

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

Broccoli is a cruciferous vegetable rich in bioactive compounds that may be beneficial against inflammatory bowel disease. 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 then a recovery period of five days. Mice remained on their diets for the duration of the study. Fecal samples were collected on day 30 and frozen at -80°C until metabolomics analysis. Liquid chromatography with a high-resolution mass spectrometry (LC-HRMS) data acquisition was performed using Waters Synapt G2, time-of-flight (TOF) XEVO. We processed the LCMS metadata in MassLynx, Progenesis QI, mzMine, and Sirius tools with built-in search engines to obtain the retention time, mass charge ratio, molecular weight, peak intensity, metabolites name, and formula using several libraries. Over 3,000 fecal metabolites were differentially abundant in feces of mice based on diet (+/- steamed broccoli sprouts) and ulcerative colitis (+/- 2.5% DSS) treatments. The steamed broccoli sprouts intervention significantly increased the concentrations of beneficial metabolites, such as sulforaphane, short-chain fatty acids, tryptophan, indoles, glutamic and polyphenolic metabolites, with positive correlations with commensal bacteria, *Bacteroides spp.*, *Intestinimonas*, *Oscillibacter* and *Lachnospiraceae* in the colon, cecal, and jejunum regions of the gut.

Introduction

Broccoli (*Brassica oleracea* var. *italica*) is a type of cruciferous vegetable in the *Brassicaceae* family, and mature broccoli, immature broccoli sprouts, fermented broccoli, and extracts of bioactives are highly effective as dietary interventions because they provide multi-faceted benefits (1–3). These benefits are primarily via anti-inflammatory and antioxidant activities (4) which reduce oxidative stress (5,6). Broccoli sprouts, in particular, contain more concentrated beneficial phytochemicals than mature broccoli heads (7–9). Diets rich in broccoli sprouts could contribute to overall well-being in healthy and disease states (10–12), and diets featuring cruciferous vegetables have demonstrated success in clinical trials, reviewed in (1). Our *in vitro* studies showed that active ingredients in broccoli sprouts have anti-inflammatory and antioxidative properties (13,14), and our mouse model studies showed that various preparations of broccoli sprouts can provide targeted reduction of inflammatory oxidative stress in the gut while promoting gut microbial diversity and intestinal structures (1,15–17).

In addition to beneficial compounds, broccoli diets increased the oxygen radical absorbance capacity and the hydroxyl radical scavenging capacity of other compounds *in vitro* (18). Broccoli diets also increased the production of anti-inflammatory cytokines Interleukin-10 (IL-10) and the cell-signaling membrane glycoprotein CD36 which can reduce antigen response; and decreased pro-inflammatory components produced by macrophages and monocytes: cytokine IL1 β , the inducible nitric oxide synthase (iNOS) enzyme, the Cyclooxygenase-2 (COX-2) enzyme, and several classes of chemokine ligands (CCLs and CXCLs) *in vitro* and in a mouse colitis model (18–20). Similarly, steamed and raw broccoli sprouts protected against colon damage via increased antioxidants; increased expression of genes for nuclear factor erythroid 2-related factors 2 (NRF2), the protein peroxisome proliferator-activated receptor- γ coactivator 1- α (PGC1 α), and the antioxidant glutathione peroxidase 2 (GPX2) and glutathione S-transferase (GSTM1) enzymes; increased the expression of tight junction proteins ZO-1, occludin and claudin-2; decreased proinflammatory cytokines IL6, IL1 β , and tumor necrosis factor alpha (TNF α); and increased microbial richness in mouse models of ulcerative colitis (dextran sodium sulfate (DSS) induced) and Crohn's disease (microbially induced in interleukin 10 knockouts (IL10 KO) (16,17).

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 classes of plant metabolites into isothiocyanates (ITC), conjugates of flavonoids, phenolic acids, short-chain fatty acids (SCFA), and vitamins (1,8,18). 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., to metabolize GSLs, such as glucoraphanin (GLR), glucoerucin and sinigrin, into ITCs, nitriles (inactive versions), erucin and indole conjugates. Some of these microbial metabolites can upregulate genes for the production of antioxidant and anti-inflammatory factors, such as glutathione (GSH), NRF2, quinone oxidoreductase 1 (NQO1), and IL10; and improve the gut mucosal barrier to alleviate IBD pathologies (21–24). Similarly, flavanol and polyphenols such as curcumin, ferulic acids, and quercetin can be metabolized by *Clostridium*, *Bacteroides*, *Ruminococcus* spp. and *Lactobacillus* spp. with the ability to decrease proinflammatory cells, increase Treg cells, antioxidants (SOD) and barrier protection such as ZO-1 and occludin (25–27). Gut microbial communities have been reported to contain phenolic-cleaving bacteria such as *Aldercruzia equolifaciens* and *Flavonifractor plautii* and enzymes such as beta-glucuronidase, *sulfotransferases* (SULT) and *catechol-O-methyltransferases* (COMT) (28,29). Microbiota such as *Bacteroides*, *Lactobacillus*, and *Eubacterium* can hydrolyze dietary fibers into SCFAs such as butyrate, propionate and acetate to stimulate anti-inflammatory responses of Treg cells to release IL10 and protect epithelial tight junction from damage, as reviewed in (30,31). Broccoli could change intestinal microbial population for tryptophan hydrolysis by *Clostridium sporogenes* and *Peptostreptococcus* spp. to increase indole metabolites with anti-inflammatory effects via inhibition of COX2 and stimulation of NRF2 pathways (32–34). Phenylalanine, a dietary amino acid

from broccoli, can be metabolized by *Enterococcus* and *Lactobacillus* spp. to increase anti-inflammatory and neuroprotective metabolites, which decreased cytokines such as interleukins (6,35–37).

Inflammatory bowel disease (IBD) is one of the major intestinal pathological conditions with a rapid rate of occurrence and global concern (38). The disease is difficult to treat due to the complex interplay between multiple pathological pathways such as inflammation, oxidative damage, dysbiosis, and gut damage (1,39), and symptom management through diet is of significant interest (1). Despite the reports that steamed broccoli sprouts could improve IBD pathologies in mouse models with decreased inflammatory markers such as IL6, TNF α , and IL1 β and maintained microbial richness along the gut biogeography (15,16), there is limited information about the role of microbiota-derived metabolites modulating these IBD pathways into healthy conditions. 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. This study pairs with previously published data on mouse health and gut bacterial communities from the same experiment (16), in which a 10% steamed broccoli sprout diet significantly reduced disease activity index (fecal occult blood, stool consistency, and weight loss/stagnation), the loss of bacterial community specific to different locations in the intestines (biogeography), and the loss of taxa with putative capacity to convert GLR to bioactive sulforaphane.

Methods

Diet preparation and mouse study design

We purchased broccoli sprouts from a local grocery store (Jonathan's Sprouts, Rochester, Massachusetts, USA), steamed them for 10 minutes over boiling water, and freeze-dried and ground them 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 our previous studies, which reported adequate conversion of GLS to ITC (15,16). The biochemical composition of broccoli sprouts has been previously described (see Table 1 in (40)). In comparison with the control chow, dried broccoli sprouts have no casein which is the protein source in AIN-93G, but has a higher protein content (~32-43% by dry weight, compared to 20% in AIN93G). Dried broccoli sprouts contain comparable amounts of lipid/oil (~3-9% by dry weight), fiber content (~5-9% fiber by dry weight, which includes cellulose), and other carbohydrate (~40-50% by dry weight) (40).

The control diet contained 0.014 g/kg of tert-butylhydroquinone, an antioxidant used to preserve the chow, and this was slightly diluted (by 10%) in the experimental diet to 0.0126 g/kg. The 10% of the diet made up of broccoli sprouts contained other anti-oxidants at a much lower dose than the potential amount of sulforaphane, for example, polyphenols (9 - 77 mg GAE g⁻¹) and flavonoids (33 - 117 μ g QE g⁻¹), compared to SFN content of raw seeds and sprouts (273 to 3632 μ g g⁻¹) (40).

Briefly, broccoli sprouts approximately 10 days post germination (Jonathan's Sprouts™, Rochester, Massachusetts, USA) were steamed for 10 minutes using a double boiler to inactivate plant myrosinase and epithiospecifier protein enzymes which hydrolyse glucoraphanin, cooled to room temperature, frozen at -80°C, freeze-dried (University of Maine Food Pilot Plant, Orono, Maine, USA), ground into a fine powder, and mixed with powdered AIN93G rodent base diet (Inotiv, Indianapolis, Indiana, USA) to a concentration of 10% by weight. Silicone molds were used to make consistently sized pellets which were dried using a food dehydrator on low (~35 °C) for up to 48 hours in a chemical safety hood, and stored in sealed plastic bags at -20°C.

The animal protocol was approved by the University of Maine Committee on the Use and Care of Animals (IACUC protocol A2020-01-04), and biosafety work was approved by the University of Maine Institutional Biosafety Committee (protocol SI-092220). Specific pathogen-free C57BL/6 male mice were purchased from Jackson Laboratory at six weeks of age, acclimated for seven days, and distributed into cages based on four treatment groups (n=10 per group) balanced by weight as reported in our study (16): 1) “Control” mice fed with AIN93G diet; 2) “2.5% DSS” mice fed with 2.5 % 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 adaptation to diets, mice were placed on 2.5% DSS treatment in drinking water for five days, then a recovery period of five days. Mice remained on their diets for the duration of the study. After two more cycles of DSS treatment and recovery, fecal samples were collected on day 30 and frozen at -80°C until metabolomics analysis. Weight change, fecal blood scores, and fecal consistency scores were used to generate Disease Activity Index (Supplemental Data), as described previously (16).

Untargeted metabolomics data acquisition and statistical analyses

In this study, we chose to focus on microbial byproducts which are largely produced in the cecum and colon, and in particular, the isothiocyanates that are produced in the colon. Many products may act locally in the colon and have demonstrable reduction of symptoms, especially antioxidants and anti-inflammatories. 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, and centrifuged at 14,000 g for 5 mins at 4°C. The supernatant (900 µl) was collected into a fresh tube and centrifuged as aforementioned, after which the supernatant (800 µl) was collected into another tube, then 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 metabolomics analysis. Liquid chromatography with a high-resolution mass spectrometry (LC-HRMS) data acquisition was performed using Waters Synapt G2, time-of-flight (TOF) XEVO machine at the mass spectrometry facility of the University of California, Irvine, with a well-established method as previously reported (41). We transferred 250 µl of each sample to the LC vials for use in the column: 10 µl injection (C18 column, 7 x 40 mm) at mobile phase conditions for metabolite analysis in an Acquity iClass UPLC and Synapt G2 system (Waters Corp., Manchester, UK) (42). We processed the LCMS metadata in MassLynx, Progenesis QI, mzMine, and Sirius tools with built-in search engines to obtain the retention time, mass charge ratio, molecular weight, peak intensity, metabolites name, and formula using several libraries (43,44).

