Amelioration of DSS induced colitis through supplementation of Red cabbage juice in mice model

**Abstract**

**Objective:** Patients with inflammatory bowel diseases (IBD) such as Ulcerative colitis and Crohn’s disease lead a decreased quality of life and have an increased chance of the development of colorectal cancer (CRC). Currently, available therapies for IBD have not provided desired outcomes and often result in high toxicities. Therefore, nutraceuticals have the potential as an alternative to conventional treatment methods which have high costs and many unwanted side effects. In this study, the nutraceutical potential of pulsed electric field-assisted red cabbage juice (RCJ) was investigated *in vivo* and *in vitro* using a mouse model and cell lines, respectively.

**Design:** In a dextran sodium sulfate (DSS)-induced colitis model, C57BL/6J mice divided into two groups, group 1 treated with RCJ for 8 weeks and other group with PBS followed by two cycles of treatment with DSS along with the RCJ/PBS. Colon cancer cell lines HCT-8 and LS174T and normal colon cell line were treated with DSS for 24 h followed or preceded by RCJ to investigate the cellular mechanistic effect of RCJ using various functional assays.

**Results:** During the chronic phase in week 11, oral DSS administration led to decreased body weight along with increased diarrhea, blood in feces, disease activity score (DAI) and survival. However, supplementation of RCJ was significantly reverted back due to enrichment of butyrate producing gut microbiome like Lachnospiraceae bacterium A4, Roseburia, Flavonifactor, butyrivibrio, ASF 500 and 502 species. In addition, butyrate produced from these bacteria attenuated the increase of inflammatory cytokines (G-CSF, GM-CSF, IL-6, IL10 and, TNF-α) in DSS group. Interestingly, colonic protective mucin MUC2 and MUC4 was significantly upregulated in RCJ group as compared group with DSS alone due to enrichment of butyrate producing gut microbiota. Mechanistically, RCJ in combination with DDS resulted in decreased expression of pSTAT-3, CD3, RORγ and F4/80 in colonic epithelium compare with DSS group. Importantly, Shotgun whole genome sequence data showed that an altered gut microbiome in DSS model, which is restored by RCJ treatment. In case of *in vitro* study, increased apoptosis was due to increased reactive oxygen species (ROS) production/accumulation in the CRC cell lines upon treatment with RCJ, while the normal cell line remained unaffected. The increase in ROS was caused by RCJ interference with the glutathione anti-oxidant system.

**Conclusion:** Overall, RCJ significantly decreased DSS–induced colitis by inhibiting inflammatory cytokines and restoring the gut microflora.

**Significance of this study**

What is already known on this subject?

What are the new findings?

How might it impact on clinical practice in the foreseeable future?

**Introduction**

The incidence and prevalence of inflammatory bowel diseases (IBD) is steadily increasing worldwide1 and has become a global emergence disease. In the U.S., 3.1 million adults have been diagnosed with IBD2, and its overall prevalence is projected to continue to exponentially increase, compounding the burden3. IBD, clinically represented by Crohn disease and ulcerative colitis, is a chronic and life-threating inflammatory disease of the gastrointestinal tract (1,2). Crohn’s disease is characterized by chronic inflammation of any part of the gastrointestinal tract, has a progressive and destructive, whereas ulcerative colitis causes irritation and inflammation of the innermost lining of the large intestine and rectum leading to ulcers or sores. The symptoms of IBD is characterized by episodes of abdominal pain, diarrhea with or without mucus, bloody stools, fecal urgency, weight loss, the abnormal innate and adaptive immune responses and their interactions that results in inflammation of the intestinal mucosa and ulceration (3,4).

The etiology of IBD is largely unknown and has been proposed it allied with genetic susceptibility of the host, alterations in intestinal microbiota, other environmental factors, and immunological abnormalities (2, 5). Patient with IBD often manifests in young adulthood and represent a lifelong relapsing and remitting course that has a negative impact on health and quality of life with symptomatic characteristic as higher levels of psychiatric distress anxiety, and somatosensory amplification (6,7). Moreover, IBD patients are at increased risk for developing colorectal cancer (CRC) approximately 3-to 5 fold more compared to patients without IBD.

Current available mode of treatments for IBD patients includes anti-inflammatory drugs like aminosalicylates and corticosteroids, immunosuppressive agents such as anti-TNF-alpha agents ((infliximab, adalimumab, and certolizumab), antibiotics, biologic agents and surgery (8, 9). Unfortunately, a substantial number of patients are not fully responsive to these conventional and adjuvant therapy and showed loss of efficacy over time (10, 11). Furthermore, risk associated with these treatments; for example anti-TNF agent is associated with adverse effects, including risks of infections leading to illness such as tuberculosis and malignancies (8, 12, 13). Due to the therapies related toxicities as well as ineffectiveness of IBD standard chemotherapy, there is an urgent need to seek an alternative and effective therapeutic approaches to the treatment of IBD. In this context, research efforts were directed to understand role of gut microbiota alteration in IBD pathogenesis, by aiming to restore the gut microbiome composition through use of nutraceuticals (prebiotics) probiotics, synbiotics, and fecal microbiota transplantation as an alternative therapies to ameliorate the intestinal inflammation of IBD. Therefore, future research activities directed to focus on development of alternatives strategies such as the use of nutraceuticals that can improve the efficacy and safety of existing therapies and might be proven as milestone for IBD patients.

Recent studies have been highlighting that alteration in gut microbiota (dysbiosis) is plays an important role in the IBD pathogenesis. It acts as a metabolic organ and contributing toward the overall health of humans by performing several physiological functions, whereas dysbiosis leads to various disease, including IBD. The gut microbial flora of human is dynamic and diverse communities of commensal bacteria, Bacteriophage (viruses) and fungi, however, more than 1000 species of bacteria constitute major proportion of gut microbiota [25–27]. Moreover, the four major phyla like Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria constitute over 90% of healthy human gut bacterial species [25, 26, 28–30]. Additionally, there is a significant difference in inter-individual microbial diversity within these major phyla [31]. The gut microbiota and human host has a mutual symbiotic relationship, in which the human host provides a nutrient-rich habitat for the microbiome, while the microbiota supports the host various physiological functions to maintain a healthy state through production of short-chain fatty acids (SCFAs), vitamins, energy production and intestinal mucosa integrity of colon epithelium, and impediment of pathogenic microbes [32–36]. Further, some of the gut microbiota members plays an important role in host immune system [31, 37, 38].

