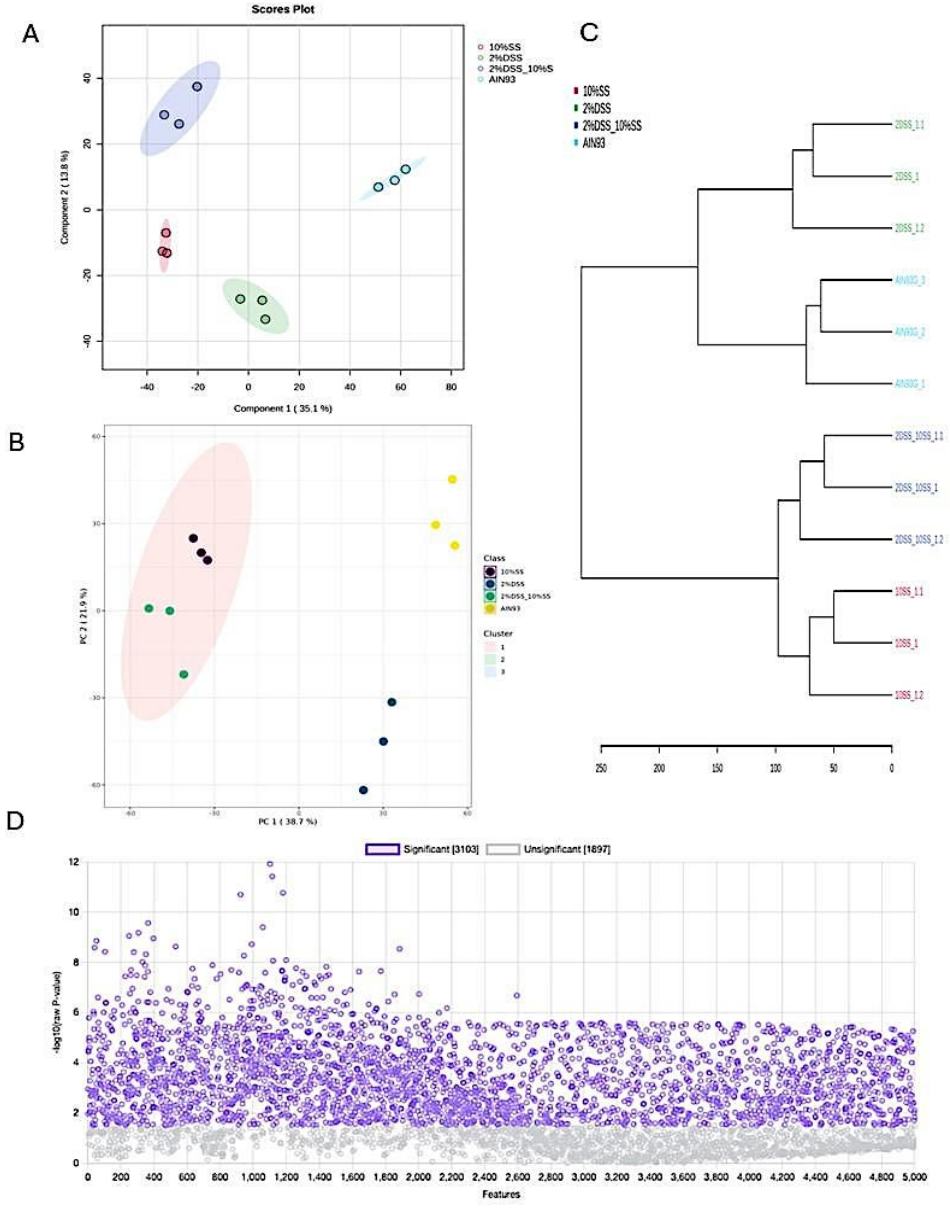


Figure 1: Fecal metabolites were differentially present by steamed broccoli sprout diet and DSS treatment. (a) PCA plots of samples with component 1 at 13.8% and component 2 at 35.1%; (b) K-mean plots with PC 1 at 21.9% and PC 2 at 38.7%; (c) Hierarchical cluster of experimental groups shown in dendrogram trees. (d) The univariate one-way ANOVA at $p < 0.05$ highlighted 3,103 metabolites differentially expressed across the experimental groups. Abbreviation: DSS: Dextran Sulfate Sodium.

Differentially expressed fecal metabolites across the four treatment groups in heat maps*:* Heat maps were used to visual metabolite concentration. In contrast, hierarchical clustering of sample metabolites showed the effect of diet (+/- 10% SS) and ulcerative colitis (+/- 2.5% DSS) treatments on all fecal metabolites in Figure 2a. Specific metabolites of interest are highlighted in Figure 2b. Open-source information regarding the metabolites of interest is reported in Table 2. The microbiota-derived metabolites of interest with differences in concentrations with broccoli diet or DSS treatment are ITCs, tryptophan metabolites, indole derivatives of tryptophan, amines derivatives of glutamine and phenylalanine, prolines, and SCFAs (Figure 2b).