The metadata was pre-processed in R to remove duplicate metabolite calls and correct for peak intensity errors before importing into MetaboAnalyst 5.0 (<http://www.metaboanalyst.ca>) (45). We normalized the data by log transformation and performed univariate One-way ANOVA analysis at $p < 0.05$ in a scatter plot, adjusted for false positive and filtered data using Benjamini-Hochberg procedure in MetaboAnalyst, and used Prism with Fisher's post-hoc test to annotate specific metabolites of interest that are statistically different across the experimental groups in bar plots. We performed unsupervised and supervised discriminant analysis for sample and group variation. The unsupervised principal component analysis (PCA) was used to make the 2-way comparison of variability and sample cluster. We performed Hierarchical clusters with heatmaps to observe the correlation between the experimental groups and metabolite peak intensity.

Correlations to microbial communities

To explore correlations to gut microbiota, we used previously published (16) 16S rRNA gene bacterial sequence table (Supplemental Data) of live bacterial communities from jejunum contents, jejunum scrapings to obtain mucosal associated communities, cecal contents, colon contents, and colon scrapings. Bacterial community data had been generated with an Illumina MiSeq, processed with the DADA2 pipeline ver. 1.26 (46) in the R

software environment ver. 4.1.1 (47). Raw sequence data (fastq files and metadata) is available from NCBI through BioProject Accession number PRJNA911821, and detailed workflows and code are available elsewhere (16). We also used previously published (16) read count data (Supplemental Data) from bacterial genes demonstrated to be involved in conversion of GSLs to ITCs identified in *Bacteroides thetaiotaomicron* VPI-5482 (48). Quantitative polymerase chain reaction (qPCR) was performed on extracted DNA from mouse gut samples, detailed previously (16). We performed Spearman correlation analyses with screening for p values 0.05 or less between some metabolites of interest and previously published microbiota richness, taxa diversity, and *B. thetaiotaomicron* genes, *BT2160-2156* operon (16).

Results

Diet and ulcerative colitis treatments induced differential metabolite profiles in feces

We compared the effects of chemical induced ulcerative colitis and steamed broccoli sprouts in four treatment groups: “Control” mice fed with AIN93G diet; 2) “2.5% DSS” mice fed with 2.5 % DSS in water to induce IBD; 3) “10% SS” mice fed with 10% steamed broccoli sprouts diet; 4) “2.5% DSS + 10% SS”. There were substantial differences in the metabolite profiles driven by diet (+/- 10% SS) and ulcerative colitis (+/- 2.5% DSS) treatments, visualized as distinct clusters using three techniques (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 and indicated similar amounts of data variation explained by the first (13.8 and 21.9%) and second (35.1% and 38.7%) axes. The K-mean clustering of the PCA plot identified inter-cluster similarity between the 10% SS and 2.5% DSS + 10% SS groups (Figure 1b); however, 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, about 7,000 were filtered out by quality control analysis in MetaboAnalyst, and 3,103 were significantly increased or decreased concentrations across the four treatment groups (one-way ANOVA at $p < 0.05$; Figure 1d), which were driving the clustering patterns.

Differentially expressed fecal metabolites across the four treatment groups in heat maps

Heat maps were used to visual metabolite concentration, and 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 and specific metabolites of interest highlighted in Figure 2b. Open-source information about the metabolites of interest are 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).

The effect of steamed broccoli sprouts on fecal metabolite concentration

To identify the effect of steamed broccoli sprouts, we performed one-way ANOVA at $p < 0.05$ using metabolite concentrations (Figures 3,4). The microbiota-derived metabolites of interest with positive significant concentrations in mice that ate broccoli sprouts (10% SS and 2.5% DSS + 10% SS) are shown in Figure 3 and Figure 4a, which highlight those metabolites also prevalent in control or 2.5% 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. We observed positive trends of polyphenolic metabolites, hexadecyl ferulate, and 2,3-Dimethoxycinnamic acid in the feces of mice that ate broccoli sprouts (10% SS), but those differences were 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 positive significant differences with the DSS treatment as shown in Figure 4b.

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, bacteria taxa, and *Bacteroides thetaiotaomicron* BT2160-2156 genes for glucosinolate conversion (16). We observed strong relationships between metabolites and a selection of the hydrolyzing microbial populations, further highlighting the importance of microbially derived metabolites during broccoli dietary management of IBD, as shown in Figures 4-8. Cecal bacterial richness had positive correlations with broccoli-increased beneficial metabolites, 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 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 have similar strong positive correlation with broccoli beneficial metabolites such as L-glutamic (strongest correlation), L-tryptophan-L-glutamic, trsptophyl-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 showed positive relationships with *Bacteroides* and *Bifidobacterium* spp. in the gut, especially from jejunum scrapings, colon content, and scrapings (Figure 5b), *Intestinimonas* spp. in jejunum scraping, colon, cecal contents and scrapings (Figure 5c), and the *Lachnospiraceae* family 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 Muribaculaceae family, and the *Turicibacter* genus (Figure 6b)

Similar to *Bacteroides* spp. in the gut, most of the broccoli-increased 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 positively correlated with *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 cecal content (Figure 8), and BT 2160 in the jejunum scraping (Figure 8).

Discussion

Broccoli sprouts diets have the potential for inflammation management due to the presence of beneficial phytochemicals such as GSLs, polyphenols, flavonoids, and fibers (1,10,49), which may act directly or can be hydrolyzed by certain gut bacteria to increase the capacity to alleviate inflammation, oxidation, microbial dysbiosis, and gut cellular damage in IBD (22,26,31,33,50), as we have previously reported (1). As part of this study on gut metabolites, we previously reported the beneficial effect of a 40-day 10% SS diet to 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 (15,16).

We found significantly increased fecal SFN-NAC levels in the 10% SS diet compared to the control and 2.5% DSS groups, which is expected as SFN-NAC is produced by microbiota in the presence of GLR but not spontaneously (49,51), and the base mouse diet does not contain GSLs. However, the administration of the diet did not significantly increase SFN-NAC in the 2.5% DSS + 10% SS group, though there was a trend of increase similar to the 10% SS group, which could indicate that GLR-converting bacteria were harmed or inactivated by the presence of DSS, or could indicate that SFN-NAC was produced but was used to combat the DSS-induced inflammation. Previous research indicated that high concentrations of NaCl can inactivate myrosinase (52), thus DSS treatment may perhaps affect bacterial myrosinase-like enzymes as well. 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 inflammatory, and activate NRF oxidative pathways (24,53–55).

The concentrations of polyphenolic metabolites, sinapate, and sinapine were enormously significant in the fecal samples of mice in the 10% SS and 2.5% DSS + 10% SS groups compared with control and DSS groups. This finding supports previous literature that sinapic metabolites are significant crucifer-derived compounds contributing to the antioxidant and anti-inflammatory benefits of the broccoli diet (56). Sinapic acid upregulates GSH and decreases markers of oxidative stress and inflammation: malondialdehyde, NFkB, IL1 β , IL-6, TNF α , iNOS, COX-2, and monocyte chemoattractant protein-1 (MCP-1/CCL2) (57,58). It protects gut barriers against damage via I-CAM expression and mitigates psychological stress during IBD (58–60). Hexadecyl ferulate and 2,3-Dimethoxycinnamic acid are phenolic metabolites with antioxidative and anti-inflammatory effects against COX-2 and NFkB pathways (61,62).

Amines, D-phenylalanine, phenylalanyl isoleucine, 3-carboxy tyrosine, and L-glutamine were 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. Phenylalanine and tyrosine metabolites can be beneficial or detrimental during IBD, depending on the conjugates and pathways engaged (63,64). For instance, O-sulfo tyrosine, upregulated in our DSS mice, has been implicated in leukocyte cell adhesion for inflammatory invasion (65). Meanwhile, conjugating a carboxyl group and tyrosine is associated with COX-2 inhibition, which was revealed as upregulated 3-carboxy tyrosine in mice that ate broccoli with or without DSS (66). In our data set, D-phenylalanine (sabinene) and phenylalanyl isoleucine increased in the 2.5% DSS + 10% SS and 10% SS groups, which could alleviate oxidative stress and recruit Tregs into the gut against inflammation (67,68). Glutamic acid was increased with 10% SS diet +/- DSS, and protects the mucosal and cellular layer of the gut from cytokines, NFkB, TNF α , and nitric oxidative stress damage and increase epithelial proliferation to maintain tight junction and prevent pathologic translocation of bacteria out of the gut (69–71).

Our data identified increased concentrations of tryptophan metabolites and indoles, L-Tryptophan-L-glutamine, tryptophyl-L-isoleucine, 7-dihydroxyindole-2-carboxylic acid, 1-methoxyindole-3-carbaldehyde with antioxidative, anti-inflammatory and gut protective benefits, after the 10% SS diet (72–74). We observed an increased level of L-tryptophan with DSS treatment compared with the control and 10% SS diet, which could be due to the activities of tryptophan to recruit T-cells to the site of damage in response to the presence of cytokines during IBD (43,75). Tryptophan and glutamine conjugate, L-Tryptophan-L-glutamine, increased with 10% SS and 2.5% DSS + 10% SS treatments, these buttress the role of glutamate rings in tryptophan/kynurenine metabolic pathway (71,76).

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 of which are gut protective, anti-inflammatory and antioxidative (77–79).

The production of other microbially-derived metabolites in the gut depends on the presence of bacteria capable of hydrolysis of dietary compounds from broccoli sprouts. We previously reported that the SS diet supports communities' richness, especially *Bacteroides* spp., which are involved in GLR hydrolysis to SFN; biogeographical patterns of bacteria richness with higher *B. thetaiotaomicron* BT 2159-2156 genes in jejunum scrapings, cecal contents and colon scrapings after the 10% SS diet +/- DSS; and the *B. thetaiotaomicron* BT2160-2156 operon were increased in cecal contents after the 2.5% DSS + 10% SS diet (16). Here, we performed correlation analysis between fecal metabolites, microbial richness, specific bacterial taxa, and *B. thetaiotaomicron* BT 2160-2156 genes. Our results revealed that beneficial metabolites 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, 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 report that GLR hydrolysis to SFN occurs majorly in the colon and cecum (15,80), primarily due to *Bacteroides* spp. enriched in the colon (48,81).