From the above context, nutraceuticals consist of bioactive compounds such as polyphenols and poly/oligosaccharides (indigestible carbohydrates by host digestive enzymes) (prebiotics) from plant that provide health benefits by modulation of gut microbiome thereby decreasing disease severity without the negative side effects9. Prebiotics can promote the growth of certain beneficial gut bacterial species, [113]. 112] through their metabolism in the colon by anaerobic gut microbiota leading to production of short-chain fatty acids (SCFA) like Acetate, propionic acid and butyrate, other bacterial metabolites like vitamins, amino acids as well as gas (CO2 and H2), which resulted in decreased pH of colon favor the growth of beneficial bacteria like Bifidobacteria, Lactobacilli, and non-pathogenic E. coli, whereas inhibiting growth of Bacteroidaceae and other pathogenic bacteria [115,209]. Fermentation products such as acetic, propionic, and butyric acids of carbohydrates are involved in the activation and regulation of multiple colon-specific and systemic pathways [210]. These SCFA plays an important role in, cell energy source (acetate), cholesterol synthesis (propionic acid) and colonocyte metabolism (butyrate) [211]. Furthermore, butyrate has been associated with anti-inflammatory actions through inhibition of IL-12, downregulation of TNF-α, and upregulation of IL-10 [212].

Red cabbage (Brassica oleracea var. capitata f. rubra), is a herbaceous, biennial, dicotyledonous flowering plant has widespread use in traditional medicine due to its antioxidant, anti-inflammatory and antibacterial properties (17, 18). It is rich sources of minerals, **oligosaccharides**, and a number of bioactive substances such as favonols, glucosinolates and phenolic compounds (15, 16). Specifically, anthocyanins being the most abundant class, a major polyphenol pigment used in treatment of symptoms associated with gastrointestinal disorders and have been reported to reduce acute and chronic colitis in mouse models11. Although, conventional extraction methods include hazardous solvents or heat that can cause thermal degradation of the bioactive compounds13,14whereas, mechanical pressing, avoids the degrading processes, but results in low yields. Thus, in this study, a novel processing technique called pulsed electric field (PEF) processing is utilized to assist in the extraction of the bioactive compounds from red cabbage. PEF processing applies short, non-thermal, electric pulses to permeabilize the plant material which naturally doubles extraction volumes without damage to the bioactive compounds. The aim of this study is to determine the nutraceutical potential of the PEF-assisted red cabbage juice (RCJ) on gut microbiome dysbiosis, colonic inflammation by using dextran sodium sulfate (DSS)-associated colitis in mouse as well as colorectal cancer cell line models.

**Methods**

**PEF assisted extraction of red cabbage juice (RCJ)**

With optimized pulsed electric field (PEF) processing parameters (1.2 kV/cm, 2 μF, 25 pulses, 3.43 kJ/kg), samples of mash derived from eightred cabbages were PEF treated and mechanically pressed (450 N, 9 min). Additionally, untreated samples were also mechanically pressed from each of the eightred cabbages. The PEF treated and untreated extracts were pooled together and stored at -80°C and utilized for further bioactivity testing and biochemical assays.

**Freezing conditions**

The initial bioactivity of the PEF treated RCJ and untreated RCJ were analyzed for the total phenolic, total anthocyanins content, and the antioxidant activity. The Folin-Ciocalteu method15was used to measure the total phenolic content; the pH differential method16 was referenced for the total anthocyanins content; and the 2, 2ʹ-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS•+) antioxidant assay17 was the basis of the antioxidant activity determination. An aliquot of each of the samples frozen at -80°Cwere used for the determination of the same bioactivity components to test the effects of freezing.

**DSS-induced colitis model in C57Bl6 mice**

To evaluate the protective role of prebiotic RCJ in DSS-induced colitis, age matched either sex 60 C57/BL/6J mice were generated in house in mouse breeding colony (UNMC), these mice (either sex) divided into two groups (30 mice (15 females+15 males)/group). The first group (30 mice) mice were served as control where 200 µL phosphate buffered saline (PBS) was given as a oral gavage daily. Whereas, in the second group (30 mice), each mice was administered with 200 µL RCJ by oral gavage daily for about 8 weeks. After end of eight weeks treatment, each group was further divided into two subgroups i.e., one group given dairy 200 µl of PBS (N=15), whereas other group given with 3% DSS in sterile water plus 200 µl PBS as an oral gavage (N=15) from the first group. The group 2 mice were further divided into two groups, one group was given 200 RCJ (N=15) and other group with DSS + RCJ groups (N=15). There were four resulting groups: PBS alone, PBS+DSS, RCJ alone, and DSS+ RCJ (**Figure 1A**). Colitis was induced by administering two cycles of 3% (W/V) DSS in drinking water as described previously18. Briefly, at start of 9th week, we started one cycle of DSS/week, followed by one week recovery period without DSS, followed by a second week of DSS. During these last three weeks (9-11), the RCJ and PBS oral gavage administration continued (Figure 1A) in respective groups as described above. At end of the experiment, mice were euthanized by CO2 asphyxiation followed by cervical dislocation. **Disease severity.** All mice (60 total) were fed *ab libtum feed and water*. The diarrhea scores, blood in feces scores and body weights of the mice were monitored twice daily throughout the experiment. The diarrhea scores and blood in feces scores were recorded daily and scored from 0 to 3 (absent, mild, moderate and severe). To access the severity of the disease, the disease activity index (DAI) was calculated as previously described 19. Briefly, DAI was calculated as a combination of scores of weight loss, diarrhea, and bleeding, resulting in a colitis score of 0 to 10 (unaffected to severe colitis). Scores for body weight were calculated based on the change in body weight from the original weight (≥0%=0; -5% to 0%=1; -10% to -6%=2; -15% to -11%=3; <-15%=4). This body weight score was summed together with the diarrhea score and blood in feces score to calculate the DAI.

