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Maize near-isogenic lines with enhanced flavonoids alleviated dextran sodium sulfate-induced murine colitis via modulation of the gut microbiota

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Abstract

The rising incidence of inflammatory bowel disease（IBD）necessitated the search for safe and effective novel therapeutic strategies. Dietary flavonoids displayed antioxidant, antiproliferative, and anticarcinogenic activities in several model systems with proven abilities to reduce inflammation and oxidative stress, thus they could be promising therapeutic agents for IBD prevention/treatment. However, understanding the role of a specific class of compounds in foods that promote health is difficult because of the chemically complex food matrices. This study aimed to utilize four maize near-isogenic lines to determine the anti-colitis effects of specific classes of flavonoids, anthocyanins and/or phlobaphenes, in a whole-food matrix. Results showed that intake of anthocyanins and phlobaphenes enriched maize diets effectively alleviated dextran sodium sulfate (DSS)-induced colitis in mice via reducing intestinal permeability and restoring barrier function. Anthocyanin diets were more effective in maintaining crypt structure, muc2 protein levels and reducing inflammation. Bacterial communities of mice consuming diets enriched with anthocyanins and phlobaphenes were more similar to the healthy control compared to the DSS control group, suggesting the role of flavonoids in modulating the gut microbiota to retrieve intestinal homeostasis. Microbiota depletion rendered these compounds ineffective against colitis. Lower serum concentrations of several phenolic acids were detected in the microbiota depleted mice, indicating that gut microbiota plays a role in flavonoid metabolism and bioavailability.

Keywords

flavonoids; anthocyanins; phlobaphenes; gut bacteria; antibiotics; inflammatory bowel disease

1. Introduction

IBD encompassing Crohn's disease and ulcerative colitis, represents chronic relapsing disorders of the gastrointestinal (GI) tract that require sustained surgical and medical interventions. The burden and incidence of IBD are on the rise, with 3.1 million cases reported in the US alone in 2015 and 6.8 million IBD cases reported globally in 2017 [1, 2]. However, precise prophylactic or therapeutic strategies have yet to be provided. Current IBD therapies have focused on interfering with the mucosal immune response, however, the use of immunosuppressants and biologicals can be accompanied by deleterious side effects [3]. The search for IBD treatments is challenged by the incompletely understood pathogenesis, which involves both environmental and genetic factors. A large body of evidence has identified gut microbiota as one of the key pathogenic players. Shifted microbial ecology, or dysbiosis can lead to an aberrant mucosal immune response that triggers or potentiates inflammation [4, 5].

Flavonoids are a large subgroup of phenolic compounds in the plant kingdom. They possess antioxidant activities and immunomodulatory properties to suppress inflammation [6]. Moreover, recent evidence has furthered our understanding of the prebiotic power of dietary flavonoids. Studies reported that flavonoid administration significantly increased the abundance of probiotics *Lactobacillus, Bifidobacterium,* and *Akkermansia* [7, 8]. In turn, gut microbiota could impact the bioavailability of flavonoids by exhibiting a diverse spectrum of functions in the bioconversion of flavonoids including deglycosylation, oxidation, demethylation, and catabolism of flavonoids into phenolic acids [9, 10].

Growing evidence has associated flavonoid intake with the alleviation of experimental colitis[11-15], with the majority of the studies using either whole-food or purified flavonoids as a source. Albeit observations of some protective effects, the complexity of the food matrix makes it difficult to pinpoint the contributions of any single component. On the other hand, isolated compounds, as opposed to their naturally occurring forms, may have altered chemical structures that could influence their bioavailability. One of the ways to address this complexity is to use isogenic, or near-isogenic, food materials [16, 17]. By standardizing the genetic backgrounds, these food materials differ only in a single gene or a few genetic loci, which allows the examination of a specific class of phytochemicals in a whole-food context with minimized background effects [18].

In this study, we utilized four maize near-isogenic lines (NILs) and conducted two independent animal experiments (conventional and antibiotics treatment) to assess the anti-colitis effects of maize phlobaphenes and anthocyanins within a whole-food matrix and the role of gut microbiota against dextran sodium sulfate (DSS)-induced colitis. For the NILs used in the present study, line A lacks both anthocyanins and phlobaphenes and shows colorless kernel pericarp (the outer layer of the ovary wall) and aleurone (the outermost unicellular layer of the endosperm); line B has a high accumulation of phlobaphenes in the kernel pericarp; line C has anthocyanin accumulation in the kernel aleurone layer; line D has an accumulation of both phlobaphenes in pericarp and anthocyanins in aleurone (Fig. 1A). Major anthocyanins detected in lines C and D were cyanidin and pelargonidin [19]. Purified diets with 25% supplementation of the four NILs respectively were used as feeding materials in the animal experiment for nine weeks. Compared

to a 3% DSS used for the conventional group of mice, a lower DSS concentration of 1.5% was used for antibiotic-treated mice to assure animal performance during DSS exposure due to their high susceptibility to epithelial damage [20] (Fig. 1B, C). The effects of the maize diets on mice fecal microbiome and serum metabolome were also evaluated. Here we report that diets supplemented with maize NILs containing phlobaphenes and anthocyanins were able to alleviate DSS-induced colitis in mice in a gut microbiota-dependent manner.