Many gut bacteria produce gut protective metabolites: *Intestinimonas* spp. (82,83) and *Lachnospiraceae* family (84–86) can hydrolyze amine metabolites for phenolic compounds, and hydrolyze fiber for SCFAs, which may protect against the risk of Crohn's disease in the ileocolic region and ulcerative colitis rectosigmoiditis (84–87). The *Oscillibacter* genus has been associated with microbial metabolism of polyphenols, decreased hyperlipidemia, recovery from colitis and increased gut protection (88–91). Meanwhile, our data showed positive correlations 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 (86). Proline residues and tryptophan are targets for *Lactobacillus* hydrolysis to increase aryl hydrocarbon receptor sensitivity and decrease gut inflammation (73,92). The *Paraclostridium* genus is associated with pathological development and severity of ulcerative colitis, such as intestinal barrier damage, colon rupture, and bloody stool (93,94). Bacterial diversity and metabolic activities prominent in the colon and cecum may be associated with less pH in regions favorable for hydrolysis of dietary metabolites (95,96).

The fecal metabolites associated with broccoli sprouts supplementation in colitis have been reported to activate cytoprotective effect and mucosal repair pathways to prevent DSS-induced oxidative protein damage, protect the intestinal cellular integrity, such as epithelial integrity and tight-junction efficiency and maintain mucosal efficiency. Metabolite SFN could activate NRF2 to support tight-junction proteins against oxidative damage and leaky gut (54). Tryptophan metabolites (97), such as indole-3-propionic acid could modulate mucosal repair via AhR/IL-22 pathway to promote transepithelial repair, resistance and integrity (98). Also, SCFAs (99), sinapic acid, ferulates, and other polyphenols protect crypts and villus architecture, reduce neutrophil infiltration, and restore the semipermeability barrier function (100) by inhibiting GPR5/HDAC (101) and NFkB/MAPK pathways (100). N-acetylcysteine and cysteine-S-Sulfate (102,103) could restore colon mucosa lining and lumen efficiency by increasing the expression of mucin, presence of goblet cells and localization of claudin-1 (104).

Conclusions

Steamed broccoli sprouts demonstrate significant potential as a dietary 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 thetaiotaomicron*, BT 2160-2156 genes,

shedding light on the intricate mechanisms underlying the beneficial effects of broccoli in IBD management. Additionally, identifying increased concentrations of crucial metabolites such as SFN-NAC and sinapic acid highlights their potential as critical mediators of the observed therapeutic effects. We highlighted these specific regions of metabolites and microbial activities in the gut.

List of abbreviations used

Chemokine ligands (CCLs and CXCLs); 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 (PGC1 α) protein; Short-chain fatty acids (SCFA); Steamed Sprouts (SS); Tumor necrosis factor alpha (TNF α).

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 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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Figure legends

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 and SS= steamed broccoli sprouts.

Figure 2: Hierarchical cluster analysis comparing the metabolites among the treatment groups (10% SS, 2.5% DSS, and 2.5% DSS/10% SS) and the control (AIN93). The heatmaps 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.

Figure 3: Selected beneficial metabolites were elevated in feces of mice consuming steamed broccoli sprout diets compared with 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. Tests that were not significant at an alpha level of 0.05 were indicated with ns. Abbreviations: Abbreviation: 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: Abbreviation: DSS= dextran sulfate sodium and SS= steamed broccoli sprouts.

Figure 5: Correlogram of fecal metabolites, bacterial diversity metrics, and IBD-associated bacterial taxa shown as PCoA plots. Panels show correlations between metabolites and a) Bacterial richness (number of sequence variants), b) *Bifidobacterium*, *Lactobacillus*, *Clostridium*, c) *Dubosi*, *Eubacteria*, *Intestinomonas*, *Escherichia*. The color scale by 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 0.05 or less.

Figure 6: Correlogram of fecal metabolites, bacterial diversity metrics, IBD-associated bacterial taxa shown as PCoA plots. Panels show correlations between metabolites and a) d) *Paraclostridium*, *Oscillobacter*, Lachnospiraceae, and b) *Colidextribacter*, Muribaculaceae, *Turicibacter*. The color scale by 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 0.05 or less.

Figure 7: Correlogram of fecal metabolites and *Bacteroides thetaiotaomicron* genes (BT 2156-2158). Panels show correlations between metabolites and a) *Bacteroides thetaiotaomicron* gene BT 2156, b) *B.thetaiotaomicron* BT 2157, and c) *Bacteroides thetaiotaomicron* gene BT 2158. The color scale by 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 0.05 or less.

Figure 8: Correlogram of fecal metabolites and *Bacteroides thetaiotaomicron* genes (BT 2159-2160). Panels show correlations between metabolites and a) *Bacteroides thetaiotaomicron* gene BT 2159, b) *B.thetaiotaomicron* BT 2160. The color scale by 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 0.05 or less.

Tables

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.

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	C ₁₁ H ₂₀ N ₂ O ₄ S ₃	340.47	NA
2	Sabiden/ D-Phenylalanine	HMDB0250791	C ₉ H ₁₁ NO ₂	165.1891	NA
3	Phenylalanyl Isoleucine	HMDB0028998	C ₁₅ H ₂₂ N ₂ O ₃	278.352	22951-94-6
4	Phenylalanyl Proline	HMDB0011177	C ₁₄ H ₁₈ N ₂ O ₃	262.309	7669-65-0
5	Isoleucyl Proline	HMDB0011174	C ₁₁ H ₂₀ N ₂ O ₃	228.292	37462-92-3
6	Valyl Proline	HMDB0029135	C ₁₀ H ₁₈ N ₂ O ₃	214.265	20488-27-1
7	N-(N-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)glycyl)-L-glutamic acid	PubChem CID 326464	C ₂₂ H ₂₅ N ₉ O ₆	511.5	71177-43-0
8	L-Tryptophan-L-Glutamine	HMDB0029081	C ₁₆ H ₂₀ N ₄ O ₄	332.3544	NA
9	Tryptophyl-L-Isoleucine	HMDB0029086	C ₁₇ H ₂₃ N ₃ O ₃	317.3828	NA
10	Leucyl- Tryptophan	HMDB0028940	C ₁₇ H ₂₃ N ₃ O ₃	317.3828	NA
11	N-oleoyl tryptophan	HMDB0241968	C ₂₉ H ₄₄ N ₂ O ₃	NA	NA
12	Indole-Propanoic OR 2,3-1H-indol-3-ylmethylamino)propanoic	PubChem CID 24802222	C ₂₀ H ₁₉ N ₃ O ₂	333.4	149724-31-2
13	3-Indoleacrylate	HMDB0000734	C ₁₁ H ₉ NO ₂	187.198	29953-71-7
14	Pacitron/L-tryptophan	HMDB0000929	C ₁₁ H ₁₂ N ₂ O ₂	204.2252	73-22-3
15	7-Hydroxyindole-2-carboxylic acid	PubChem CID 20039897	C ₉ H ₇ NO ₃	177	84639-84-9
16	1-Meoxyindole-3-carbaldehyde	HMDB0040972	C ₁₀ H ₉ NO ₂	175.184	67282-55-7
17	L-Tyrosine, O-Sulfo-	HMDB0155722	C ₉ H ₁₁ NO ₆ S	261.252	956-46-7
18	3-carboxy tyrosine	PubChem CID 53728607	C ₁₀ H ₁₁ NO ₅	225.2	NA
19	2,6-Difluoropyridine-3,5-diboronic acid pinacol ester	PubChem SID 458703181	C ₁₇ H ₂₅ B ₂ F ₂ NO ₄	366.2	1204333-58-3
20	Lysophosphatidylethanolamine (15:0/0:0)	HMDB0011502	C ₂₀ H ₄₂ NO ₇ P	439.523	NA

S/N	Name	HMDB ID or PubChem CID	Chemical Formula	Average Molecular Weight	CAS Registry number
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	HMDB0002271	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
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