**Cytokine analysis**

Pro-inflammatory cytokines including IFN-γ, IL-1α, IL-1β, IL-1ra, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IL-23, and TNF-α were identified to be associated with IBD19,20,21. These cytokines were analyzed with a cytokine array kit according to the manufacturer’s protocol. (Proteome Profiler Mouse Cytokine Array Kit, Panel A, R&D Systems, Minneapolis, MN, USA). Plasma samples (100 μL/group) were pooled within each group with five samples in the PBS group and six samples in each of the other groups. The results were quantified by analyzing the pixel density of each spot on the developed film with ImageJ software (http://rsb.info.nih.gov/ij).

### Histology and immunohistochemistry

Colon tissue samples harvested from the mice were fixed in 10% buffered formalin. The samples were then processed and embedded in paraffin blocks. From each block, 4μm sections were prepared. The prepared colon tissue sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) from all treatment groups (N=6/group). The H&E stained tissues were scored based on inflammatory cell infiltration (0-3) and intestinal architecture change (0-3). The two scores were summed for a final score out of a possible 6. The PAS stained tissues were analyzed by counting the PAS positive cells. The number of cells per crypt were reported with a minimum of 20 crypts counted for each section. For immunohistological analysis, the prepared tissue sections were heated overnight and then de-paraffinized in xylene (2x, 5 min). The sections were then hydrated through graded alcohol. Then citrate buffer (0.01M, 95°C, pH 6.0) was used for antigen retrieval. Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol for 1 h. After PBS washing, sections were blocked with 2.5% horse serum (ImmPRESS kit; Vector Labs, Burlingame, CA, USA) for 2 h. Next, sections were incubated with primary antibodies and kept at 4°C for overnight. Following PBS containing 0.01% tween 20 (PBST) washing, the sections were incubated for 30 minutes with secondary antibody (peroxidase-labeled anti-mouse/anti-rabbit IgG (ImmPRESS kit, Vector Labs, Burlingame, CA, USA)). The sections were washed with PBST (3x, 5 min), then developed using DAB substrate kit (Vector Labs, Burlingame, CA, USA). Counterstaining was performed with hematoxylin. Graded alcohol was used to dehydrate the slides, followed by xylene (2x, 5 min). After drying, the slides were mounted with toluene and imaged.The following antibodies from various vendors were used for immunohistochemistry study: MUC1 (Cat. #???), MUC2 (Cat. #???), MUC4 (Cat. #???), MUC5AC (Cat. #???), TNF-α (Cat. #???), pSTAT3 (Cat. #???), CD3 (Cat. #???), RORγ (Cat. #???), F4/80 (Cat. #???), pNF-κB (Cat. #???), SOD (Cat. #???), Glutathione (Cat. #???) and 4-OH enol (Cat. #???).

**Microbiome analysis**

Mouse fecal samples were collected before experiment, during RCJ/PBS treatment, DSS treatment with/without RCJ/PBS and during necropsy aseptically and frozen at -80oC until fecal microbial DNA isolation. Microbial DNA was isolated from mice fecal samples (DNeasy PowerSoil Kit, Qiagen, Hilden, Germany). A pooled sample from before the treatments began and a sample from each group during the treatments were analyzed. From the end of the experiment, samples were analyzed from three animals from each group. The DNA samples were purified using Zymo Spin columns (Zymo Research ). Libraries (total of 17, of which 3 samples from each group) n =17) were prepared using Nextera Flex DNA Kit from illumina. The concentrations of the libraries were measured in Qubit30 using high Sensitivity kit. The quality (Size distribution) libraries were checked in Agilent 2100 Bioanalyzer. The libraries were pooled in equimolar ratio and was denatured in the presence of NaoH. The loading concentration was 1.5 pm and the sequencing was done in Nextseq NS500. It was a 150 base pair paired end run and a high output flow-cell was used. The sequencing run was monitored in Basespace ( illumina). FASTQ files for each sample were aligned to the reference genome using Diamond (*Nature Methods* **12**, 59-60 (2015)). The alignment files were processed using MEGAN 6(PLoS Computational Biology, 12(6):e1004957). The bar plots were generated based on the output from the MEGAN analysis. The groups were compared using the linear discriminant analysis (LDA) effect size (LEfSe) method (*Genome Biol. 2011 Jun 24; 12(6):R60* ). A LDA score of >2 was used to identify features that significantly discriminated among groups. The cladogram was generated based on different abundance values. The box plots were generated using Statistical Analysis of Metagenomic Profiles (STAMP) (*Bioinformatics*, 30, 3123-3124 ) analysis.

YAMP pipeline (), was used for profiling the metabolic functions of the samples directly using the short reads. All the samples had good qualities and none of the samples were discarded after the quality check and trimming step. YAMP leverages FastQC(), and MultiQC() for quality check, BBmap() for removing contamination, MetaPhlAn() for taxonomic profiling, and HUMAnN() for functional profiling of the metagenomics raw reads. HUMAnN outputs were normalized to count per million, CPM, and then MaAsLin2 was used to extract the correlations between Pathway, and gene counts with different treatments, RCE only, DSS Only, and RCE plus DSS only. Finally, a python script was used to plot the significant results of MaAsLin2. MultiQC outputs, YAMP configurations, Pipeline versions, MaAsLin R script, and the python script made for data analysis are provided in (https://github.com/chan-csu/RCE\_Megtagenomics)

**Cell culture**

The human colorectal adenocarcinoma cell lines HCT-8 and LS174T and normal colorectal epithelial cell line NCM460 were cultured in Dulbecco's Modified Eagle Medium (DMEM) media containing 10% fetal bovine serum, 100 U/mL penicillin, and 10 ug/mL streptomycin. The cells were incubated at 37°C with 5% CO2 overnight before beginning experiments. It is important to note that NCM460 was originally derived from normal human colon mucosal epithelium, the cell line has since developed some transformed characteristics22.