2. Materials and Methods

2.1 Chemicals and reagents

Dextran sulfate sodium (DSS, molecular weight 4KDa) salt and butyric acid (≥99%) were obtained from Alfa Aesar (Haverhill, MA, USA). Ampicillin sodium salt, neomycin trisulfate salt, fluorescein isothiocyanate-dextran (4kDa), L-methionine, protocatechuic acid, chlorogenic acid and ferulic acid were obtained from Sigma (St.Louis, MO, USA). Other chemicals were obtained from Fisher Scientific (Hampton, NH, USA) unless otherwise mentioned.

2.2 Plant materials

Four maize NILs [19] were planted and self-pollinated in summer at the Penn State Russel Larson Agronomy Research farm, Rocsplings, PA, USA. 45 days after pollination (DAP) maize cobs were harvested and wind-dried for 2 weeks. Dried kernels were removed from the cob, ground and stored at 4°C before being sent for diet preparation.

2.3 DSS-induced-colitis model and diet treatments

Wet chemistry analysis of the maize materials was performed by DairyOne (Ithaca, NY, USA) and the results were considered during diet formulation. 7% corn oil diet (control)(TD.95092), 25% maize line A supplemented diet (A)(TD.190202), 25% maize line B supplemented diet (B)(TD.190203), 25% maize line C supplemented diet (C)(TD.190204) and 25% maize line D supplemented diet (D)(TD.190205) were manufactured by Envigo Teklad (Madison, WI, USA) using diet formulas shown below (Table 1). Wild-type male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed at Purdue University (West Lafayette, IN, USA). All animal procedures were performed in accordance with the Purdue University guidelines for animal care and use and experiments were approved by Purdue Institutional Animal Care and Use Committee (protocol no. 1810001817). Two experiments, conventional (n=66) and antibiotics treated (n=72), were conducted independently. Mice were randomly assigned to six treatment groups after one week of acclimatization to receive a designated diet for a total of nine weeks. For the conventional experiment, the control group mice continued on a control diet with no DSS added in drinking water throughout the experimental period. The remaining mice groups were fed with either control diet (referred to as DSS group in this study) or A, B, C and D diet respectively and exposed to 3% DSS (w/v) in the drinking water for 5-7 days at the beginning of the ninth week. For antibiotics experiment, the control group mice continued on the control diet

with 1 g/L ampicillin and 0.5 g/L neomycin added to drinking water throughout the experimental period, no DSS was added in this group. The remaining mice groups were fed with designated diets and antibiotics-containing water as described above. At the beginning of the ninth week, 1.5% DSS (w/v) was added to drinking water for a total of 5-7 days, water bottles were replaced every three days during DSS exposure. Body weight and food intake were recorded weekly for the first eight weeks and daily during the final week.

Table 1 Animal experiment diets composition

Diet	Control	A	B	C	D
Ingredient (g/kg)					
Casein	200.0	165.8	168.7	171.8	165.9
L-Cystine	3.0	3.0	3.0	3.0	3.0
Corn Starch	397.5	213.7	214.7	202.9	212.8
Maltodextrin	132.0	132.0	132.0	132.0	132.0
Sucrose	100.0	95.3	92.3	91.5	91.3
Corn Oil	70.0	58.6	57.1	60.8	59.0
Cellulose	50.0	37.13	34.8	40.5	38.5
Mineral Mix	35.0	37.1	35.0	35.0	35.0
Vitamin Mix	10.0	10.0	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5
TBHQ, antioxidant	0.014	0.014	0.014	0.014	0.014
Corn A	0	250.0	0	0	0
Corn B	0	0	250.0	0	0
Corn C	0	0	0	250.0	0
Corn D	0	0	0	0	250.0

2.4 Animal experiment sample processing

At the end of the ninth week, mice were euthanized in carbon dioxide chambers. Blood collected through cardiac puncture was placed into a capillary blood collector (Becton Dickinson, Franklin Lakes, NJ, USA) and settled for 30 minutes, followed by 2-minute centrifugation at 3000 rpm for serum collection. Mice serum was stored at -80°C until further use. Mice cecum and colon samples were harvested immediately after sacrifice, rinsed with sterile PBS and weighed before and after content removal. Cecum and colon digesta were carefully squeezed into a 1.5 ml microcentrifuge tube (Axygen, Corning, NY, USA) and snap frozen in liquid nitrogen. Tissues were then washed again with PBS and recorded for colon length. Cecum was snap frozen, whereas the colon was cut open and divided into distal and proximal parts. Distal and proximal colonic tissues were evenly cut into three small pieces respectively: the first piece was kept in RNAlater and used for gene expression assay; the second piece was snap frozen and used for protein extraction; the third piece was preserved in 10% formalin and used for colon histology scoring.