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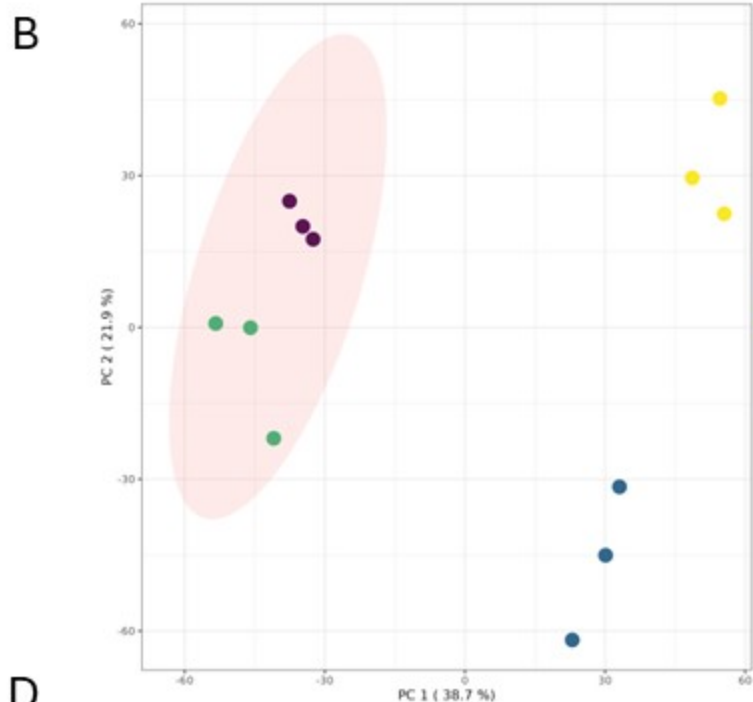
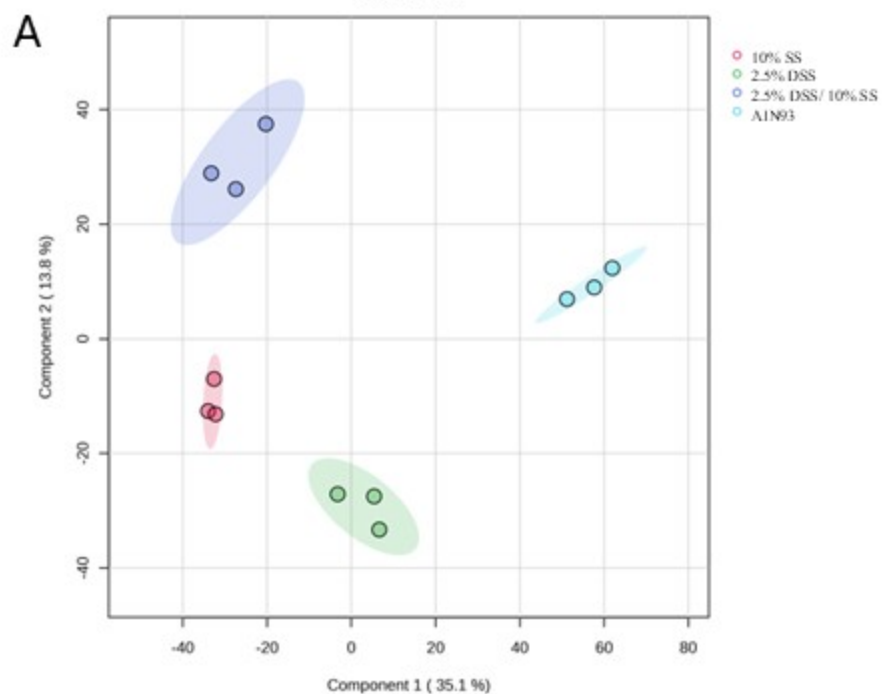
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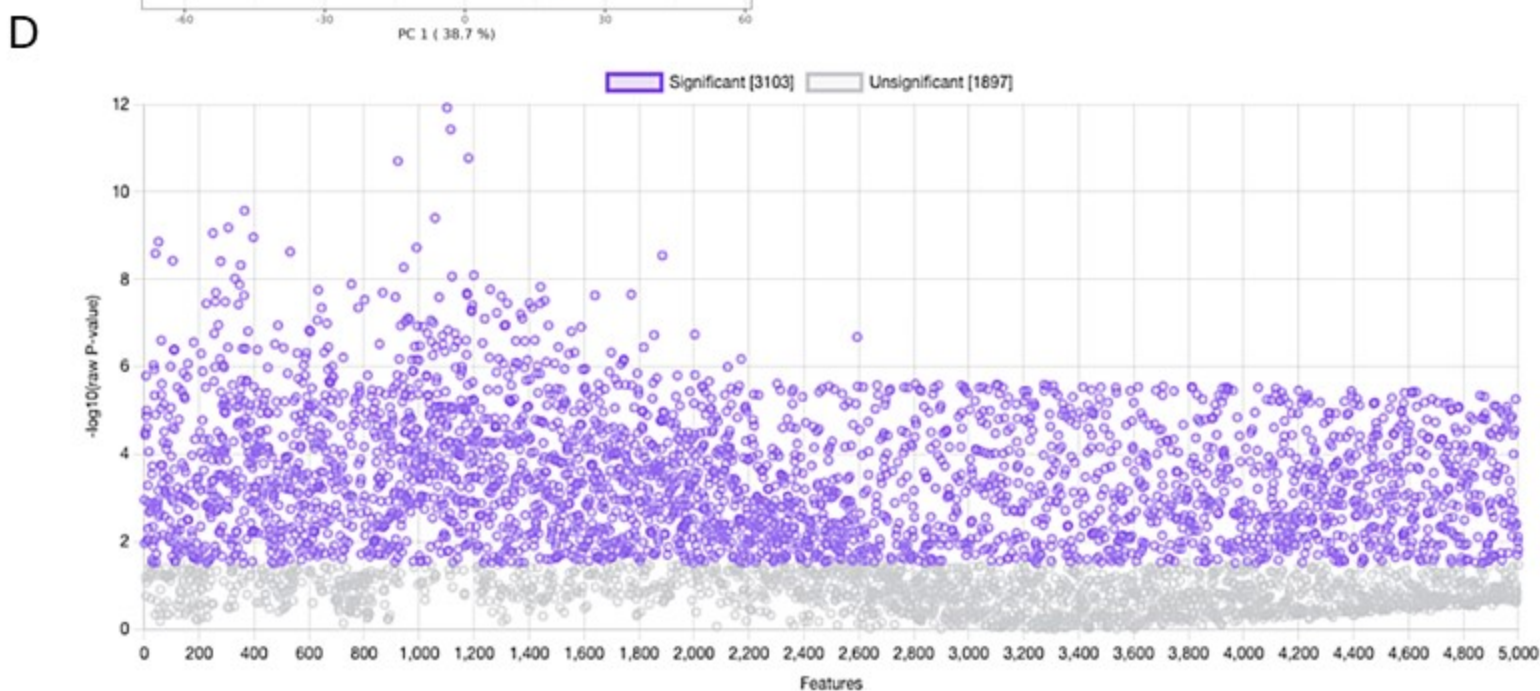
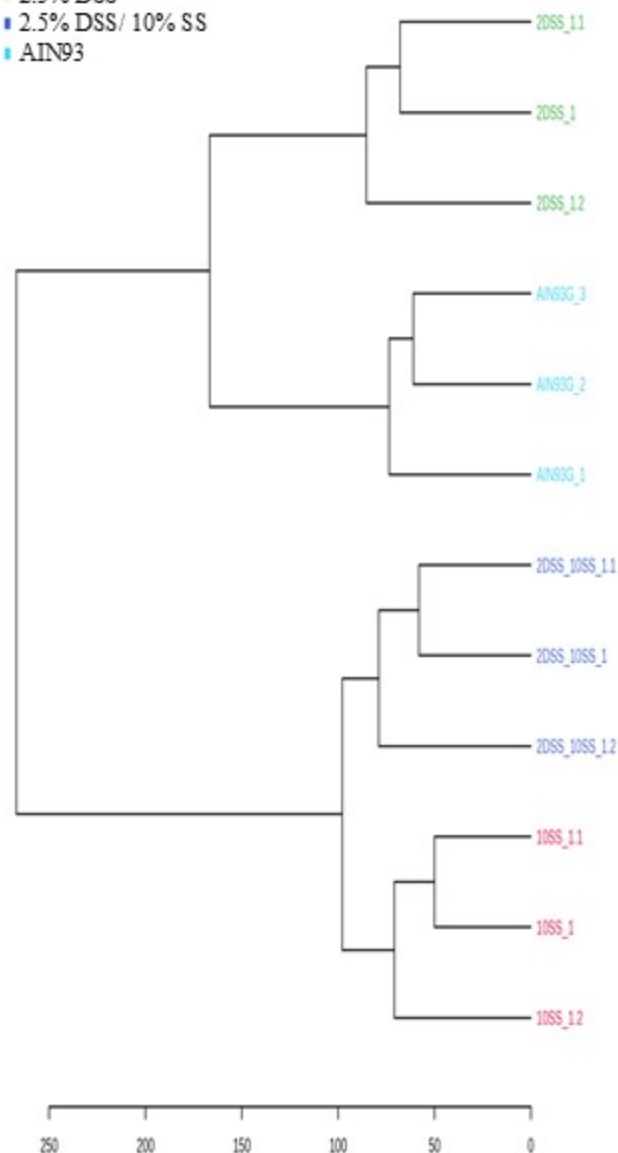
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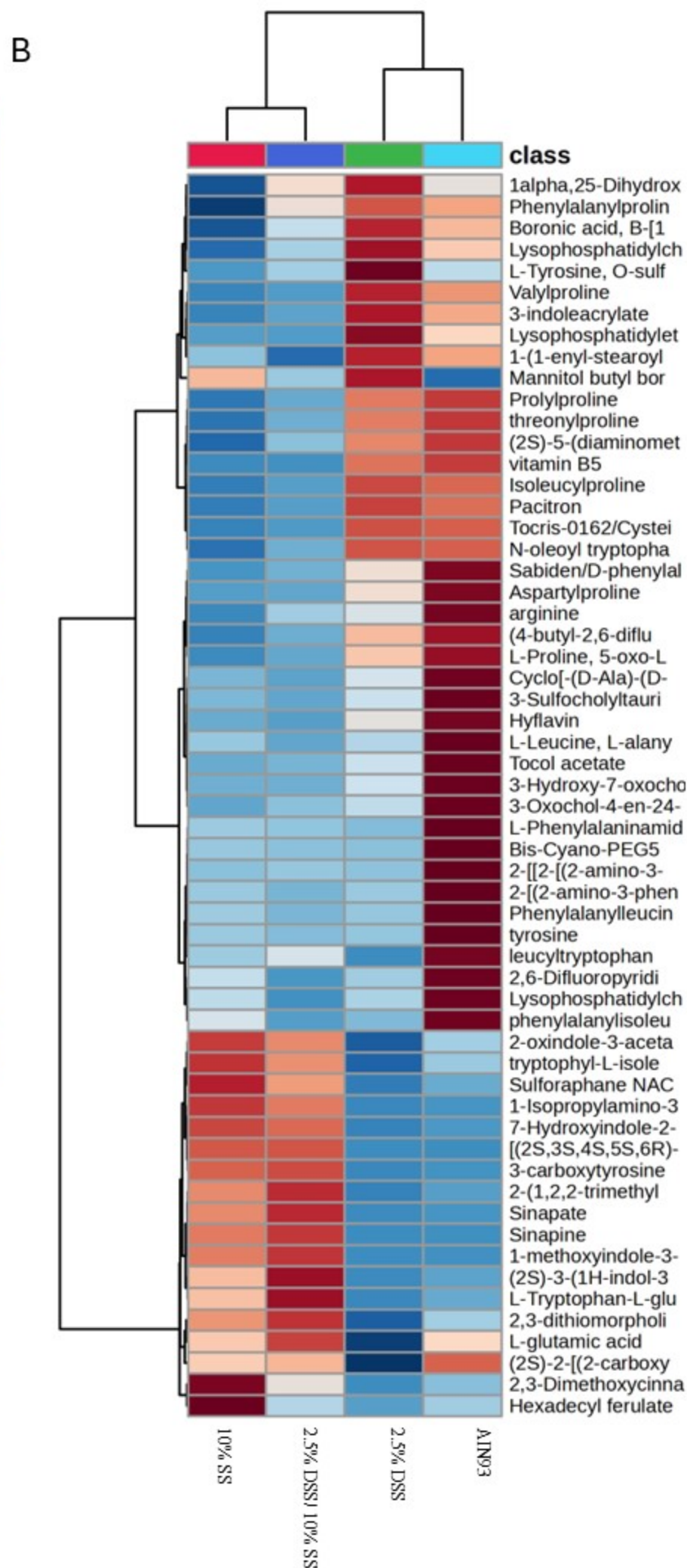
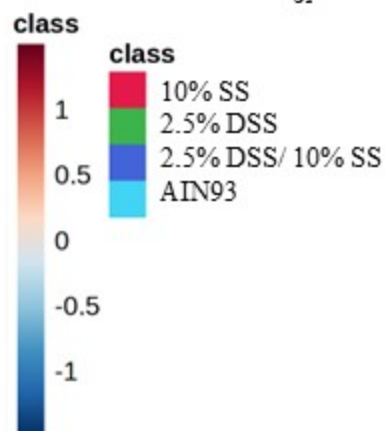
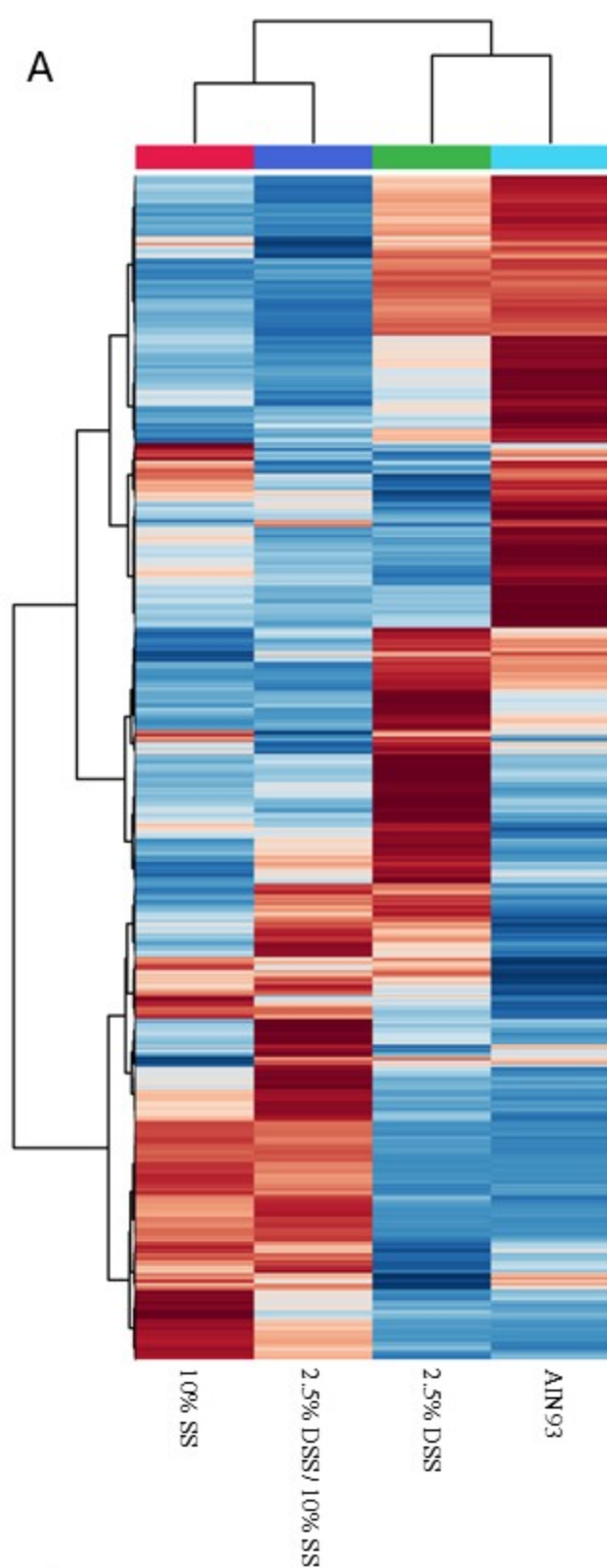
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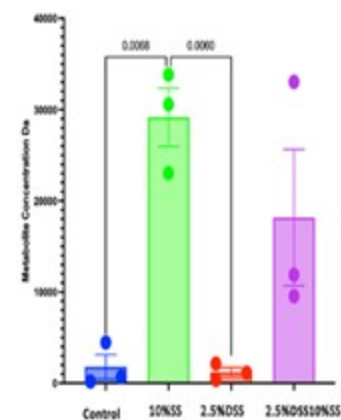
C