**Optimal RCJ dose determination by MTT assay**

Each cell line (HCT-8, LS-174T andNCM460) was seeded in 96-well culture plates with 2000 cells per well. The plates were incubated at 37°C with 5% CO2, overnight. Next day, cells were treated with RCJ at 1%, 3%, and 5% (V/V) for different time (24, 48 and 72 h) intervals.

For each time point, media was removed from each well; then, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (5 mg/mL) was mixed with fresh media (1:10) and added to each well (100 μL) and incubated for 4 h at 37°C. MTT was removed and DMSO was then added to dissolve the formazan crystals and the plates were at 560 nm and 670 nm (SpectraMax 190, Molecular Devices, San Jose, CA, USA). The absorbance values were normalized to the untreated- control.

**treatment of colorectal cancer cell lines with RCJ *in vitro***

To induce inflammation, all the cell lines were treated with 3% DSS (W/V) (DB001-32, TdB Consultancy, Uppsala, Sweden) was dissolved in fresh culture media for 24 h. After 24 h, cell lines were treated with RCJ (3% V/V) for 48 h. For the pre-RCJ treatments, DSS treatment was done for 24 h before the RCJ treatment. For the post-RCJ treatment, DSS was treated after the RCJ treatment.

**Immunoblotting analysis**

Cell lines were treated following the pre-RCJ and post-RCJ treatment schemes; along with DSS control. Protein was isolated from the cells using radio-immunoprecipitation assay buffer (50 mM Tris-HCl pH-7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) containing complete protease inhibitor cocktail (St. Louis, MO, USA) along with 1 mM PMSF, 2 mM Na3VO4, and 10 mM NaF inhibitors to lyse the cells. The samples were centrifuged at 13,000 rpm for 20 min at 4oC. The isolated proteins were denatured and separated on 10-12% SDS-polyacrylamide gel. The proteins were then blotted onto polyvinylidene difluoride membranes (Millipore, Massachussets, MA, USA). The membranes were probed overnight at 4°C with specific antibodies. They were then washed and probed with the corresponding secondary antibody. The probed bands were visualized by chemiluminescence. The primary antibodies were listed as below:phospho-cMyc (Ser62), c-Myc, glutathione peroxidase 1 (GPx-1), superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-β), nuclear factor kappa-light-chain-enhancer of activated B cells subunit p65 (NFκB p65), p53, Bcl-1 associated X apoptosis regulator (Bax), B-cell lymphoma 2 (Bcl-2), p27 KIP, and p21 Waf1/cip. β-actin was used as an internal loading control.

**Cell cycle analysis**

For cell cycle analysis, flow cytometry was performed following previously described procedures23. For 48 h, cells were serum starved. Then, the cells were treated according to the pre-RCJ, post-RCJ, and DSS only treatment schemes along with a control without treatment. Following treatment, the cells were harvested with PBS and fixed in 70% ethanol. After fixation, the cells were washed and then stained with Telford reagent, which contained 50 µg/mL propidium iodide, 90 mM EDTA, 0.1% Triton X-100, and 1 µg/mL RNase A. The DNA content was analyzed by using a FACS flow cytometer.

**Colony formation assay**

For this assay, 500 cells per well were seeded in a six well plate. Following the pre-RCJ, post-RCJ, DSS only, and control treatments, fresh media was added. After 14 days, media was removed and the cells were washed with PBS. The cells were then fixed with methanol and stained with 0.5% crystal violet and further dissolved in 10% (v/v) acetic acid, and absorbance was recorded at 590 nm 24.

**Apoptosis assay**

The cells were treated following the DSS pre-RCJ, DSS post-RCJ, DSS only, and control treatments. The treated cells were washed in PBS and then re-suspended in binding buffer andanalyzed by flow cytometry.by staining with Annexin V and propidium iodide.

**Intracellular reactive oxygen species (ROS) measurement**

The intracellular reactive oxygen species (ROS) generated in the control, DSS only, DSS pre-RCJ, and DSS post-RCJtreated cells were measured with a fluorometric intracellular ROS kit (Sigma-Aldrich, St. Loius, MO, USA). The fluorescence intensity was measured with an excitation wavelength of 640 nm and an emission wavelength of 675 nm. The fluorometric product recorded was proportional to the amount of ROS present.

**Statistical methods**

Statistically significant differences were analyzed by using Student’s t-test with a 0.05 significance level (p<0.05). Linear mixed effects models were used to analyze changes in body weight and DAI scores over time. Treatment group, time, and treatment by time interactions were included in the model. The percent change in body weight model was adjusted for baseline weight. Pairwise comparisons were adjusted for multiple comparisons with Tukey’s method. The Kaplan-Meier method was used to estimate the survival curve from induced colitis and survival times were calculated as the days from treatment initiation to death from colitis of last study date. Animals that died from causes other than colitis were treated as censored. Toxicity data was summarized descriptively over time using boxplots. SAS software version 9.4 was used for data analysis (SAS Institute Inc., Cary, NC).

**Results**

**Bioactive compounds’ retention during freezing and PEF treatment**

To understand whether PEF treatment, as well as freezing and thawing, would have any significant effect on red cabbage juice (RCJ) biological activity, we characterized different biological parameters and found that freeding had no significant effect (p<0.05) on the bioactivity of the RCJ (**S Figure 1** ). Further, there was no significant change in total phenolic content, antioxidant activity, or total monomeric anthocyanins of the RCJ (**S Figure 2)** due to freeze thawing.

Then, chemical parameters analysis was carried out using four red cabbages (total fresh weight of 3.8kg), 1.4 L of juice were obtained using a food processor and filtered with a cheesecloth. The pH of RCJ is 6.42±0.05 with dissolved solids of approximately 6.4±0.2 gm. RCJ juice contained 18.5±0.2 gms of glucose, 15.1±0.2 gms of fructose, 0.8±0.1 gms of citric acid, 3±0.3 gms of malic acid with 34.2±2.1 gms of unknown acids per liter of RCJ (Table 1).