2.5 16S rRNA sequencing and data analysis

Total DNA was isolated from cecum digesta of the conventional group mice using DNeasy

PowerSoil Kit (Qiagen, Hilden, dyer Germany) following the manufacturer's protocol. DNA concentration was determined using the Take3 Micro-Volume Plate (BioTek, Winooski, VT, USA) and further diluted to a final concentration of 5 ng/ml. Diluted DNA materials were sent to The University of Toledo Microbiome Core Facility (Toledo, OH, USA) for library preparation and sequencing. The 16S library preparation and processing followed the Illumina User Guide for 16S Metagenomic Sequencing Library Preparation section 'Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System (Part No. 15044223 Rev. B) for 16S library preparation, clean-up, normalization, and pooling'. PCR reactions were carried out using MiSeq V3 Reagent Kit (600 cycle) targeting the V3-V4 region (341F and 806R). Demultiplexed fastq files were loaded onto Quantitative Insights Into Microbial Ecology2 (Qiime2) version 2019.1.0 for quality filtering, downstream phylogenetic diversity analyses and taxonomic analysis. Sequence quality control was performed using Qiime2 plugin Deblur, sequences were truncated at the first quality-dropping base (position 294). Alpha rarefaction plotting was performed prior to alpha diversity analysis to assure the capture of OTUs and decide optimal sampling depth. Taxonomy assignment was performed at the genus level using Silva 132 database [21]as the reference database.

2.6 Gene expression assay

Total RNA was isolated from mice colonic tissue preserved in RNAlater (ThermoFisher, Waltham, MA, USA) using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and was reverse transcribed using SuperScript IV VILO Mater Mix (Thermofisher). The expression levels of IL-6, TNF-α, IL-1β and MUC2 were quantified by quantitative real-time PCR (qRT-PCR) using pre-designed duplex Taqman gene expression assays (Thermofisher) Mm01210733_ml (IL-6), Mm00443258_ml (TNF-α), Mm00434228_ml (IL-1β) and Mm01276696_ml (MUC2). β-Actin (Mm02619580_gl) was used as a reference. Results were analyzed by ΔΔCt method and expressed on a log2 scale.

2.7 In vivo permeability assay

The methodology for *in vivo* permeability assay was previously described [22]. Briefly, mice were orally gavaged with 600 mg/kg body weight of FITC 4kDa after a 4-hour fasting period. Blood was collected from the submental vein 3 hours post-gavage. Serum fluorescence intensity was determined (λ_{ex}=490 nm; λ_{em}=520 nm) using a BioTek Cytation1 microplate reader (Winooski, VT, USA). A standard curve was constructed using serial dilutions of FITC.

2.8 Short Chain Fatty Acid (SCFA) extraction and quantification

Fecal samples collected at week 8 were used for SCFA quantification. Heptanoic acid was used as internal standard (IS). Briefly, around 50 mg of fecal pallets were transferred to a reinforced tube pre-filled with silica beads (Fisher Scientific, Hampton, NH, USA). 600 µl of 0.5% (v/v) phosphoric acid-containing millipore water was added per 50 mg of fecal sample. Sample homogenization was performed using a Bullet Blender Gold (Next Advance, Troy, NY, USA) setting 8 for 2 minutes. Samples were then re-homogenized by vortexing for 1 minute and

centrifuged for 10 minutes at 4°C, 15,000 rpm. Supernatants were collected and added with 1:1 (v/v) IS-containing ethyl acetate, vortexed for 2 minutes and centrifuged using the same settings described above. The ethyl acetate phase was stored in a 2 ml autosampler vial. A standard curve was prepared by serial dilution of an IS-containing mixture of acetic acid, butyric acid and propionic acid using 3, 1.5, 0.75, 0.375 and 0.1875 mM concentrations. SCFAs were quantified using an Agilent 7890A gas chromatogram system (GC-FID 7890A, Santa Clara, CA, USA) fused with a silica capillary column (Nukon supelco No.40369-03A, Bellefonte, PA, USA).