- 10% SS
- 2.5% DSS
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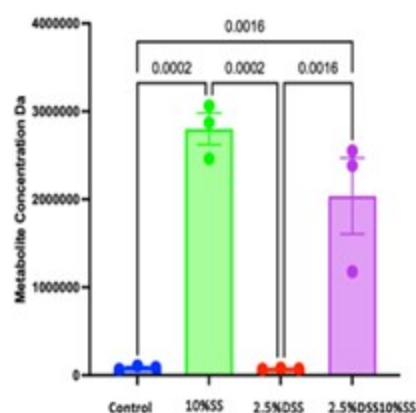




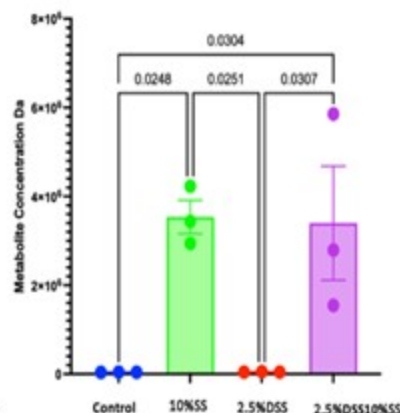
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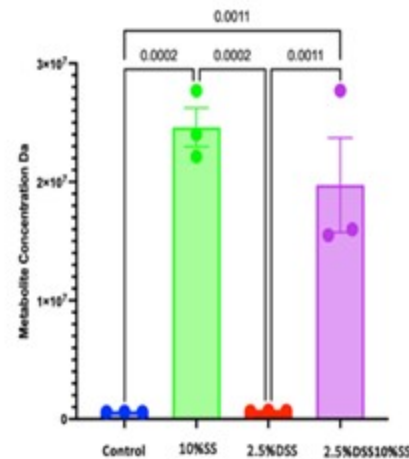
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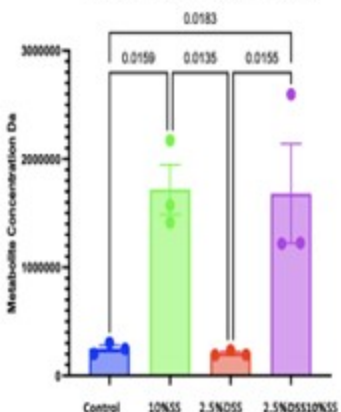
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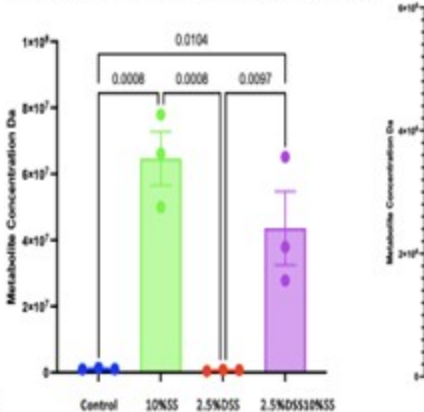
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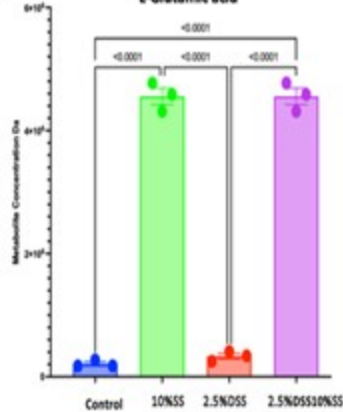
2,3-dithiomorpholin-4-ylpropanoic acid



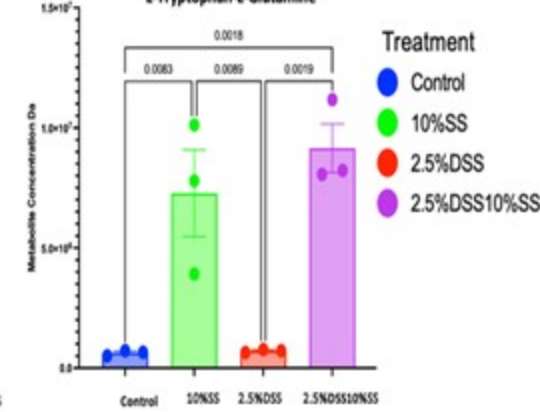
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L-Glutamic acid



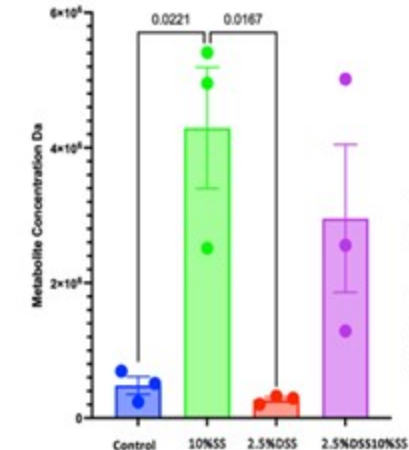
L-Tryptophan-L-Glutamine



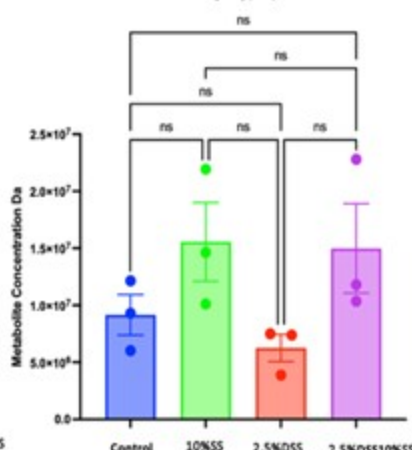
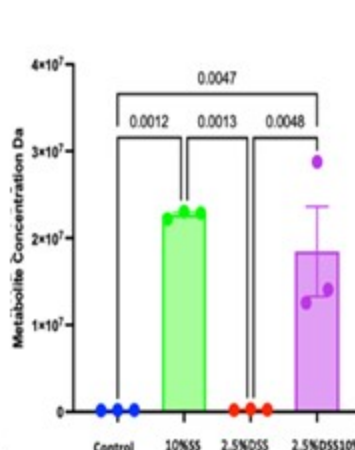
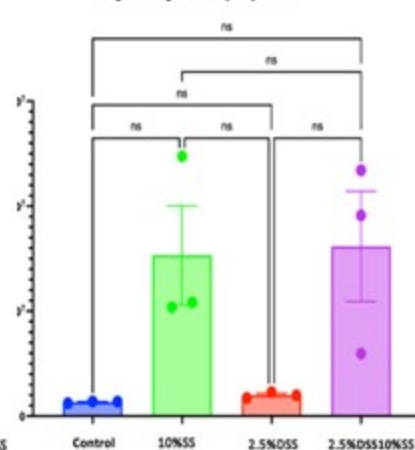
Treatment



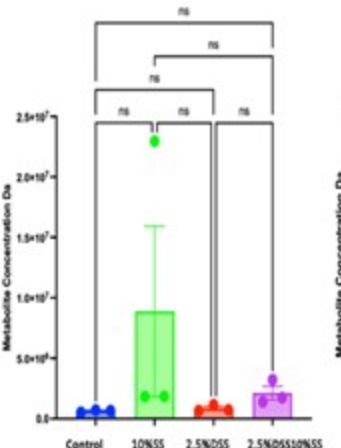
Tryptophyl-L-isoleucine



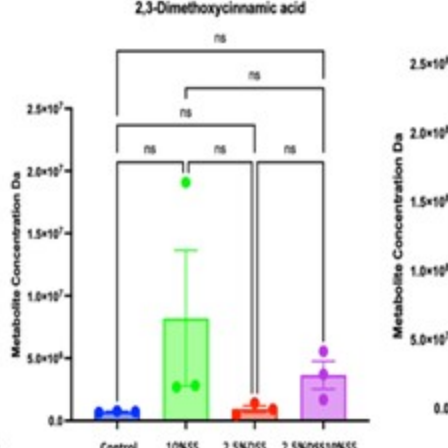
LeucylTryptophan

3-acetamido-4,5-diacetyloxy
-6-ethylsulfanyloxan-2-ylmethyl acetateIndole-Propanoic-2,3-1H-indol
-3-ylmethylaminopropanoic