Spectrophotometry analysis of RCJ revealed that 382.5±93.5mg of total phenolic compound and 257.7±3.1 mg of anthocyanins were present in 1 liter of RCJ (**Table 1** ). Next, we also performed HPLC-DAD method to determine the composition of polyphenols present in the RCJ, we identified that approximately 8 monomeric polyphenols were present in RCJ (**Table 2**). In addition to polyphenols, RCJ also contains 254±23.6 and 55±2.3 mg of free anthocyanins and total hydroxyl cinnimic acid per Litre of red cabbage juice. After the characterization of organic acids and sugars present in the red cabbage juices, we also observed high concentration of an inorganic acid compound.

Due to a high fraction of organic acid fraction, we further analyzed glycosyl composition (sugar residue analysis) to quantify the monosaccharide composition of polysaccharides, oligosaccharides, or glycoproteins present in RCJ by GC-MS method using TMS-derivatized monosaccharides. Our results revealed that the carbohydrates in the RCJ are mainly composed of glucose residues, with galactose, fructose, arabinose, and mannose as the minor sugar components.

**Prophylactic RCJ intervention alleviated DSS-induced colitis in mice**

To directly assess the effect of RCJ in DSS-induced colitis development, C57Bl/6 mice were divided into two groups, group one (N=30) administered PBS (200 µl) as a oral gavage, whereas group two was administered with RCJ groups ((200 µl) for 8 weeks. After 8 weeks, each group further divied into PBS alone, PBS+DSS group, RCJ alone, and RCJ+DSS group. The respective treatments were given by oral gavage daily as described in the materials and methods section (**Figure 1A**). During the experimental period, mice that received DSS alone had the highest diarrhea scores, blood in feces scores and displayed a significantly lower (p<0.001) bodyweight out of the four groups. As compared to DSS group, mice administered with DSS + RCJ group mice showed a lower disease activity index (DAI) score, hematochezia, and better colon length. The disease activity index (DAI) is usually considered to assess the severity and inflammation of the colon in clinical settings. Therefore, our DAI analysis reavealed that DSS received mice had the highest DAI scores compared to the other treatment groups (p<0.001).

We analyzed the survival benefits due to RCJ in combination with DSS, the Kaplan-Meier Survival curve revealed that greater peecentage of deaths due to severe colitis in the mice group treated with DSS compared to the other groups (p<0.001, **Figure 1B** ). whereas there is no death were observed in the PBS, RCJ and RCJ+DSS groups. Times were censored at the end of the study due to deaths unrelated to colitis, such as mis-administration of oral gavage.

In addition, the colon of DSS-administered mice was markedly shorter colon length (p<0.01) than that of PBS control (**Figure 1C ),** H&E staining moreover depicts DSS+RCJ treated mice showed increased crypts depth, Lesser hyperplasia of crypts, macroscopic spaces between crypts, submucosal edema, low epithelial cells damage and damaged brush borders (villi), reduced mononuclear cell infiltration in the submucosa and reduced colon inflammation (**Figure 1D**). Thus, it decreased the overall histology score (parameters for the histology score were 1. Architectural damage 2. Extent of inflammation 3. Chronic inflammatory infiltrate) in DSS treated mice compared to those in the DSS+RCJ. RCJ consistently alleviated DSS-induced damage to brush borders and tight junctions in the colon **TEM images** (**Figure 1E**).

**RCJ ameliorates colitis by regulating the intestinal barrier function**

From our initial data, we found disruption of crypt structure in the DSS treated mice group and improved in the case of DSS+RCJ mice group. Thus to understand how RCJ enhances the intestinal barrier function, FITC-dextran permeability was assessed and ZO-1.

First, we performed a FITC-dextran permeability assay to assess the functional consequence of tight junction protein. It was seen that DSS group showed the highest levels of FITC-dextran in the serum but reduced its level significantly in the DSS+RCJ treated group (p < 0.05) (**Figure** ). These results indicated that RCJ treatment help to regain the lost intestinal barrier function. Tight junction protein (ZO-1, Occludin, and Claudin-1) plays a vital role in maintaining the intestinal barrier function,and once such essential protein which is decreased at protein levels is,tight junction scaffolding protein zonula occludens-1 (ZO-1) in colitis. Thus we checked for ZO-1 protein expression by IHC and found that ZO-1 makes critical, a tight junction was less in DSS group, and while DSS+ RCJ group had significantly elevated (p < 0.001) (**Figure** ).

Previous reports have shown that ZO-1 down-regulation may be one cause of ineffective mucosal healing in patients with IBD (**34478742**).

The H&E staining (Figure ) also shows that the PBS control group had displayed intact colonic mucosa, crypts, stroma, and submucosa, with no inflammatory cells infiltrated in submucosa and ulceration. While the DSS group showed intense, severe epithelial cell damage, submucosal edema, inflammatory lesions, including shortening and hyperplasia of crypts, macroscopic spaces between crypts, and severe inflammatory cellular infiltration in the submucosa. Nevertheless, the treatments with RCJ notably ameliorated the colon inflammatory symptoms, including less inflammatory cell infiltration, submucosal edema relative, intact surface epithelium, normal crypt glands, and mild submucosal edema; the condition of the colon was close to that of PBS mice. Thus, to assess the effect of RCJ on the colonic mucosal barrier, mucin-secreting goblet cells in the colonic epithelia were measured using Alcian blue staining. Where the DSS s treated, group showed a significant reduction in the thickness of colonic epithelial mucosa, which was attenuated by RCJ to near normal (**Figure** ).

Further, we checked for specific mucins (MUC1, MUC2, MUC4, and MUC5AC), to understand the relation between mucin, RCJ, and DSS-induced colitis. Mucins are an important player in protecting the gastrointestinal tract to eliminating bacterial toxins. Recent studies are suggesting that mucins are involved in the initiation of inflammatory bowel disease that would lead to cancer progression (**21913981**). In the present study, we observed that DSS treatment showed a reduction in the MUC1, MUC2, and MUC4 expression, whereas upregulation of MUC5AC expression (**Figure** ). However, supplementation of RCJ showed a significant (p<0.001) increase in MUC1, MUC2, and MUC4 expression (**Figure** ). Thus our data corroborated that the protective role of RCJ in DSS-induced mice associated with a modulatory effect on mucins such as increased expressions of MUC1, MUC2, and MUC4 and decreased MUC5AC.