2.9 Colonic tissue protein extraction and MUC2 quantification

Around 20 mg snap frozen colonic tissues were thawed on ice and added with 400 µl sterile PBS, followed by two freeze-thaw cycles to disrupt the cell membrane. Samples were then homogenized using Bullet Blender Gold at setting 6 for 4 minutes, followed by centrifugation for 10 minutes at 4°C, 12,000 rpm. Supernatants were stored at -80°C until further use. Total protein quantification was performed using Pierce BCA Protein Assay Kit (ThermoFisher) whereas MUC2 quantification was performed using Mouse MUC2 ELISA Kit (Aviva Systems Biology, San Diego, CA, USA) according to manufacturers' protocols. Results are expressed as MUC2 to total colonic protein ratio.

2.10 Colon histology

Distal colon tissue sections were stained with hematoxylin and eosin and microscopically examined by a board-certified veterinary pathologist based on a published scoring system that considered mucosal hyperplasia, epithelial cell death, mononuclear infiltrate, polymorphonuclear leukocyte infiltrate, crypt architectural distortion and submucosa involvement [23]. The pathologist was blinded to the groups and compared distal colon sections to positive controls (DSS). To determine the extent of mouse colonic lesions, a semi-quantitative method was used that included the amount of inflammatory infiltrate and necrosis. Briefly, a 0-3 scoring scheme was employed to score each parameter, yielding a maximum score of 18. To grade mucosal hyperplasia, mononuclear infiltrate and polymorphonuclear leukocyte infiltrate, the following histomorphological scale was used: 3=marked, 2=moderate, 1=mild and 0=normal. To grade crypt architectural distortion and submucosa involvement, the following histomorphological scale was used: 3=50% or greater, 2=10%-50%, 1=<10% and 0=normal. To grade epithelial cell death, the following histomorphological scale was used: 3=ulcerations, 2=erosions, 1=superficial epithelial sloughing/single cell necrosis, 0=normal.

2.11 Serum extraction for targeted metabolomics

Sample extraction was performed by Bligh-Dyer method using methanol containing 0.1% formic acid, millipore water containing 0.1% formic and chloroform [24]. L-methionnine sulfone was used as an internal standard (IS). Briefly, 320 µl acidified methanol was added to 80 µl serum sample followed by vigorous vortex and sonication, then 160 µl acidified water and 320 µl chloroform were added. Samples were vortexed for 2 minutes and centrifuged at 4°C, 5,000 rpm for 30 minutes.

The aqueous phase was collected and evaporated to dryness in a vacuum concentrator (Eppendorf, Hauppauge, NY, USA). The dried fraction was reconstituted in 75 µl 80% methanol containing 0.1% formic acid, followed by 2 minutes vortex and 8 minutes of centrifugation at 12,000 rpm. The reconstituted samples were transferred to HPLC autosampler vials for downstream analysis. For standard curve construction, standard mixes containing target compounds were prepared at following concentrations: 1,333, 666.5, 133.3, 13.33, 1.333 ng/ml via serial dilution using the same solvent as for sample preparation. An Agilent 6460 QQQ coupled to an Agilent 1200 Rapid Res LC system was used for the analysis (Palo Alto, CA, USA). LC separation was performed by using a Water's Crop Xbridge reversed-phase C18 2.1×100 mm, 3.5 µm column (Milford, MA, USA) with a flow rate of 0.35 ml/min. 5 µl of sample was injected and eluted with a mixture of phase A (5% acetonitrile, 95% ddH_2O with 0.2% formic acid) and phase B (100% acetonitrile with 0.2% formic acid). The biphasic gradient was as follow: 0 minutes, 100% A; 0.5 minutes, 94% A; 2 minutes, 91% A; 3 minutes, 87% A; 4.5 minutes, 65% A; 5.2 minutes, 100% A; 6-10 minutes, 100% A. Electrospray (ESI) interface operated in both positive and negative modes were used for compound characterization and quantification. Data were acquired in multiple reaction monitoring mode (MRM). The source parameters for MS were set as follows: gas temperature 325°C, drying gas flow rate 8.0 L/min, nebulizer pressure 45 psi, sheath gas temperature 250°C, sheath gas flow 7.0 L/min, capillary 3800 V, ΔEMV of +300 V, nozzle 1000 V(+) and 500 V(−). Data analysis was performed by Agilent MassHunter Quantitative analysis software (v6.0). The peak area was normalized to IS and concentration was calculated based on the pre-constructed standard curve.