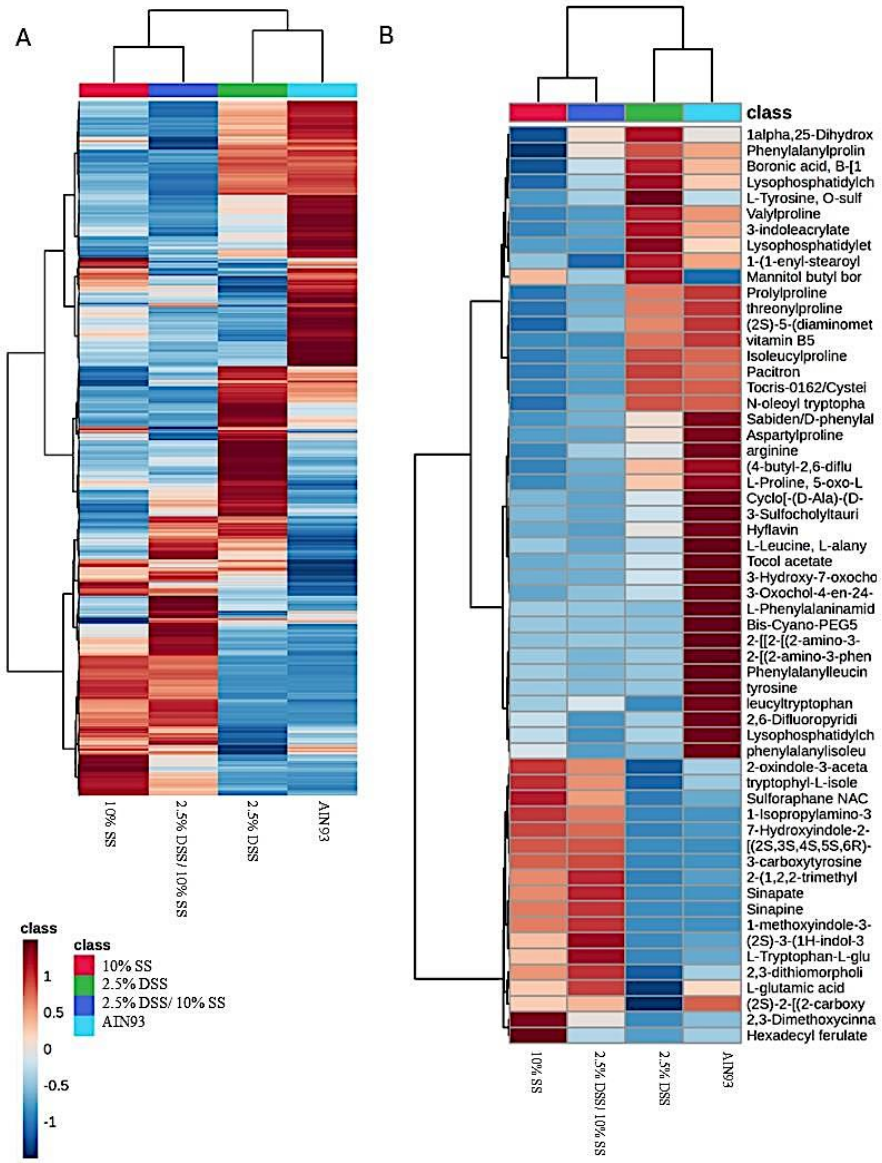


Figure 2: Hierarchical cluster analysis comparing the fecal metabolites among the treatment groups (10% SS, 2.5% DSS, and 2.5% DSS/10% SS) and the control (AIN93). The heat maps showed that (a) all differentially changed fecal metabolites and (b) specific differentially changed fecal metabolites of interest between the groups. The red color denotes an increase in metabolite concentration, while the blue color shows a decrease. Abbreviations: DSS: Dextran Sulfate Sodium and SS: steamed broccoli sprouts.

The effect of steamed broccoli sprouts on fecal metabolite concentration: To identify the impact of steamed broccoli sprouts, a one-way ANOVA was performed at $p< 0.05$ using metabolite concentrations (Figures 3,4). The microbiota-derived metabolites of interest with positive significant concentrations in mice

ate broccoli sprouts (10% SS and 2.5% DSS/10% SS), which ***are*** shown in Figure 3 and Figure 4a. These figures highlight the metabolites prevalent in 2.5% of DSS mice. These include sulforaphane N-acetyl cysteine (SFN-NAC), D-phenylalanine (sabiden), phenylalanyl isoleucine, L-glutamic acid, L-tryptophan-L-glutamine, tryptophyl-L-isoleucine, indoles, 3-carboxytyrosine, sinapic acid (polyphenol), derivatives of boronic acid, propanoic acid, and acetic acid. Positive trends were observed between polyphenolic metabolites, hexadecyl ferulate, and 2,3-dimethoxycinnamic acid in the feces of mice that ate broccoli sprouts (10% SS). However, these differences are not significant. Likewise, tryptophan metabolites N-oleoyl tryptophan, leucyl tryptophan, and indole propanoic acid showed a positive trend in mice that ate broccoli (10% SS) without any considerable difference. Meanwhile, L-tryptophan, indole acrylate, O-sulfotyrosine, cysteine-s-sulfate, pentanoic acid, and proline metabolites had significant differences with the DSS treatment as shown in Figure 4b.

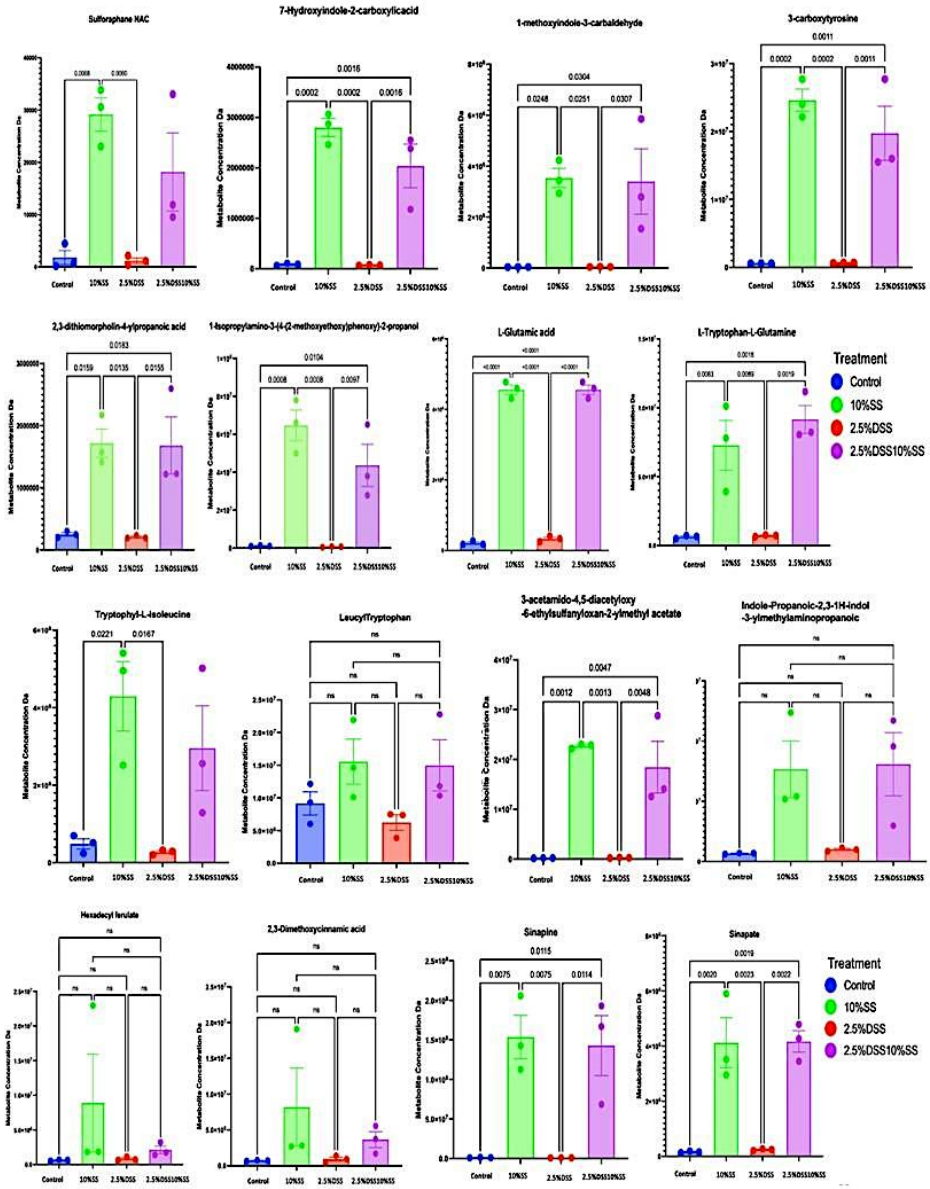


Figure 3: Selected beneficial metabolites were elevated in feces of mice consuming steamed broccoli sprout diets compared with the control of ulcerative colitis treatment groups (n=3 mice/group). Numbers topping the box indicate significance levels of a post-hoc test following a one-way analysis of variance. Insignificant tests at an alpha level of 0.05 were indicated with ns. Abbreviations: DSS: Dextran Sulfate Sodium and SS: steamed broccoli sprouts.