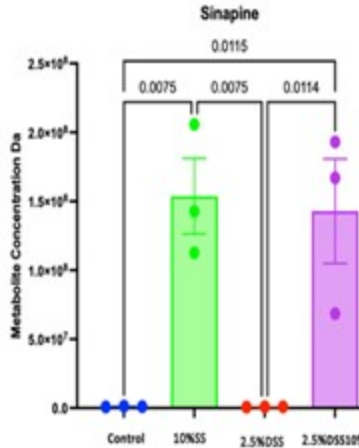
Hexadecyl ferulate



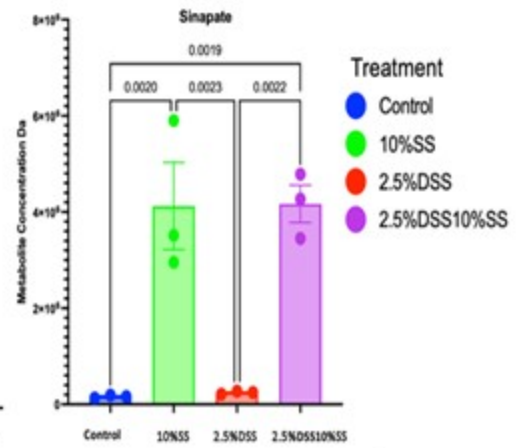
2,3-Dimethoxycinnamic acid



Sinapine



Sinapate

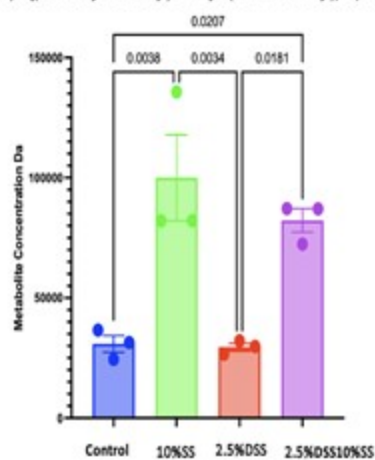


Treatment

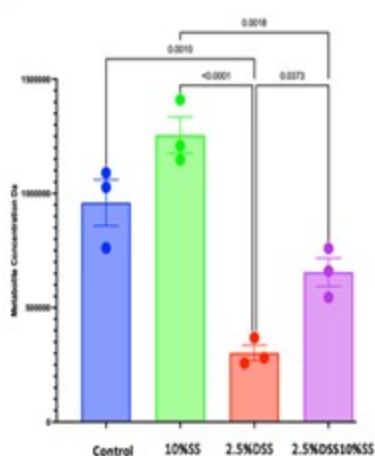


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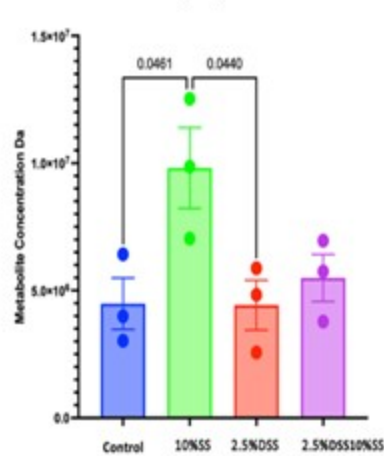
2S)-2-[(2-carboxy-2-oxoethyl)amino]-3-(1H-imidazol-5-yl)propanoic acid



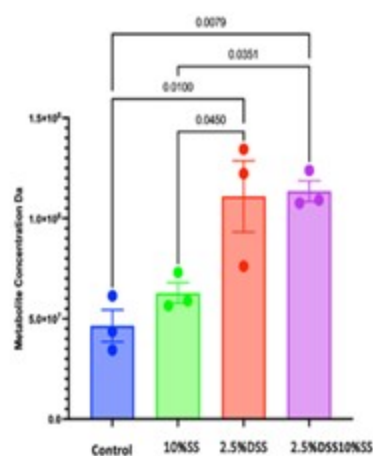
Sabiden/D-Phenylalanine



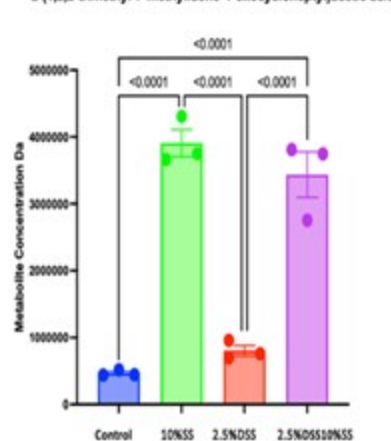
Phenylalanylserine



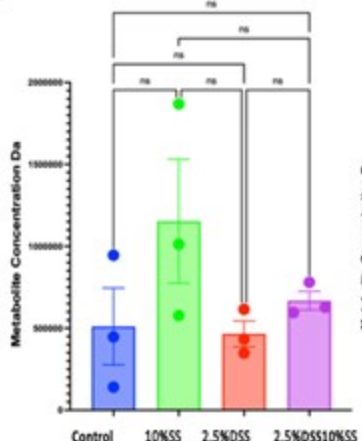
Phenylalanylproline



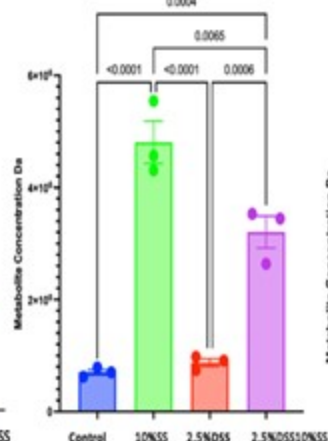
2-(1,2,2-trimethyl-7-methylidene-4-oxocycloheptyl)acetic acid



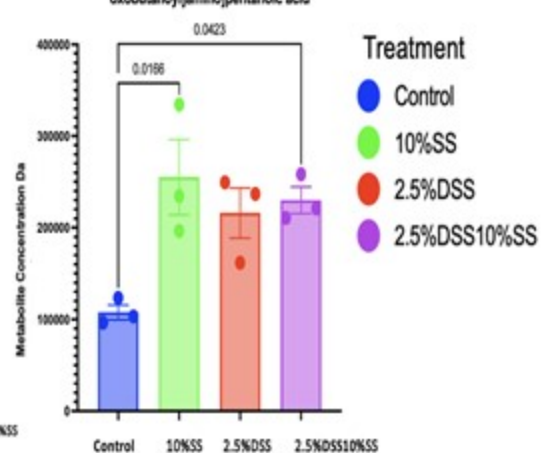
N-Oleoyl Tryptophan



2-oxindole-3-Acetate

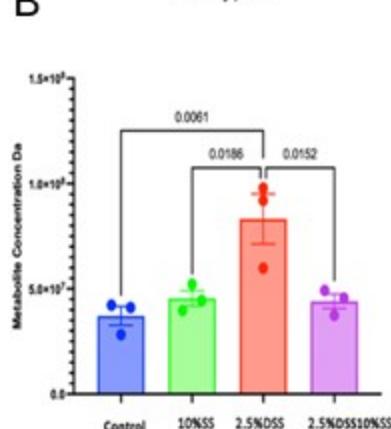


(2S)-4-(diaminomethylideneamino)-2-[(2S)-2,4-diamino-4-oxobutanoyl]amino]pentanoic acid

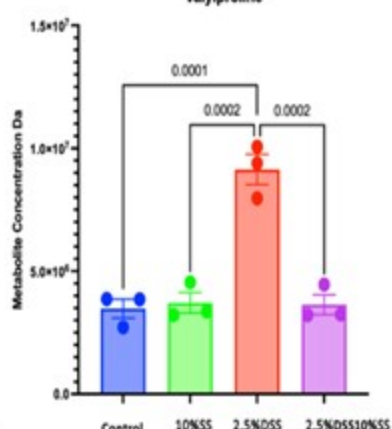


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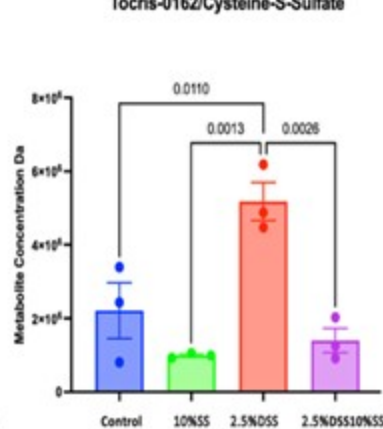
Isoleucylproline