2.12 Statistical analysis

Statistical tests were performed using SPSS 26.0, GraphPad Prism 8 and Qiime 2 software. Statistical differences were determined using one-way ANOVA with Tukey's multiple comparison test, Fisher's LSD multiple comparison test, or non-parametric Kruskal-Wallis test with Dunn's multiple comparison test. Differences in bacterial beta-diversity were analyzed using permutational multivariate analysis of variance (PERMANOVA) in Qiime2. Differences in serum phenolic acid levels were analyzed using two-tailed Student's t-test. Values were considered significant when $P < 0.05$.

3. Results

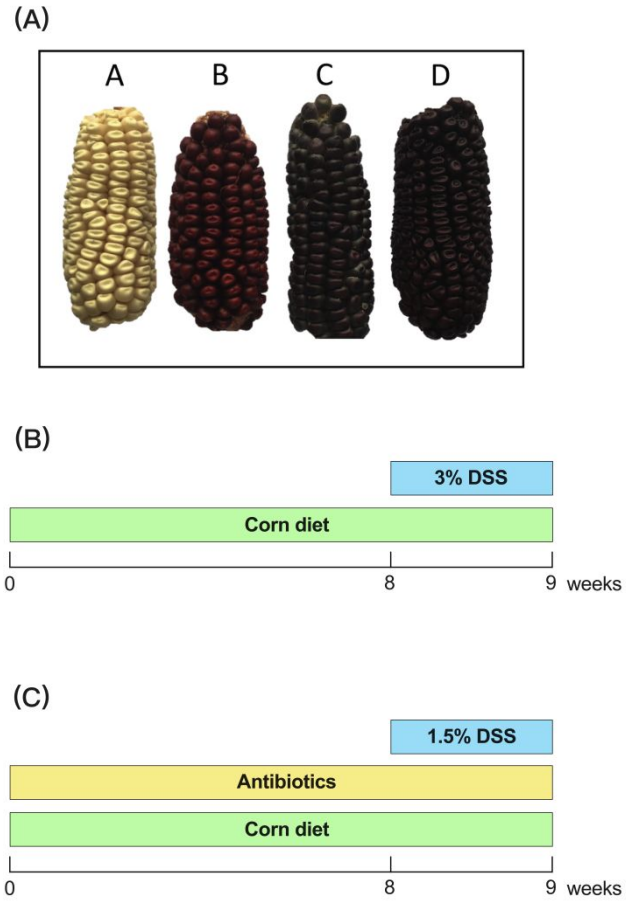

Fig. 1 Maize near-isogenic lines (NILs) phenotypes (A) and animal experiment timeline for (B) conventional and (C) antibiotics treatment.