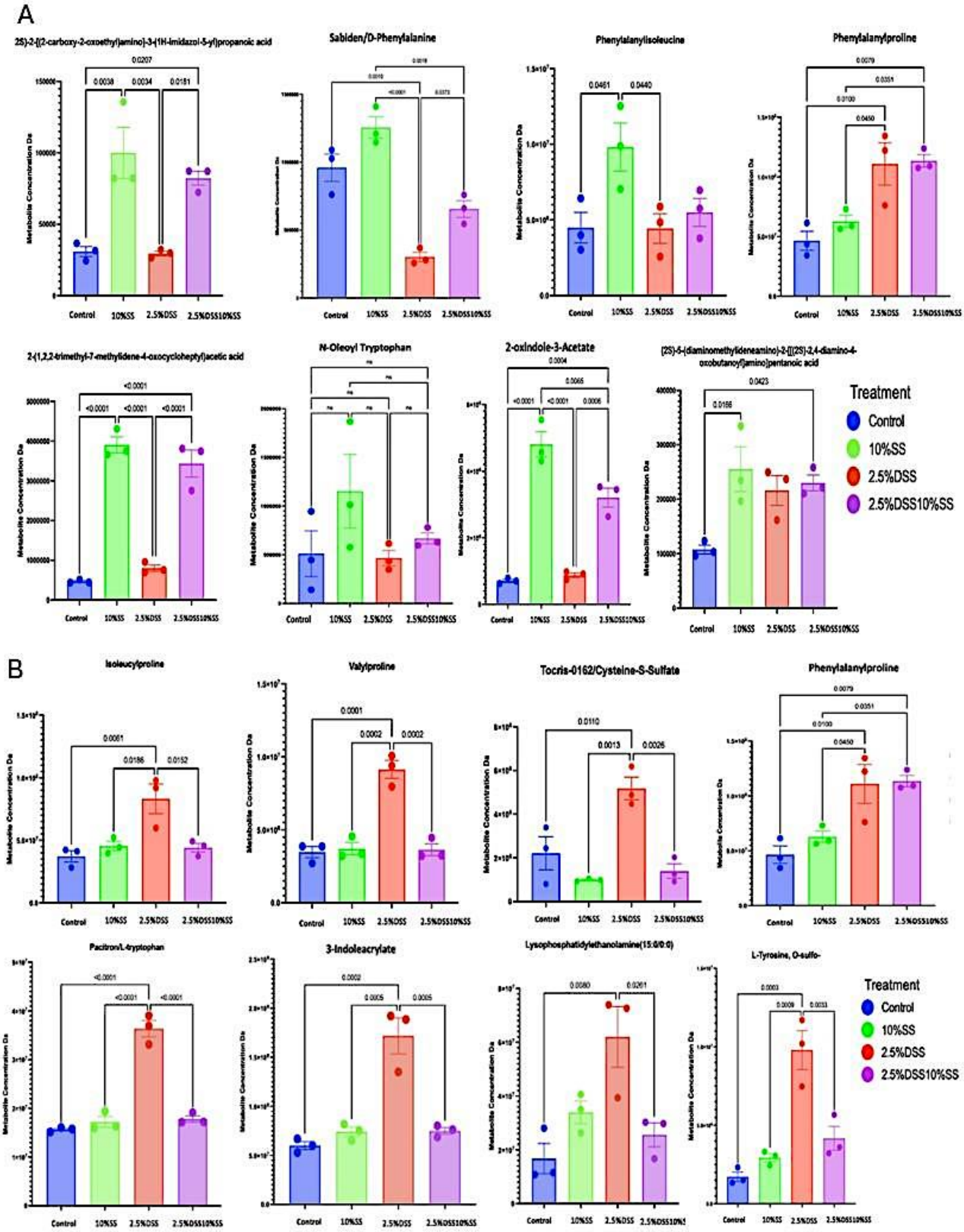


Figure 4: Concentrations of fecal metabolites associated with a) beneficial effects and elevated in mice consuming steamed broccoli sprouts or b) dextran sodium sulfate induced ulcerative colitis in mice (n=3 mice/group). Numbers topping the box indicate significance levels of a post-hoc test following a one-way analysis of variance. Tests that were not significant at an alpha level of 0.05 were indicated with ns. Abbreviations: DSS: Dextran Sulfate Sodium and SS: steamed broccoli sprouts.

Table 2: Open source information about annotated fecal metabolites of interest, Human Metabolomics Database or PubChem identification number, chemical formula, average molecular weight and CAS number.

S/N	Name	HMDB ID or PubChem CID	Chemical Formula	Average Molecular Weight	CAS Registry number
1	Sulforaphane-N-acetyl-cysteine	HMDB0240561	C11H20N2O4S3	340.47	NA
2	Sabiden/D-Phenylalanine	HMDB0250791	C9H11NO2	165.1891	NA
3	Phenylalanyl Isoleucine	HMDB0028998	C15H22N2O3	278.352	22951-94-6
4	Phenylalanyl Proline	HMDB0011177	C14H18N2O3	262.309	7669-65-0
5	Isoleucyl Proline	HMDB0011174	C11H20N2O3	228.292	37462-92-3
6	Valyl Proline	HMDB0029135	C10H18N2O3	214.265	20488-27-1
7	N-(N-(4-(((2,4-Diamino-6-pteridinyl)methyl)methylamino)benzoyl)glycyl)-L-glutamic acid	PubChem CID 326464	C22H25N9O6	511.5	71177-43-0
8	L-Tryptophan-L-Glutamine	HMDB0029081	C16H20N4O4	332.3544	NA
9	Tryptophyl-L-Isoleucine	HMDB0029086	C17H23N3O3	317.3828	NA
10	Leucyl-Tryptophan	HMDB0028940	C17H23N3O3	317.3828	NA
11	N-oleoyl tryptophan	HMDB0241968	C29H44N2O3	NA	NA
12	Indole-Propanoic OR 2,3-1H-indol-3-ylmethyl amino)propanoic	PubChem CID 24802222	C20H19N3O2	333.4	149724-31-2
13	3-Indoleacrylate	HMDB0000734	C11H9NO2	187.198	29953-71-7
14	Pacitron/L-tryptophan	HMDB0000929	C11H12N2O2	204.2252	73-22-3
15	7-Hydroxyindole-2-carboxylic acid	PubChem CID 20039897	C9H7NO3	177	84639-84-9
16	1-Meoxyindole-3-carbaldehyde	HMDB0040972	C10H9NO2	175.184	67282-55-7
17	L-Tyrosine, O-Sulfo-	HMDB0155722	C9H11NO6S	261.252	956-46-7
18	3-carboxy tyrosine	PubChem CID 53728607	C10H11NO5	225.2	NA
19	2,6-Difluoropyridine-3,5-diboronic acid pinacol ester	PubChem SID 458703181	C17H25B2F2NO4	366.2	1204333-58-3
20	Lysophosphatidylethanolamine (15:0/0:0)	HMDB0011502	C20H42NO7P	439.523	NA
21	2,3-dithiomorphin-4-ylpropanoic acid	ChemWhat Code 997648	C11H20N2O2S2	276.42	691410-93-2
22	1-Isopropylamino-3-(4-(2-methoxyethoxy)phenoxy)-2-propanol	PubChem CID 193122	C15H25NO4	283.36	30311-37-6
23	(2S)-2-[(2-carboxy-2-oxoethyl)amino]-3-(1H-imidazol-5-yl)propanoic acid	HMBD0002271	C6H8N2O2	140.058	1074-59-5
24	(2S)-5 (diaminomethyl ideneamino)-2-[[(2S)-2,4-diamino-4-oxobutanoyl]amino]pentanoic acid	PubChem CID 92266784	C10H20N6O4	288.3	NA