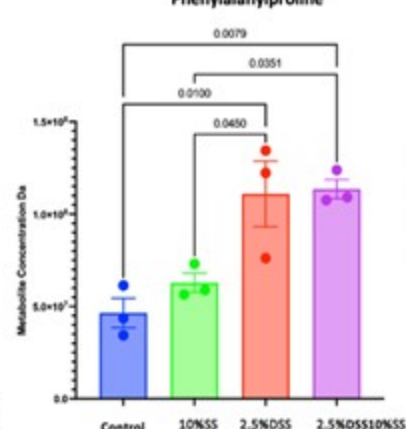
Valylproline



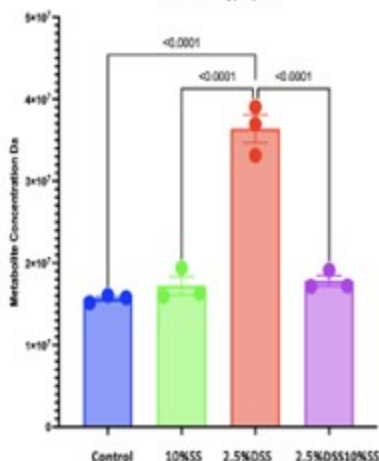
Tocris-0162/Cysteine-S-Sulfate



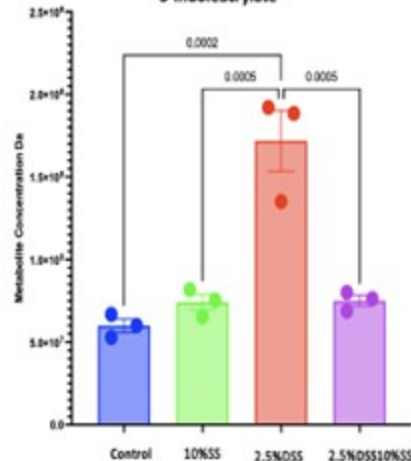
Phenylalanylproline



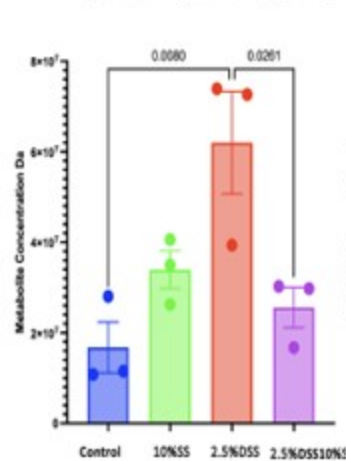
Pacitron/L-tryptophan



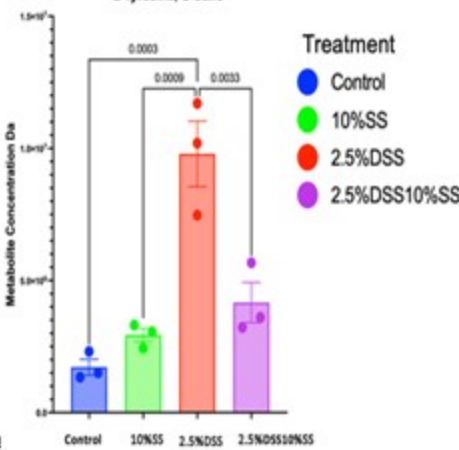
3-Indoleacrylate



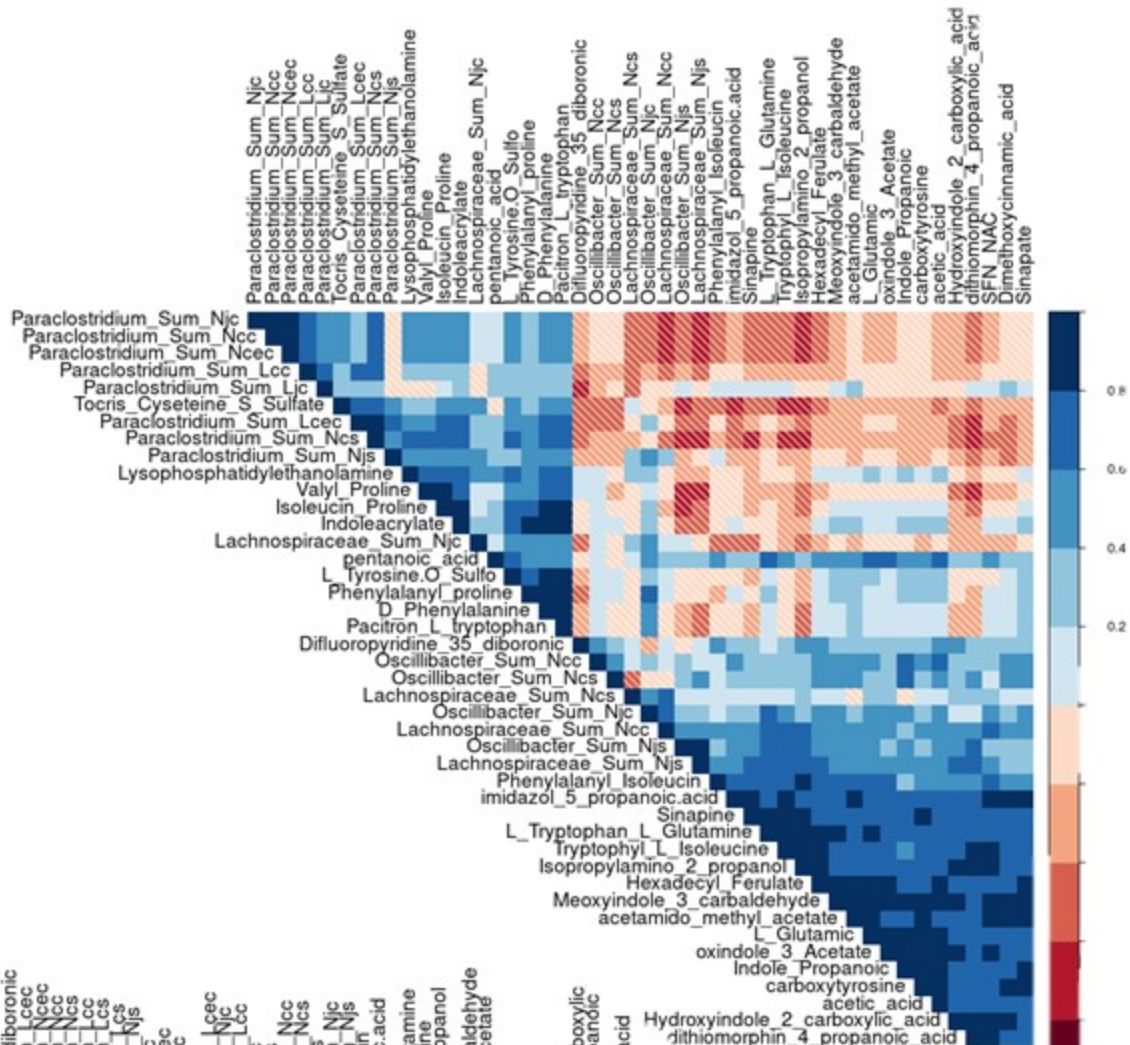
Lysophosphatidylethanolamine(15:0/0:0)