The healthy epithelial cell is one of the key factor for enhanced intestinal barrier function. Proliferation and apoptosis are the key two factors in determining healthy epithelial cells. DSS induced epithelial cell apoptosis, which is demonstrated by reduced terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positive nuclei in the colonic epithelium (**Figure)**. The DSS+ RCJ group significantly reduced TUNEL positive nuclei. Further, the colonic epithelial cell proliferation was assessed by Ki67 staining, where DSS+ RCJ group showed a significantly higher number (p < 0.05) of Ki67 cells positive when compared to DSS group (**Figure)**. The above overall results depict that RCJ could improve the integrity of the intestinal epithelial barrier, reduce intestinal permeability and restore intestinal barrier function.

**Prophylactic RCJ intervention alleviated DSS-induced** oxidative stress in intestinal mucosa, **colonic proinflammatory status.**

To assess the influence of RCJ on oxidative stress, total superoxide dismutases (T-SOD), catalase (CAT), and glutathione peroxidase (GSH-px) in the colon tissue were measured. As compared to the DSS group, DSS+RCJ group suppressed concentrations of CAT, T-SOD, and GSH-px with colitis compared with PBS controls were attenuated mainly by RCJ (**Figure** ). Taken together, these results showed that RCJ treatment markedly ameliorated DSS induced colitis.

To illuminate how RCJ remitted the colitis severity, we quantified the expression of pro- and anti-inflammatory cytokines and chemokines using a cytokine array (IL-1α, IL-1β, IL-3, IL-6, IL-10, IL-16, IL-17, IL-23, IL-27, CCL1, CXCL-1, CXCL-9, CXCL-10, CXCL-11, G-CSF, GM-CSF, TNF-α, IFN-γ). We observed that DSS-treated mice had elevated levels of several crucial pro-inflammatory cytokines like TNF-α, IFN-γ, IL-1β, and IL-6 and chemokines like CXCL1, CXCL 9- 11 (**Figure** ). In contrast, both RCJ and DSS+RCJ treatment showed significantly (p>0.005) lower levels of various pro-inflammatory cytokines and chemokines. Further, iNOS and COX 2 were also reduced in both RCJ and DSS+RCJ treatment groups (**Figure )**. These experimental results suggested that RCJ can diminish the pro-inflammatory cytokine response in DSS-induced colitis mice.

**Prophylactic RCJ intervention modulates microbial diversity and regulates microbiota structural segregation in mice, and alters gut microbiota metabolites (SCFAs) production in DSS-induced colitis.**

Further, to understand the release of inflammatory cytokines and chemokines, which could be due to abnormal gut microbiota composition, this would induce an altered immune response, including the release of inflammatory factors and the aggregation of inflammatory cells. In our chemical parameter analysis, we observed oligo- and poly-saccharide content in the RCJ, so we evaluated the prebiotic effect of RCJ on the gut microbiome with and without DSS treatment. We thus performed 16S rDNA sequencing to examine the alteration of gut microbiota (contents of three randomly selected mice from each group before treatment, during the first cycle of DSS treatment as well as at the time of euthanasia) using cecum contents collected at the end of the experiment.

Followed shotgun metagenomic sequencing was performed.

First, we measured the effect of RCJ administration on colon microbial alpha diversity using Shannon indices. It is a measure of community diversity at the species level in the colon. We observed that treatment with DSS resulted in a decrease in alpha diversity (**Figure** ). The existing carbohydrates/poly-saccharides in RCJ could be responsible for promoting higher alpha-diversity observed in subjects treated with RCJ and RCJ + DSS. In total, 1716787 quality-filtered amplicon sequences and 2662 ASVs were obtained from 35 samples.

we performed non-metric multidimensional scaling (NMDS), with Bray-curtis as a distance measure, to see how the different treatments affected the gut microbial community composition. The two optimal axes NMDS1 and NMDS2 show a clear separation between the subjects of each treatment significantly. Moreover, RCJ with DSS group are between DSS group and control group. This means that subjects treated with RCJ after being treated with DSS, have a microbiome more similar to the control subjects than subjects that are treated with DSS. As a result, RCJ has effectively ameliorated the effects of DSS treatment (**Figure** ). A number of 171 ASVs were present in all groups, but some ASVs were particular to a specific group (256 in the control group, 459 in the DSS group, 205 in the DSS+RCJ group, 410 in the RCJ)

Further, we analyzed the impact of RCJ on both caecal mucosa and caecal luminal microbiota composition by MEGAN analysis. Our analysis revealed increased abundances of phylums Bacteroidetes, Chlamydiae, Chordata and decreased Firmicutes at phylum level in DSS treated group of mice compared with PBS, RCJ, and RCJ+DSS groups. In case of RCJ in combination with DSS group, we observed an increased abundance of Firmicutes, Deferribacteres phylum with decreased Bacteroidetes, and chordata (**Figure** ). Our data analysis also has shown that increased Firmicutes-to-Bacteroidetes (F/B) ratio, which is indicative of high SCFA production in RCJ+DSS group in comparison to DSS group. Therefore, our results revealed that RCJ treatment impacted both caecal mucosal-associated and luminal microbiota composition at phylum level.

we analyzed metagenomic data at the family level, we observed increased abundances of families like Bacteroidaceae, Prevotellaceae, Rikenellaceae, Helicobacteriaceae, Enterobacteriaceae, Chlamydiaceae. In contrast, decreased Clostridiaceae, Eubacteriaceae, lactenospiraceae Oscilliospiraceae families in DSS treated group of mice compared with RCJ+DSS group. Our results clearly show that administration of RCJ in combination with DSS resulted in increased enrichment of butyrate-producing families Eubacteriaceae, lactenospiraceae, Oscilliospiraceae, and Clostridiaceae (**Figure** ). Our data analysis revealed that treatment of RCJ in combination with DSS resulted in increased enrichment of butyrate-producing genus like Dorea, Kineothrix, Lachnocostredium, Roseburia, Oscillibacter, Eubacterium, and Faecalibacterium(**Figure** ).