S/N	Name	HMDB ID or PubChem CID	Chemical Formula	Average Molecular Weight	CAS Registry number
25	2-(1,2,2-trimethyl-7-methylidene-4-oxocycloheptyl)acetic acid	PubChem CID 16720154	C13H20O3	225.1	NA
26	[(2S,2S,4S,5S,6R)-3-acetamido-4,5-diacetyloxy-6-ethylsulfanyloxan-2-yl]methyl acetate	PubChem CID 4347027	C16H25NO8S	391.4	49810-41-5
27	Hexadecyl Ferulate	HMDB0039317	C26H42O4	418.609	158306-36-6
28	2,3Dimethoxy cinnamic acid	HMDB0034315	C11H12O4	208.211	14737-89-4
29	Sinapine	HMDB0029379	C16H24NO5	310.365	84123-22-8
30	Sinapate	HMDB0032616	C11H12O5	224.51	7362-37-0
31	2-oxindole-3-Acetate	HMDB0035514	C10H9NO3	191.058	2971-31-5
32	Tocris-0162/ Cysteine-S-Sulfate	HMDB0000731	C3H7NO5S2	201.221	1637-71-4

Correlation analysis between fecal metabolites, microbial richness, bacterial taxa, and *B. thetaiotaomicron BT2160-2156* genes. We further performed correlation analyses between the metabolites of interest and previously published data from this study on microbial richness, bacterial taxa, and Bacteroides thetaiotaomicron BT2160-2156 genes for glucosinolate conversion [18]. A strong relationship was observed between metabolites and the selection of the hydrolyzing microbial populations. This highlights the importance of microbially derived metabolites during broccoli dietary management of IBD, as shown in Figures 4-8. Cecal bacterial richness was positively correlated with increased beneficial metabolites such as: SFN-NAC, phenylalanyl-isoleucine, L-glutamic acid, tryptophyl-L-isoleucine, indoles, 3-carboxy tyrosine, sinapic acid, derivatives of propanoic acid and acetic acid, hexadecyl ferulate, and 2,3-Dimethoxycinnamic acid. Meanwhile, cecal microbial richness had a zero or negative correlation with the DSS-increased metabolites such as L-tryptophan, indole acrylate, O-sulfotyrosine, cysteine-s-sulfate, and prolines (Figure 5a). Bacterial richness in colon scraping and content has a similar strong positive correlation with broccoli beneficial metabolites such as L-glutamic (strongest correlation), L-tryptophan-L-glutamic, tryptophyl-L-isoleucine, indoles, 3-carboxytyrosine, sinapic acid, derivatives of propanoic acids, boronic acid, and acetic acids, hexadecyl ferulate, and 2,3-Dimethoxycinnamic acid (Figure 5a).

Metabolites SFN-NAC, phenylalanyl-isoleucine, L-glutamic, L-tryptophan-L-glutamic, tryptophyl-L-isoleucine, indoles, 3-carboxytyrosine, sinapic acid, derivatives of propanoic acids, boronic acid and acetic acids, hexadecyl ferulate, and 2,3-Dimethoxycinnamic acid dispalyed positive relationships with Bacteroides and Bifidobacterium spp.. in the gut, This was especially seen in jejunum scrapings (Figure 5b), Intestinimonas spp. was found in jejunum scraping, the colon, cecal contents, and scrapings (Figure 5c), while the Lachnospiraceae family is found in jejunum scraping, colon scrapings and cecal contents.

There were negative correlations between these metabolites and *Paraclostridium* spp. in the cecal contents and colon scrapings (Figure 6a), *Dubosiella* spp. in the colon and cecal contents (Figure 5c), *Escherichia* spp. in the jejunum and colon scrapings and cecal content (Figure 5c). Positive correlations were observed between D-Phenylalanine, L-tryptophan, indole acrylate, O-sulfotyrosine, cysteine-s-sulfate, and prolines and *Lactobacillus* spp. in jejunum and colon scrapings (Figure 6a), cecal and jejunum contents, *Paraclostridium* spp. in the cecal contents and colon scrapings (Figure 6a). Additional correlations were observed between fecal metabolites and bacteria in the *Colidextribacter* genus, the Muribaculaeceae family, and the *Turicibacter* genus (Figure 6b)