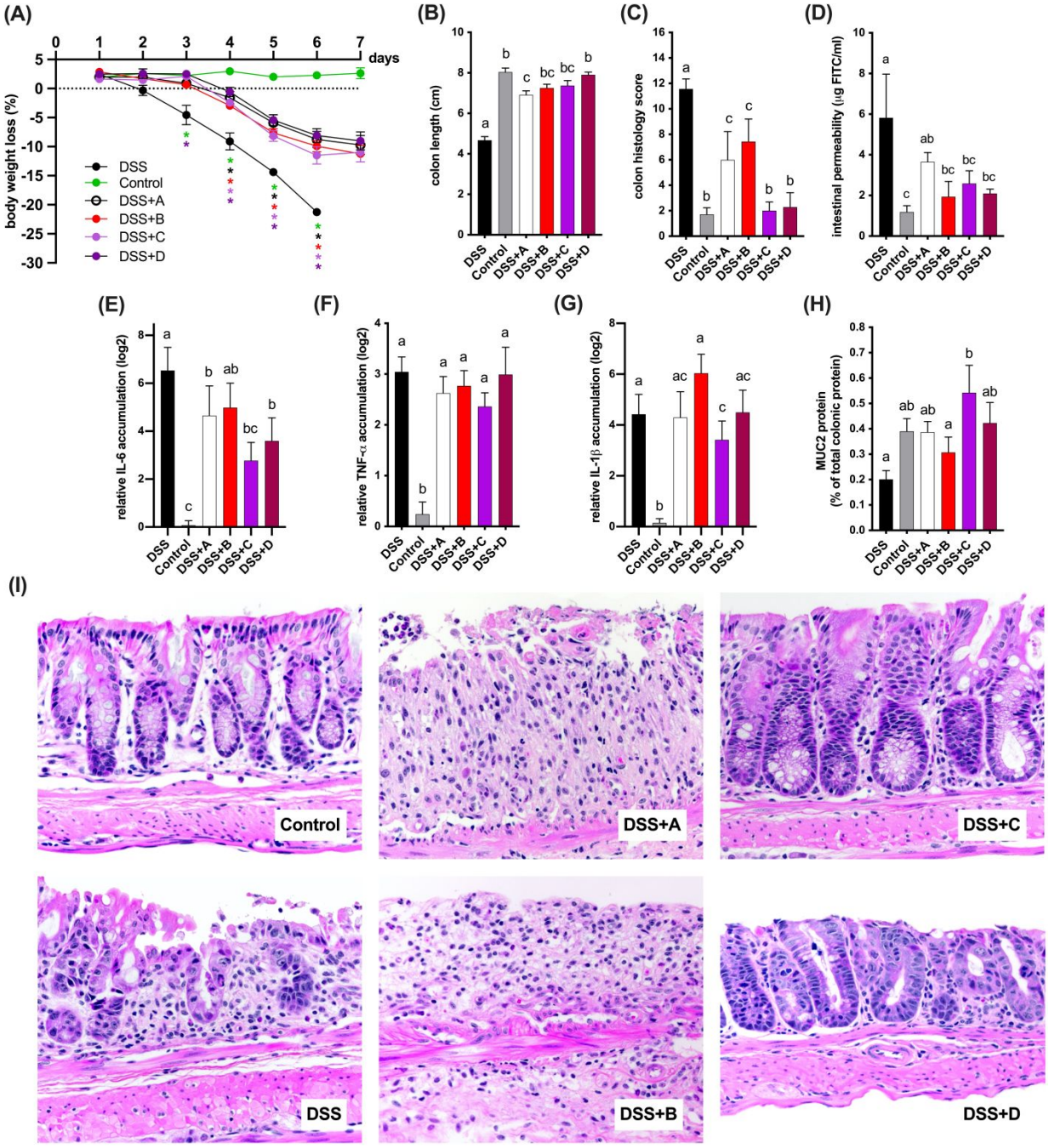

Fig. 2 Phlobaphenes and anthocyanins enriched maize diets ameliorated DSS-induced colitis in wild-type mice. (A) Daily monitored body weight loss in mice after DSS exposure. (B) Endpoint mice colon length. (C) Accumulative distal colon histology score. (D) Intestinal permeability was measured using FITC. Relative mRNA expression levels of pro-inflammatory cytokine (E) IL-6, (F) TNF-αand (G) IL-1β in colonic tissues. (H) Colonic MUC2 protein quantification. (I) Representative histological photomicrographs of hematoxylin and eosin (H&E)-stained paraffin longitudinal section of distal colon tissues. Data are expressed as means ± SEM (n = 10 or 11 mice per group). Significance level ($P < 0.05$) was determined by one-way ANOVA followed by Fisher's LSD. Colored asterisks

indicate that the mean value of the corresponding treatment group was significantly different from that of the DSS group. Values assigned to different letters are significantly different.

3.1 Flavonoid-rich maize diets ameliorated acute colonic inflammation in mice

Exposure to DSS resulted in abrupt body weight loss and macroscopic damage to the colon in mice consuming the control diet. Histological examination of the colon also revealed crypt loss, massive immune cell infiltration, and tissue edema in the control group mice, which collectively represent the onset of colonic inflammation. Mice consuming flavonoid-rich maize diets showed a reduction in DSS-induced body weight loss, colon shortening, and colon histological alterations. Among all the diets, D diet was found to be the most effective in restoring colon length, and C and D diets were more effective at protecting and maintaining mucosal architecture (Fig. 2A-C, I). All four maize diets were shown to be effective in reducing intestinal permeability, corresponding to a well-preserved intestinal barrier function (Fig. 2D). Pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β are common biomarkers of colitis with elevated productions in the context of intestinal inflammation. The mRNA expression levels of IL-6 were downregulated in mice consuming C diet and D diet (Fig. 2E). The primary mucus building block mucin-2 glycoprotein (MUC2) was quantified to explore the effect of maize diets on fortifying intestinal barrier function. We found that the anthocyanin-rich C diet exhibited the most potent effect in up-regulating the MUC2 protein levels (Fig. 2H).