Further, MetaStat analysis revealed that RCJ across the different groups significantly reversed bacterial abundance. In the DSS group, we saw a significant increase in Chlamydia abortus, and Bacteroides bacterium 52\_46. In contrast, there was a decrease in Oscillibacter 1-3, Dorea 5-2, Lachnospiraceae bacterium 10-1 and Acetatifactor muris compared with RCJ+DSS group (**Figure** ). In case of RCJ alone treated group, there is a significant abundance of Muribaculum intestinal, and Bacteroides CAG927 compared to other groups. In contrast, RCJ treatment resulted in the correction of these bacterial groups, which may contribute to the re-establishment of intestinal equilibrium

LEfSe analysis was performed to detect bacterial organisms differentially abundant among the four treatment groups. We considered species with a significant difference when the LDA score was greater than the default score of 4.0. The histogram length represents the LDA score, which indicates the degree of influence of species with significant differences between different groups. The biomarker demonstrating substantial differences between the RCJ+DSS group and the other three groups were Lachnospiraceae, Oscillibacter, Dorea, Roseburia, whereas Provotella\_CAG485 Clostredium sp \_CAG557, Lachnospiraceae,bacterium A2 were the dominant phylotypes detected in the RCJ group (**Figure** ). While Bacteroidaceae, Bacteroides, Bacteroides sartori, Romicoccus were the dominant phylotypes seen in the DSS group contributing to the differences between the intestinal microbiota of the control and DSS groups (**Figure** ). While the phylum Trichomonas is more predominant in the control group (**Figure** ).

Further, we also performed LefSe analysis to reveal the significant ranking of abundant taxa in all four treatment groups. The cladogram showed significant differences in 106 taxa’s among four treatment groups (PBS, RCJ, DSS and DSS+RCJ) (**Figure**). Red, green, blue, and purple indicate different groups, with the species classification at the phylum level, class, order, family, and genus shown from the inside to the outside. The red, green, blue and purple nodes in the phylogenetic tree represent microbial species that play an important role in the PBS, RCJ, DSS and DSS+RCJ groups, respectively (**Figure** ). Yellow nodes represent species with no significant difference. At the species levels, *Roseburia intestinalis, Roseburia hominis, Faecalibacterium prausnitzii,*are enriched in DSS+ RCJ group compared to DSS alone.*, Lactobacilli johnsonii ,* and *Bifidobacteria pseudolongum*  were all the bacterias that are increased in RCJ group showing specificity to RCJ treatment..

SFA - Microbial products were beneficial to host health, among which SCFAs improve inflammation to maintain colonic health, resulting in attenuating IBD pathogenesis. As shown in (**Figure** ), the contents of butyrate, propionate, and acetate in the DSS group were 15.20, 4.75, and 2.29 μM/g, respectively. However, all RCJ treatments significantly improved the contents of butyrate, propionate, and acetate in colitic mice (p < 0.001). Similarly, microbiota transplantation with RPTtreated mouse feces significantly increased acetate, propionate, and butyrate in comparison with the DSS group (**Figure** ). The above results showed that RCJ could increase the contents of SCFAs in DSS-induced mice, which in turn might promote the anticolitis properties of RCJ.

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Spearman correlation analysis was further performed to understand the association between differentially enriched microbes and anti-oxidative, inflammatory parameters, or SCFAs profiles

Pathway analysis

**RCJ reversing the dysregulation of immunological responses in DSS-induced colitis mice.**

As we saw, the release of the pro-inflammatory cytokines was reduced when treated with RCJ and thus, the overall inflammation was also reduced, which was induced by DSS. We wanted to understand the role of the immune cells in regulating these events. First we checked for two major immune cells that are macrophages and T cells population, which were stained by F4/80 CD3 markers and found that both of these were elevated in DSS groups, and interestingly, the DSS+RCJ both showed significantly (p>) lower macrophages and T cell populations (**Figure** ) .

Due to intestinal barrier function lost in DSS treated animals, loss in tight junction protein and epithelial cell damage would lead to leakiness. This, would allow microbial translocation (lipopolysaccharides (LPS) from gram-negative bacteria), and other bacterial endotoxins will be released from the colonic lumen to lamina propria.. These events trigger the maturation of T helper 17 (Th17) and recruitment of neutrophils in the lamina propria, which aggravates oxidative stress and secretion of G-CSF, which was confirmed with our cytokine array analysis. G-CSF stimulates the bone marrow to produce more neutrophils. Thus, ROR-g a specific marker for Th 17 T cells subtype and MPO specific marker for neutrophil cells were satined. We found that the DSS group had a higher ROR-g and MPO expressing cell population when compared to the DSS+RCJ group, which signifies (p>) that RCJ treatment could reduce Th17 T cell maturation and neutrophil recruitment (**Figure** ).

Previous studies have shown that colonic Foxp3+ regulatory T cells (Tregs), which are anti-inflammatory subsets of CD4+ T cells that maintain immune homeostasis. Thus, we checked for the Tregs population in the colonic region and found that the DSS group had a significantly lower Foxp3+ Treg cell population when compared to the DSS+ RCJ group representing that RCJ could increase the T reg levels, which is crucial for immune cells as its secrets IL10, a vital anti-inflammatory cytokine.

Further, the LPS can also cause inflammation by activating TLRs, on the macrophages present in the lamina propria. This, in turn, activates the NFkB pathway, NF-κB is a well-characterized, ubiquitous transcription factor and primary mediator of the inflammatory response during inflammation and increases pro-inflammatory cytokine levels TNF-α, IL6, IL-1β, and COX2 levels in these macrophages. Increases in TNF-α act as autocrine signaling for activation of NFkB, and an increase in IL6 causes activation of the STAT3 pathway. The pNFkB, TNF-α, and pSTAT3 levels were increased in the DSS group while it was significantly (p>) reduced in the case of the DSSS+ RCJ group (**Figure** ). The overall results show that RCJ treatment inhibits the recruitment of immune cells and lowers the inflammation in DSS-induced colitis.