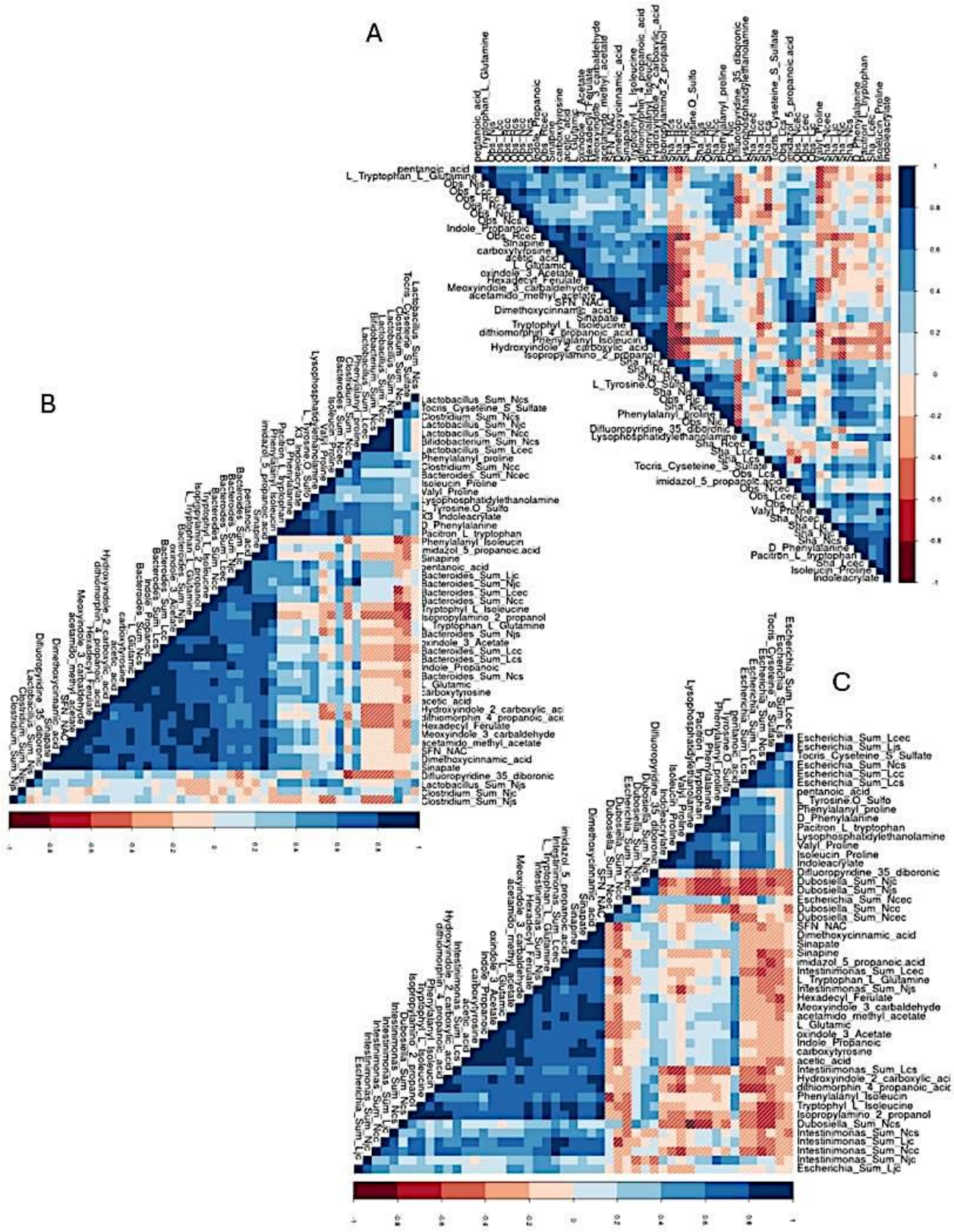


Figure 5: Correlogram of fecal metabolites, bacterial diversity metrics, IBD-associated bacterial taxa, and B. thetaiotaomicron genes (BT 2160-2156). The color scale in the plot illustrates the strength and direction of the pairwise association, which is determined by the correlation coefficient between each pair of variables at p values of 0.05 or less. Panels show correlations between metabolites and a) bacterial observed (Obs) richness (number of sequence variants), b) *Bifidobacterium, Lactobacillus, Clostridium*, and c) *Dubosi*, *Eubacteria, Intestinomonas, Escherichia.*

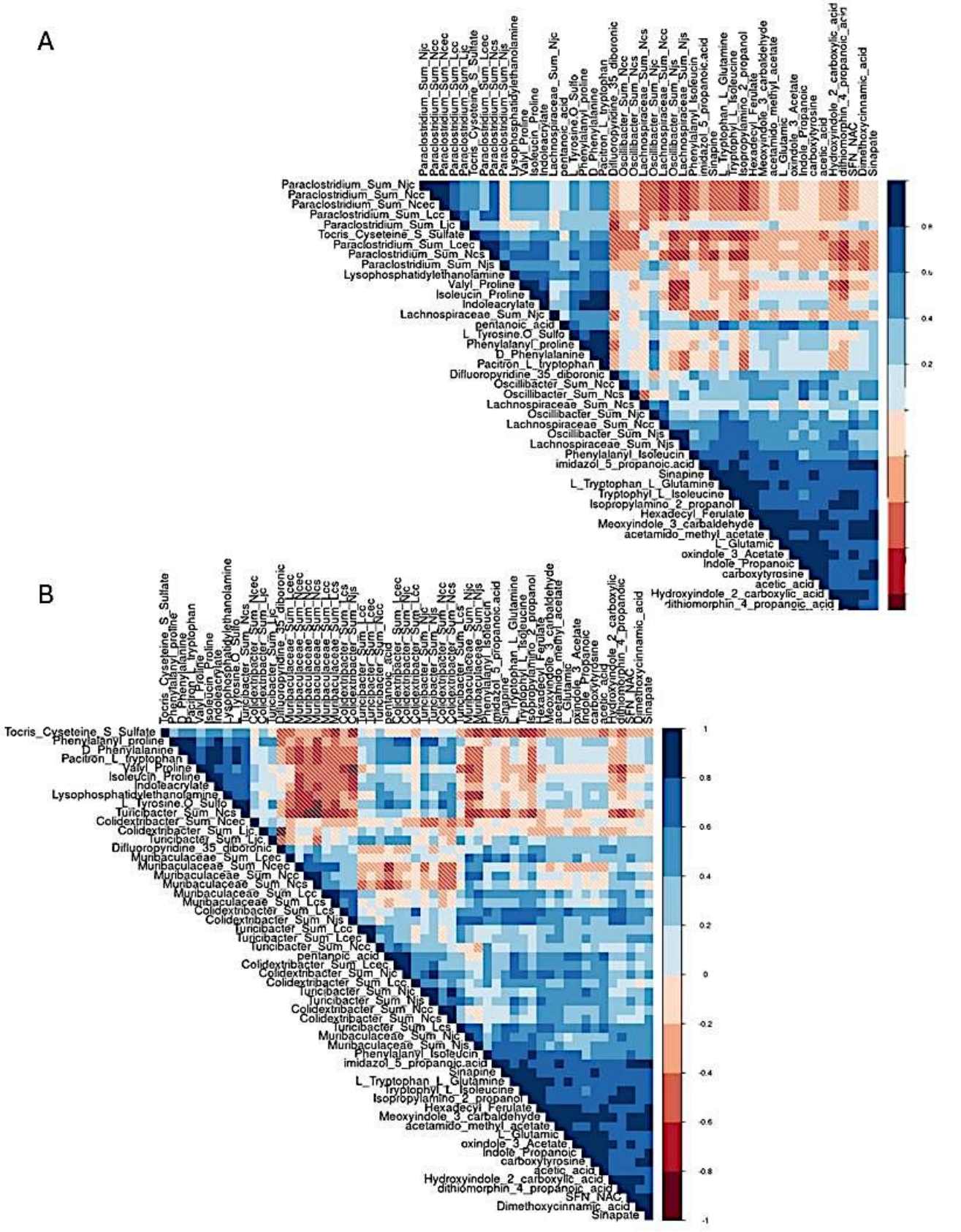


Figure 6: Correlogram of fecal metabolites, bacterial diversity metrics, IBD-associated bacterial taxa, and B. thetaiotaomicron genes (BT 2160-2156). The color scale in the plot illustrates the strength and direction of the pairwise association, which is determined by the correlation coefficient between each pair of variables at p values of 0.05 or less. Panels show correlations between metabolites and a) *Paraclostridium*, *Oscillobacter*, Lachnospiraceae, and b) *Colidextribacter*, Muribaculaceae, *Turicibacter*.