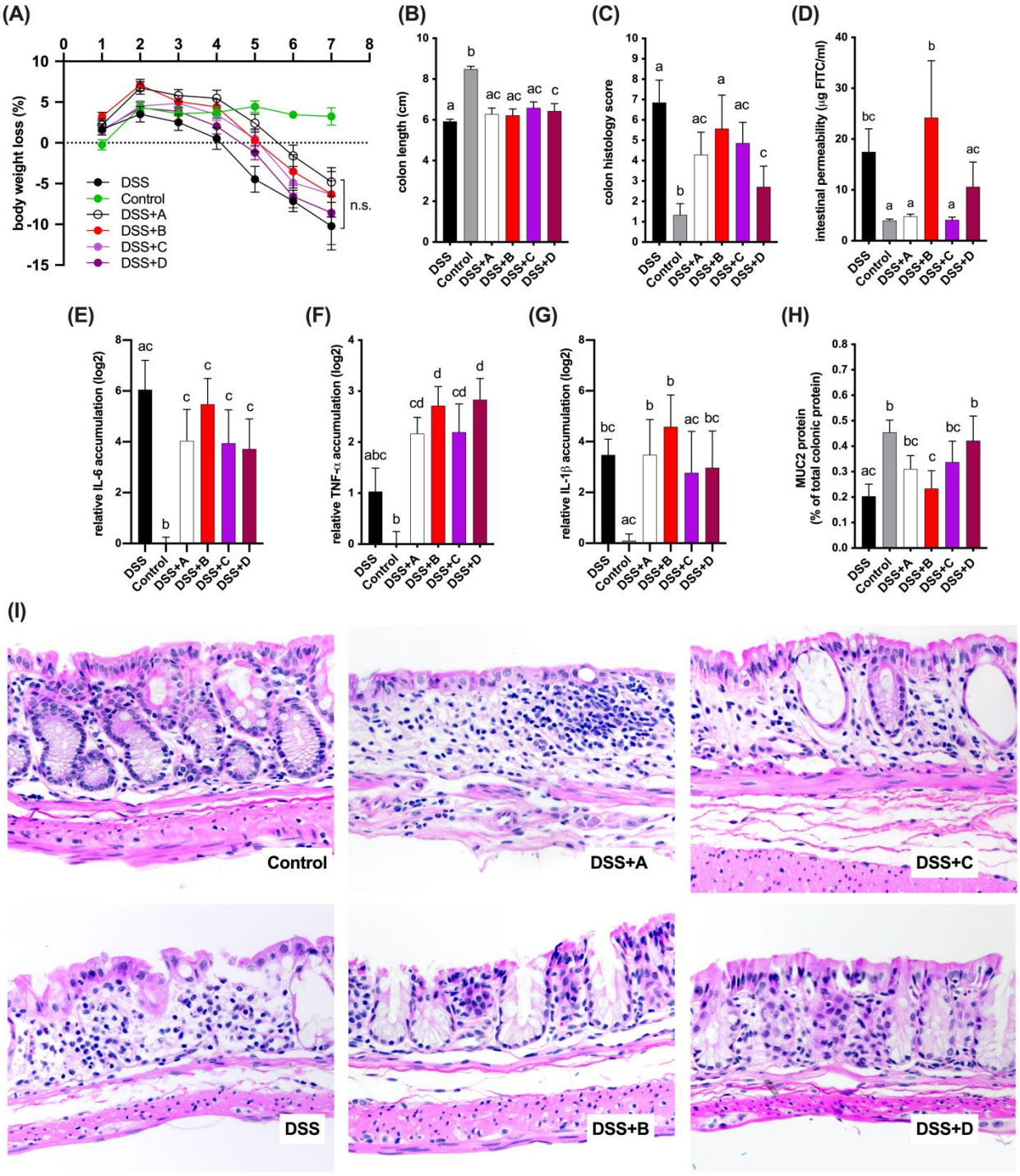

Fig. 3 Phlobaphenes and anthocyanins enriched maize diets became ineffective in antibiotic-treated wild-type mice. (A) Daily monitored body weight loss in mice after DSS exposure. (B) Endpoint mice colon length. (C) Accumulative distal colon histology score. (D) Intestinal permeability was assayed using FITC. Relative mRNA expression levels of pro-inflammatory cytokine (E) IL-6, (F) TNF-α and (G) IL-1β in colonic tissues. (H) colonic MUC2 protein quantification. (I) Representative histological photomicrographs of H&E-stained paraffin longitudinal section of distal

colon tissues. Data are expressed as means ± SEM (n = 11 or 12 mice per group). Significance level ($P < 0.05$) was determined by one-way ANOVA followed by Tukey's post-test. Colored asterisks indicate the mean value of the corresponding treatment group was significantly different from that of the DSS group. Values assigned to different letters are significantly different.