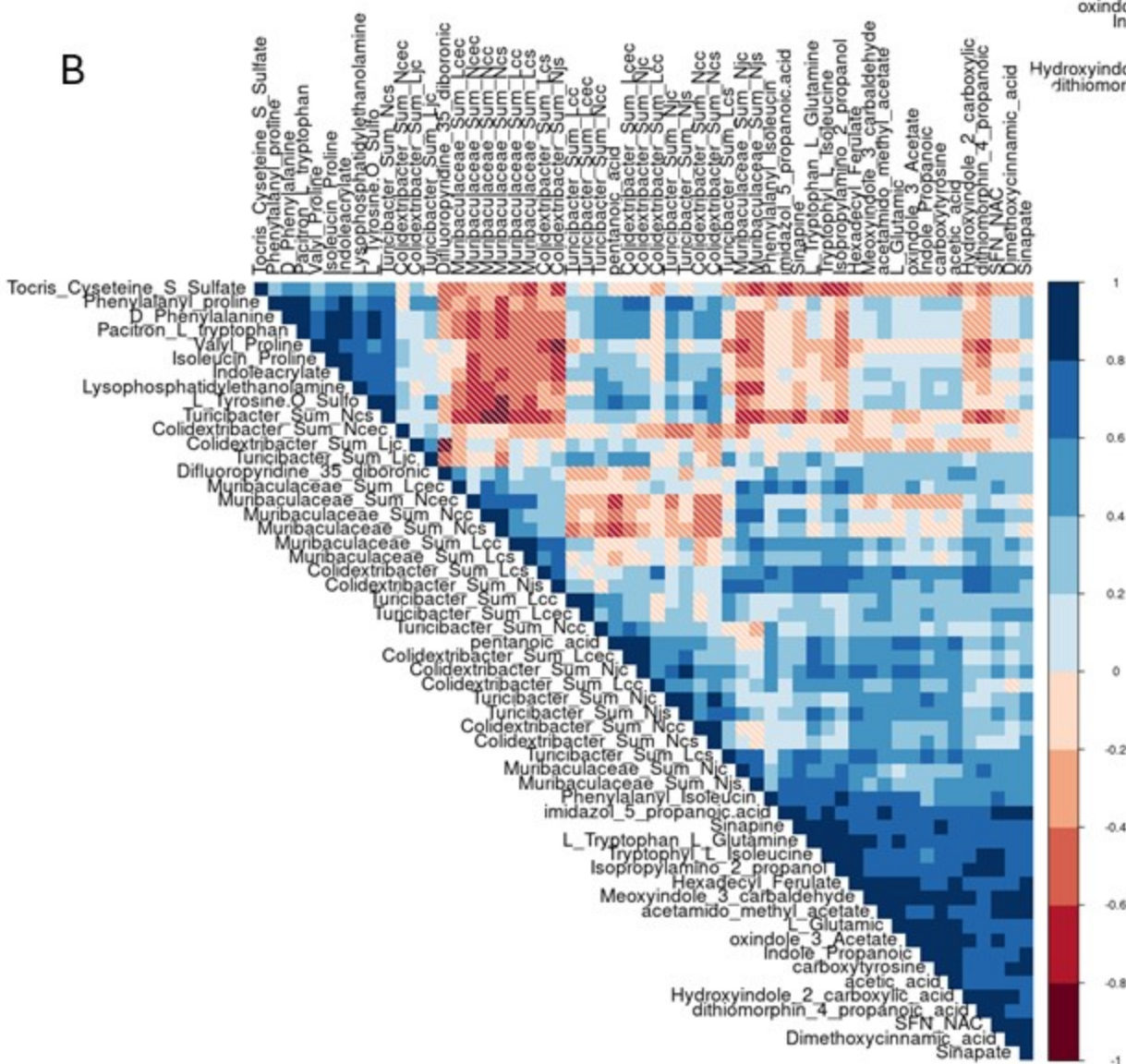
L-Tyrosine, O-sulfo-



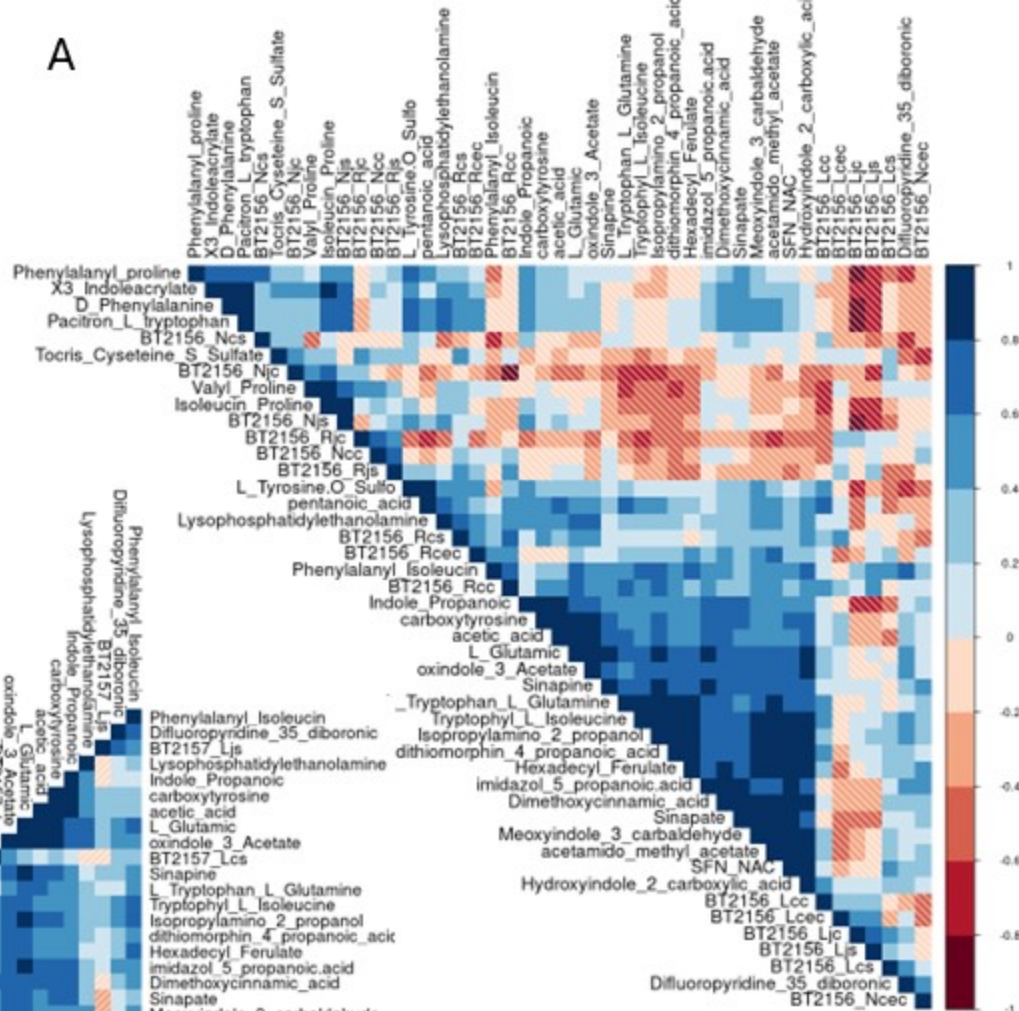
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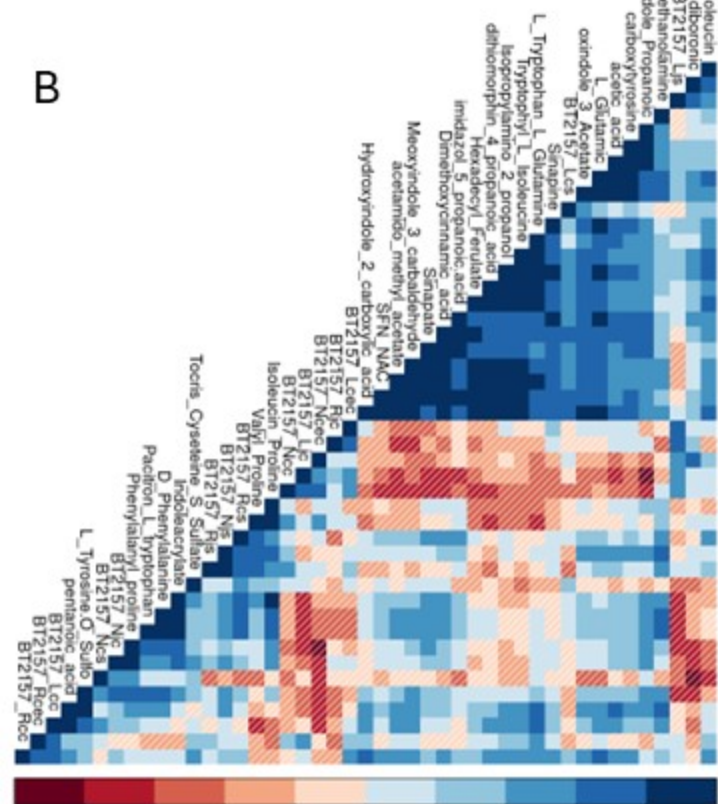
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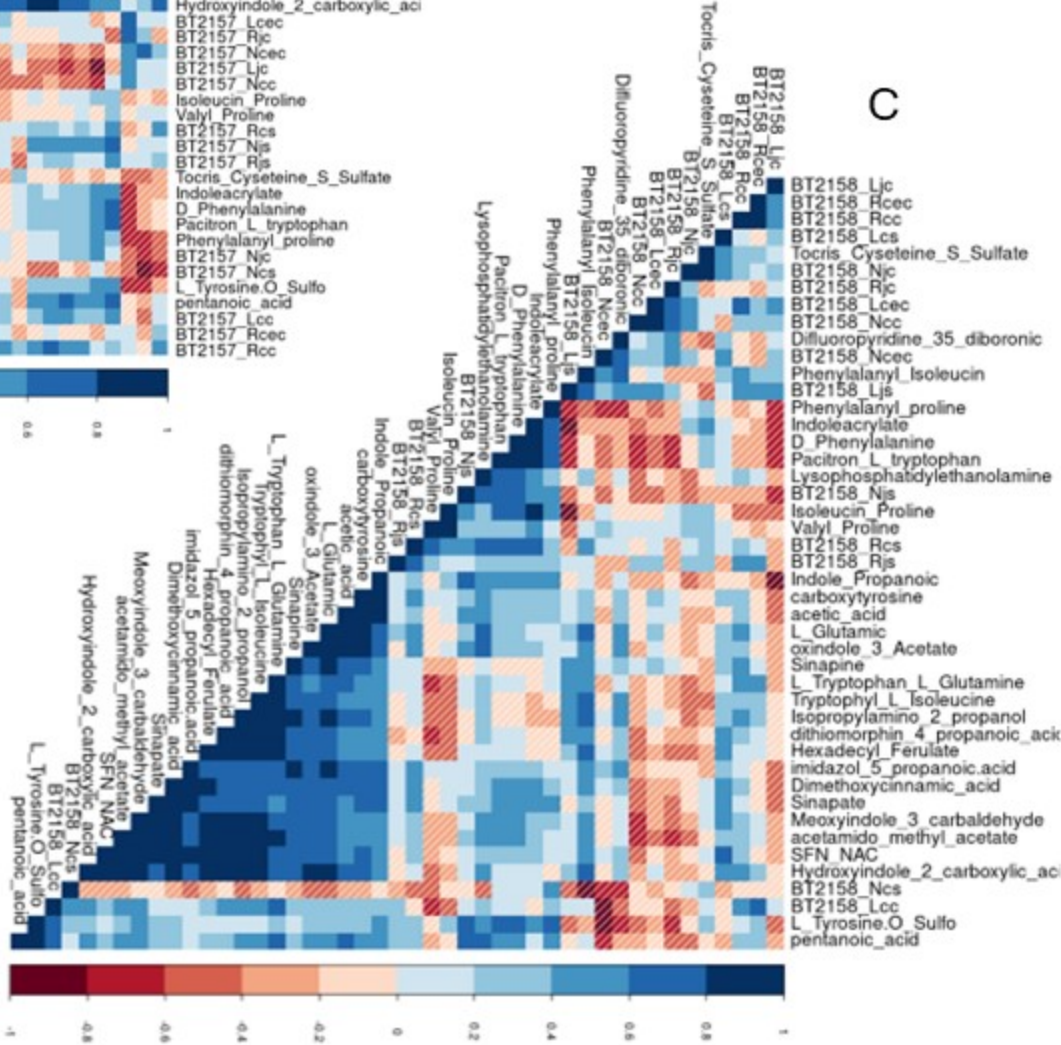
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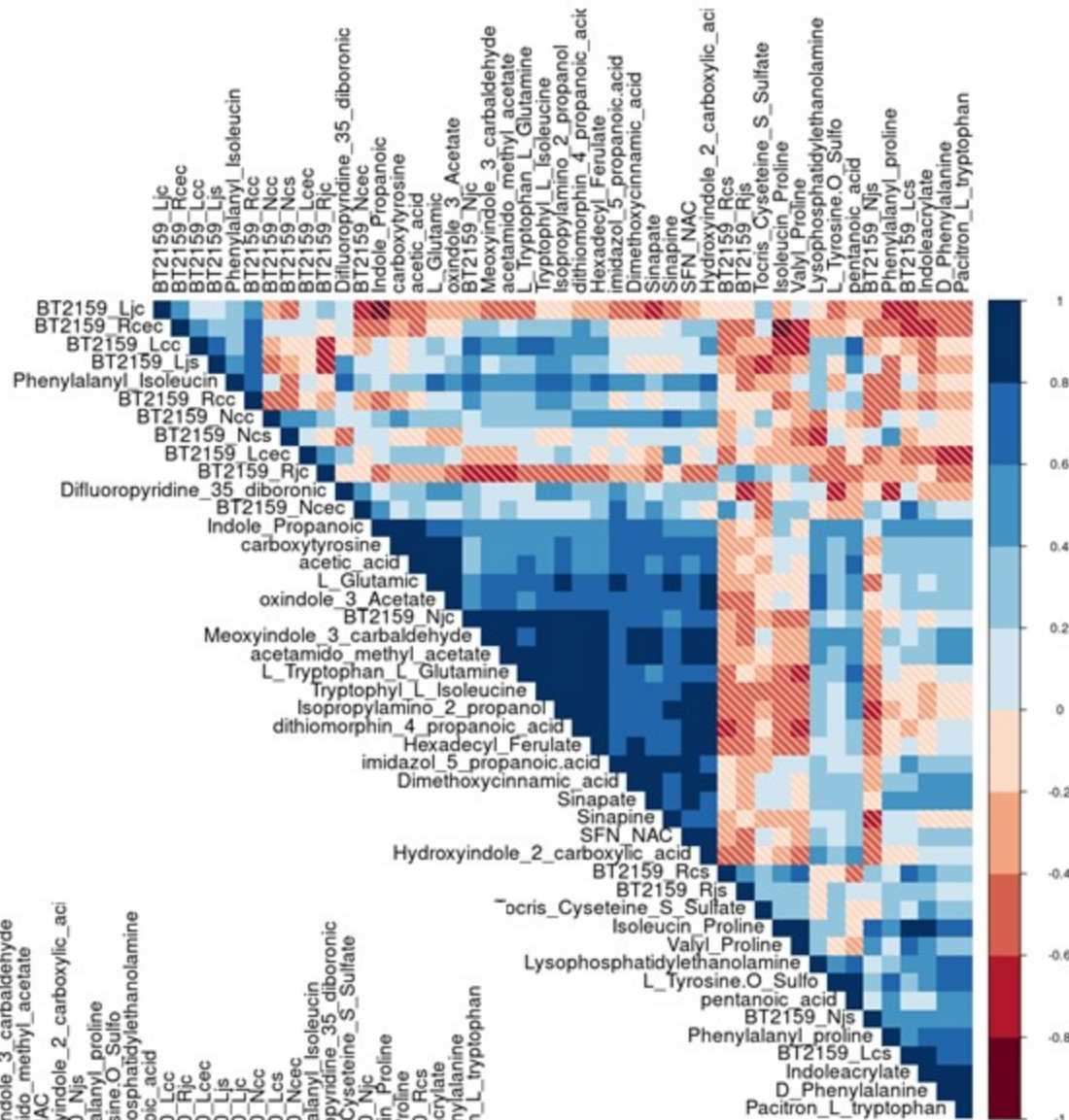
B



C



A



B