Similar to Bacteroides spp. in the gut, most broccoli-increased metabolites such as: SFN-NAC, phenylalanyl-isoleucine, L-glutamic, L-tryptophan-L-glutamic, tryptophyl-L-isoleucine, indoles, 3-carboxytyrosine, sinapic acid, derivatives of propanoic acids, boronic acid and acetic acids, hexadecyl ferulate, and 2,3-Dimethoxycinnamic acid was positively correlated with the Bacteroides thetaiotaomicron gene BT 2156 in the cecal content (Figure 7), BT 2157-2158 in the cecal content and jejunum scrapings (Figure 7), BT 2159 in the cecal content (Figure 8), and BT 2160 in the jejunum scraping (Figure 8).

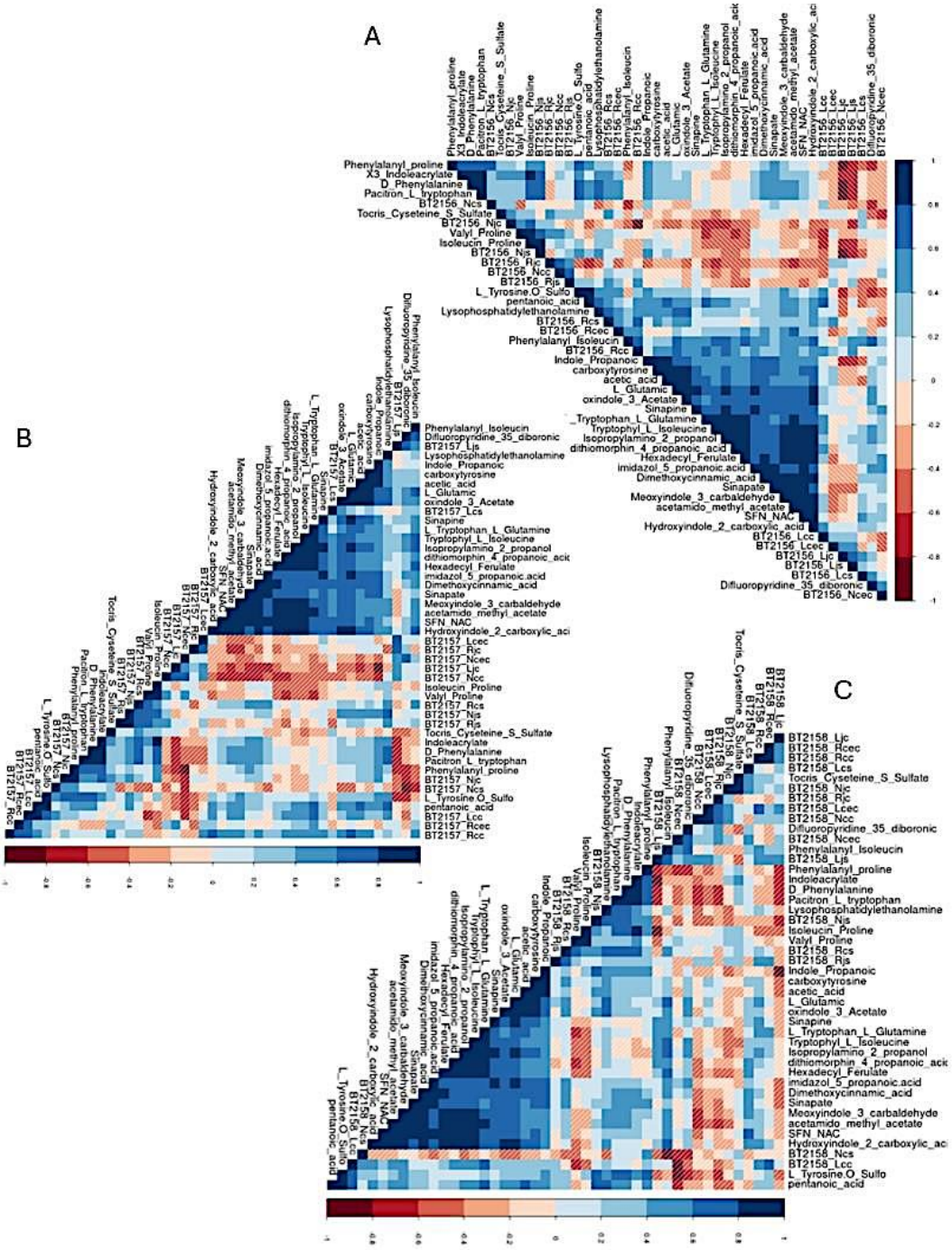


Figure 7: Correlogram of fecal metabolites and *Bacteroides thetaiotaomicron* genes (BT 2156-2158). The color scale in the plot illustrates the strength and direction of the pairwise association, which is determined by the correlation coefficient between each pair of variables at p values of 0.05 or less. Panels show correlations between metabolites and a) *Bacteroides thetaiotaomicron* gene BT 2156, b) *B.thetaiotaomicron* BT 2157, and c) *Bacteroides thetaiotaomicron* gene BT 2158.