**Discussion**

Inflammatory bowel disease (IBD) in humans, including Crohn's disease and ulcerative colitis, is a complex chronically relapsing inflammatory disorder of the gastrointestinal tract. Current IBD treatments have poor tolerability and insufficient therapeutic efficacy, therefore, several alternative therapeutic approaches are being explored. Recently, a number of dietary supplements have emerged as promising interventions. Red cabbage contains abundant bioactive compounds such as anthocyanins, is one of the most abundant polyphenols in the human diet and has been shown to inhibit inflammatory responses in intestinal cells.  10,12.

Minimal differences in bioactivity (phenolic content, anthocyanin content, and antioxidant activity) were observed between the RCJ with PEF treated and the RCJ without PEF treatment. Further, there was no difference in the bioactivity of the RCJ after the freeze/ thaw cycle. Due to this retained bioactivity and the increased extraction yields with PEF treatment, the RCJ prepared by PEF treatment was utilized to evaluate the nutraceutical value in preventing DSS induced colitis model.

Intestinal dysfunction may affect the absorption of nutrients and, consequently, body weight, colon shortening and led to colon cancer. This study evaluated the mitigating effects of RCJ on colon damage and the bacterial profile in a mouse model of dextran sulfate sodium (DSS)-induced colitis. The oral administration of RCJ markedly ameliorated dextran sulfate sodium salt-induced mouse colitis, as demonstrated by a reduction in weight loss, the higher survival rate and the severity of bleeding. In addition, mice were shown to recover when continuing to receive RCJ during the rest period after the first cycle of DSS. The recovery was marked by lowering of the diarrhea and blood in feces which indicated that RCJ may have beneficial effects on treatments for colitis. This preliminary study indicated that RCJ may function synergistically with probiotics to provide a novel and effective strategy to prevent colitis.

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The DAI is a good indicator of the severity of disease, and it is comparable to clinical representation of IBD19. Treatment with RCJ showed reduction in the disease severity in DSS group. Notably, the treatment group that received RCJ followed by the DSS treatments did not show colon length shortening effect where the reduction of colon length is a classic indication of experimental colitis27... toAlthough, reports showed that immunoregulatory compound such as recombinant Sj16  (rSj16) and anti-inflammatory drug sulfasalazine has protective effects on DSS-induced colitis, but the limitation was observed. In study, by using rSj16 s28.the treatment had some impact on DSS-induced colitis in mice, but it did not return the animal to normal and by using sulfasalazin in combination with herbal medicine it offered a better response for DSS-induced ulcerative colitis mice for human UC than sulfasalazine aloneRCJresults28. and ce29. In current study, the RCJ showed more effective response, attenuated clinical activity i.e., the colon length shortening, decreased body weight and the higher DAI of DSS-induced colitis mice than the immunoregulatory compound and anti-inflammatory drug.

Cytokines are small peptide proteins produced mainly by immune cells, wherein they act as key pathophysiological regulators that governs the occurrence, development and, ultimately led of inflammation that characteristic features of IBD. n19. In IBD, the innate immune response plays a pivotal role and DSS-mediated stimulation of - pro-inflammatory cytokines are involved colitis30,19.,In our study, we were able to demonstrate that DSS supported this trend by increasing many pro-inflammatory cytokines and RCJ administration attenuated these inflammatory response. Furthermore, H&E staining revealed the greatest inflammatory cell infiltration, intestinal architectural change, a loss of villi and necrosis of intestinal surface in the DSS- treated mice.Whereas. both control and RCJ groups did not show such kind of effect.Although the supplementation of RCJ had some loss of villi, but not as significant as the DSS group. Similarly, the PAS staining showed the greatest loss of mucins in the DSS treated group while other groups retained the mucin with increase expression of Muc2 that suggested RCJ has a protective role against inflammatory colitis in mice model.

**Next**, inhibitory effect of RCJ on the cellular proliferation of colon cancer cells without affecting normal colon cells was observed This inhibitory effect was associated with stimulation of apoptosis. Furthermore, RCJ –treated colan cancer cells showed increased ROS production, as it is well known ROS production is a hall mark of apoptosis, therefore, it is clearly evident that RCJ –induced apoptosis is ROS –dependent apoptosis. n. It was also evident that CRC cell lines are more sensitive to ROS accumulation compared to normal cells, 31 resultant of Warburg effect 32. Hence, increased oxidative stress selectively kills cancer cells while not affecting the normal cells. Several findings have been shown that anthocyanins has ability to induce apoptosis by increasing production/accumulation of ROS in various *in vitro* CRC cell lines such as Caco-2, LoVo, drug-resistant LoVo/ADR, and leukemic cell line HL-6033,34,35. Here, we observed the increased expression of antioxidant enzymes (SOD1 and SOD2) and down-regulation of GPx-1 in the CRC cells but not in the normal cell line after treatment with RCJ. Previous studies have been proposed, that anthocyanins mediated apoptotic pathway interfere with the glutathione antioxidant system35,36. With this view, inhibition of GPx-1 by RCJ treatment supports further accumulation of ROS 37Therefore, these results corroborated that RCJ is acting as a pro-oxidant and leading to ROS production and it is also interfered with the glutathione antioxidant system..Moreover, we observed that accumulation of ROS directed to upregulation of the p53 and modulation of the Bcl-2 family of proteins, and eventually led to apoptosis(**Supplementary Figure 4B**)

In conclusion, RCJ was shown to have potential as a nutraceutical for colitis and IBD treatment. Our data also showed that administration of RCJ restore the altered gut microbiota. *In vitro*, RCJ acted through ROS-induced apoptosis and interfered with the glutathione antioxidant system in CRC cell lines.

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**Funding**

Funding was provided by the Nebraska Research Initiative and the National Science Foundation Graduate Research Fellowship Program under Grant No. 1610400.