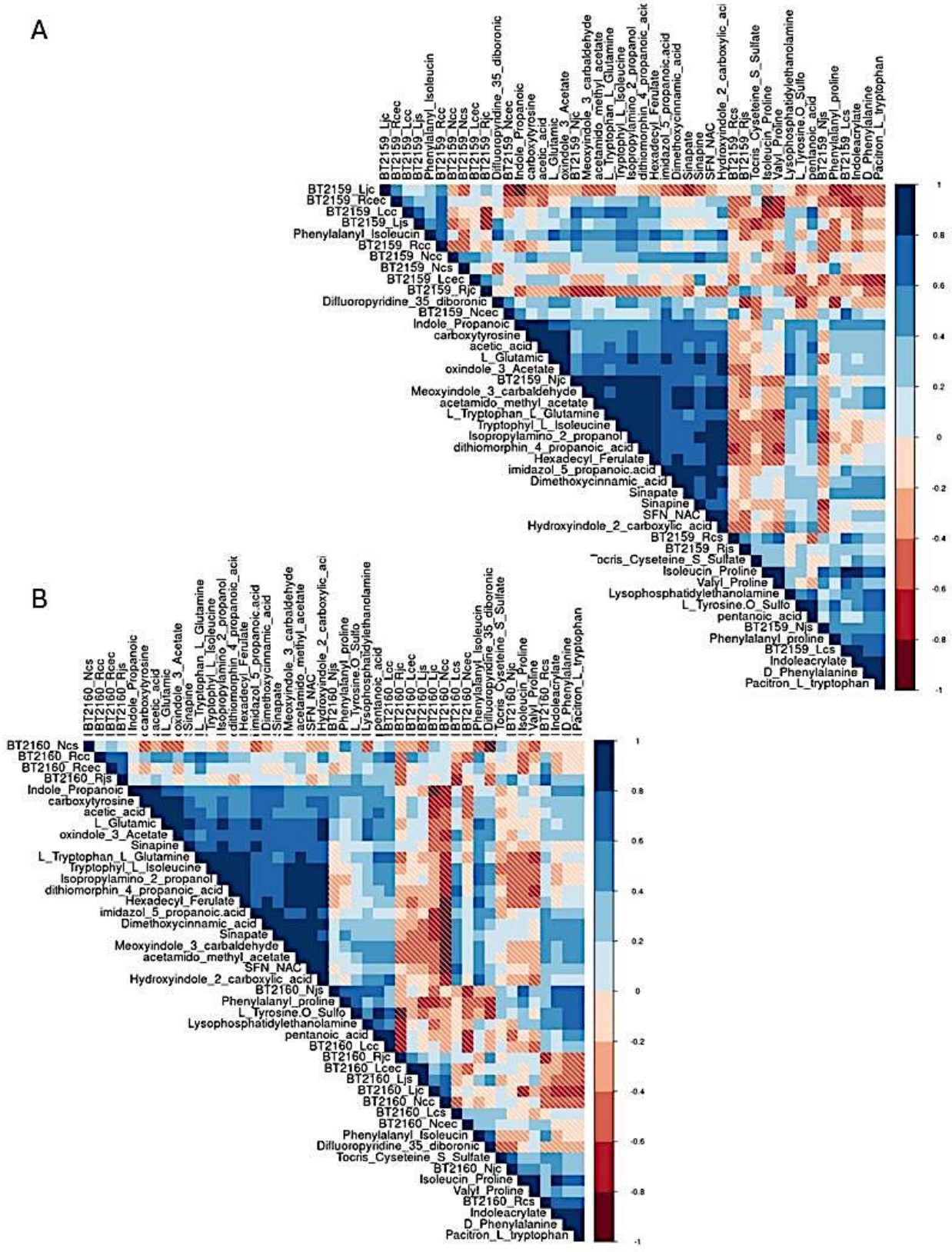


Figure 8: Correlogram of fecal metabolites and *Bacteroides thetaiotaomicron* genes (BT 2159-2160). The color scale in the plot illustrates the strength and direction of the pairwise association, which is determined by the correlation coefficient between each pair of variables at p values of 0.05 or less. Panels show correlations between metabolites and a) *Bacteroides thetaiotaomicron* gene BT 2159, b) *B.thetaiotaomicron* BT 2160.

DISCUSSION

Broccoli sprouts diets have the potential to manage inflammation due to high levels of phytochemicals such as GSLs, polyphenols, flavonoids, and fibers [1,10,51]. This may act directly or be hydrolyzed by certain gut bacteria to alleviate inflammation, oxidation, microbial dysbiosis, and gut cellular damage in IBD [24,28,33,35,52], as reported [1]. Previous studies reported that a 40-day 10% SS diet can restore IBD-related weight loss, decrease disease activity index and inflammatory markers, increase the metabolism of GLR to anti-inflammatory SFN, and preserve healthy biogeographical patterns of diverse bacteria in the gut [17-18].

Fecal SFN-NAC levels increased significantly for mice following the 10% SS diet, compared to the control and 2.5% DSS groups. This finding was expected as SFN-NAC is produced by microbiota in the presence of GLR, which cannot be done spontaneously [51,53], since mouse diets do not contain GSLs. However, the diet administration did not significantly increase SFN-NAC in the 2.5% DSS + 10% SS group. However, there was an increase similar to that of the 10% SS group, which may indicate that GLR-converting bacteria were harmed or inactivated by the presence of DSS. SFN-NAC was also produced but not used to combat DSS-induced inflammation. Previous research showed that high concentrations of NaCl can inactivate myrosinase [54]. Thus, DSS treatment may affect bacterial myrosinase-like enzymes. SFN-NAC possesses antioxidant and anti-inflammatory properties in the gut, with the ability to transform proinflammatory monocytes into macrophages, upregulate Treg cells to release IL-10, and modulate NFkB, STAT3, and activate NRF oxidative pathways [13-14,26,55–57].

The concentrations of polyphenolic metabolites, such as sinapate and sinapine, were significant in the fecal samples of mice in the 10% SS and 2.5% DSS + 10% SS groups compared to controls and the DSS groups. This finding supports previous findings of sinapic metabolites significantly contributing to crucifer-derived compounds that provide antioxidant and anti-inflammatory benefits in high-broccoli diets [58]. Sinapic acid upregulates GSH and decreases markers of oxidative stress and inflammation: malondialdehyde, NFkB, IL1β, IL-6, TNFα, iNOS, COX-2, and monocyte chemoattractant protein-1 (MCP-1/CCL2) [59-60]. It protects gut barriers against damage via I-CAM expression and mitigates psychological stress during IBD [60–62]. Hexadecyl ferulate and 2,3-dimethoxycinnamic acid are phenolic metabolites with antioxidative and anti-inflammatory effects against COX-2 and NFkB pathways [63-64].

Amines, D-phenylalanine, phenylalanyl isoleucine, 3-carboxy tyrosine, and L-glutamine significantly increased in the 10% SS when compared with the control and 2.5% DSS, and in the 2.5% DSS/10% SS mice when compared with the 2.5% DSS mice. Depending on the conjugates and pathways engaged, phenylalanine and tyrosine metabolites may be beneficial or detrimental during IBD [65-66]. For instance, O-sulfotyrosine is upregulated in our DSS mice, but may also influence leukocyte cell adhesion for inflammatory invasion [67]. Meanwhile, conjugating a carboxyl group and tyrosine is associated with COX-2 inhibition, which upregulates 3-carboxytyrosine in mice that ate broccoli with or without DSS [68]. In this data set, D-phenylalanine (sabiden) and phenylalanyl isoleucine increased in the 2.5% DSS/10% SS and 10% SS groups, which may alleviate oxidative stress and recruit Tregs into the gut against inflammation [69,70]. Glutamic acid increased with the 10 SS diet +/- DSS, and protected the mucosal and cellular layer of the gut from cytokines such as NFkB and TNFα. This also protects against nitric oxidative stress damage while increasing epithelial proliferation, maintaining tight junctions, and preventing pathologic bacteria translocation from the gut [71–73].

Our data identified increased concentrations of tryptophan metabolites and indoles, L-Tryptophan-L-

glutamine, tryptophyl-L-isoleucine, 7-dihydroxyindole-2-carboxylic acid, and 1-methoxyindole-3-carbaldehyde with antioxidative, anti-inflammatory, and gut protective benefits, after the 10% SS diet [74–76]. An increased level of L-tryptophan was observed with the DSS treatment, compared with the control and SS diet. This could be due to tryptophan recruiting T-cells to the site of damage in response to the presence of cytokines [45,77]. Tryptophan and glutamine conjugate, L-Tryptophan-L-glutamine, increased with 10% SS and 2.5% DSS/10% SS treatments. These display the role of glutamate in the tryptophan/kynurenine metabolic pathway [73,78].

The SS diet is fiber-rich, which serves as a substrate for microbial production of SCFAs, and identified propanoic/propionic, boronic acid, and acetates, such as 2-oxindole-3-acetate, 1-isopropylamino-3-(4-(2-methoxyethoxy)phenoxy)-2-propanol, 2,6-difluoropyridine-3,5-diboronic acid pinacol ester, 2,3-dithiomorpholin-4-ylpropanoic acid and 2-(1,2,2-trimethyl-7-methylidene-4-oxocycloheptyl) acetic acid. All these are protective to the gut through their anti-inflammatory and antioxidative properties [79–81].

The production of microbially-derived metabolites in the gut depends on the presence of bacteria capable of hydrolysis of dietary compounds from broccoli sprouts. It has been previously reported that the SS diet supports communities' richness, especially Bacteroides spp. These are involved in GLR hydrolysis to SFN. Biogeographical patterns of bacteria richness were higher in B. thetaiotaomicron BT 2159-2156 genes from jejunum scrapings, cecal contents, and colon scrapings after the 10% SS diet +/- DSS. The B. thetaiotaomicron BT2160-2156 operon increased in cecal contents after the 2.5% DSS/ 10% SS diet [18]. A correlation analysis was performed between fecal metabolites, microbial richness, specific bacterial taxa, and B. thetaiotaomicron BT 2160-2156 genes. The results revealed that beneficial metabolites were upregulated with the SS diet, such as SFN-NAC, L-glutamic, L-tryptophan-L-glutamic, tryptophyl-L-isoleucine, indoles, 3-carboxy tyrosine, sinapate, derivatives of propanoic acids, boronic acid, and acetic acids, hexadecyl ferulate, and 2,3-Dimethoxycinnamic. Metabolites were positively correlated with observed richness, commensal bacteria taxa, Bacteroides spp., Intestinimonas spp., Oscillibacter spp., and the Lachnospiraceae family, mainly in the colon, cecal, and jejunum of the gut. These findings support the hypothesis that GLR hydrolysis to SFN occurs most commonly in the colon and cecum [17,82]. This is primarily due to the rich population of Bacteroides spp. in the colon [50,83].

Many gut bacteria produce gut-protective metabolites: Intestinimonas spp. [84,85] and metabolites of the Lachnospiraceae family [86–88] that hydrolyze amines for phenolic compounds. These metabolites can also hydrolyze fiber for SCFAs, which may protect against the risk of Crohn's disease in the ileocolic region and ulcerative colitis rectosigmoiditis [86–89]. The Oscillibacter genus is associated with microbial metabolism of polyphenols, decreased hyperlipidemia, recovery from colitis, and increased gut protection [90–93]. Meanwhile, this data showed a positive correlation between cecal and colonic-located Lactobacillus and Paraclostridium genera with broccoli-enriched metabolite, D-Phenylalanine, and DSS-enriched metabolites, L-tryptophan, indole acrylate, O-sulfotyrosine, cysteine-s-sulfate, and prolines [88]. Proline residues and tryptophan are targets for Lactobacillus hydrolysis, which increase aryl hydrocarbon receptor sensitivity and decrease gut inflammation [75,94]. The Paraclostridium genus is associated with pathological development and severity of ulcerative colitis, such as intestinal barrier damage, colon rupture, and bloody stool [95,96]. Bacterial diversity and metabolic activities prominent in the colon and cecum may be associated with lower pH in regions favorable for hydrolysis of dietary metabolites [97-98].

Scientific innovations and Practical implications: An in-depth annotation and understanding of the metabolites and microbial interactions associated with consuming broccoli and sprouts is necessary to create personalized dietary recommendations that improve health. This is specifically important for the management of gut inflammation. This study used untargeted metabolomic approaches to identify novel dietary and microbial anti-inflammatory, antioxidant, prebiotic, and gut-protective metabolites associated with a steamed broccoli sprout diet. The study evaluated bacterial community changes due to diet composition, which may be an effective tool against ulcerative colitis in mouse models. Further research should focus on targeted nutritional interventions with broccoli-derived metabolites that interact with the gut microbiota to mitigate IBD pathology in diverse age groups. Additionally, investigations should translate these findings into human trials to validate the efficacy of steamed broccoli sprouts for clinical IBD management.

CONCLUSIONS

Steamed broccoli sprouts demonstrate significant potential as a nutritional intervention for managing IBD by modulating fecal metabolites, such as sulforaphane N-acetyl cysteine, amines, indoles, phenolic metabolites and SCFAs, associated with anti-inflammatory, antioxidative, and gut-protective effects. This study elucidates novel microbially derived metabolites and their associations with specific bacterial communities, such as Bacteroides spp., Intestinimonas, Oscillibacter, Lachnospiraceae, and Bacteroides thetaiotaomicro BT 2160-2156 genes. This highlights intricate mechanisms that provide the beneficial effects of broccoli for IBD management. Additionally, identifying increased concentrations of crucial metabolites such as SFN-NAC and sinapic acid highlights their potential to modulate therapeutic effects. The specific regions of metabolites and microbial activities in the gut are highlighted within this research.

List of abbreviations used: Chemokine ligands (CCLs and CXCCLs); Cyclooxygenase-2 (COX-2) enzyme; Dextran sodium sulfate (DSS); Glucoraphanin (GLR); Glucosinolates (GSL); Glutathione (GSH); Glutathione peroxidase 2 (GPX2) enzyme; Glutathione S-transferase (GSTM1) enzyme; Inducible nitric oxide synthase (iNOS) enzyme; Inflammatory Bowel Disease (IBD); Interleukin (IL); Isothiocyanates (ITC); Monocyte chemoattractant protein-1 (MCP-1/CCL2); Nuclear factor erythroid 2-related factors 2 (NRF2); Peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1α) protein; Short-chain fatty acids (SCFA); Tumor necrosis factor alpha (TNFa).

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: Conceptualization was done by T.E.A., T.Z., Y.L., and S.L.I. Methodology was done by T.E.A., T.Z., Y.L., and S.L.I. Formal analysis, data visualization, and data curation were done by T.E.A., and the Investigation was done by T.E.A., Y.L., and S.L.I.. Resources and funding were provided by S.L.I., Y.L., and T.Z.. The original draft was written by T.E.A., and revisions were done by T.E.A., T.Z., Y.L., and S.L.I.. Supervision and project administration were provided by Y.L. and S.L.I.

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