3.2 The protective effects of flavonoid-rich maize diets are dependent on gut microbiota

Accumulating evidence has indicated that health benefits conferred by flavonoids were likely the results of the reciprocal interactions between flavonoids and the gut microbiota [25, 26]. We hypothesized that bacterial metabolism was essential for the health benefits of anthocyanins and/or phlobaphenes. To test this, broad-spectrum antibiotics ampicillin and neomycin were added to mice drinking water throughout the experiment to deplete gut microbiota in all groups. Bacteria-depleted mice were more susceptible to DSS treatment and flavonoid-rich maize diet supplementation failed to ameliorate the colitis symptoms. During DSS exposure, all the maize diet groups showed no difference in terms of mice body weight loss compared to the DSS group (Fig. 3A). Flavonoid-rich diets also failed to prevent DSS-induced crypt loss and epithelial damage (Fig. 3B-C, I). Anthocyanin-rich maize diets ameliorated intestinal permeability but not phlobaphene-rich maize diets (Fig. 3D). Flavonoid-rich diets also failed to reduce DSS-induced increase in pro-inflammatory cytokine gene expression levels. Albeit IL-1β and TNF-α expression levels were elevated in mice consuming flavonoid-rich maize diets (Fig. 3F-G). In addition, the mucus protection effect found in the anthocyanin-rich C diet was attenuated in bacteria-ablated mice (Fig. 3H). Collectively, these data suggest that the health-beneficial effects conferred by flavonoid-rich diets were dependent on gut microbiota.

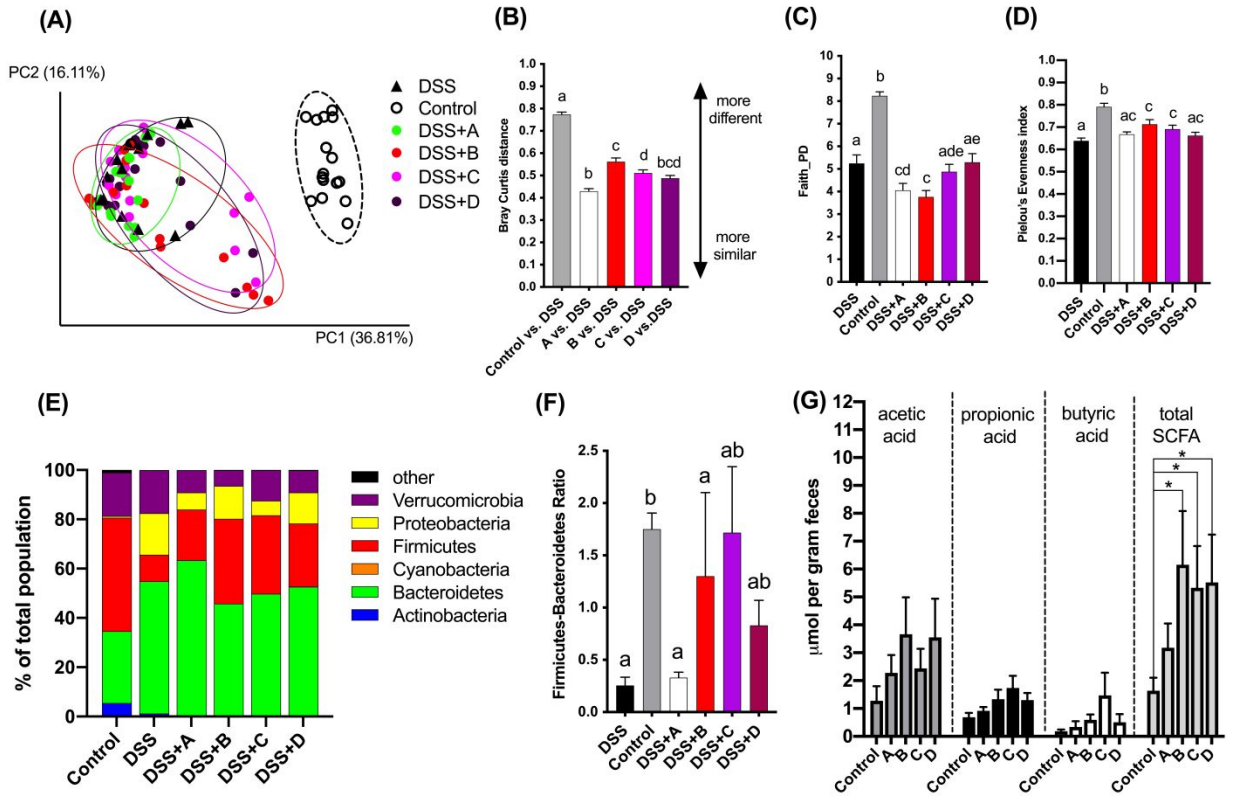

Fig. 4 Phlobaphenes and anthocyanins enriched maize diets modulated gut microbiota in wild-type mice to retrieve gut homeostasis. (A) Principal coordinates analysis (PCoA) of the Bray-Curtis distance matrix of wild-type mice. (B) Bar plot of β-diversity determined by Bray-Curtis distances. (C) Microbiota richness is determined by Faith's Phylogenetic Diversity. (D) Microbiota evenness is determined by Pielou's Evenness. (E) Microbiota composition at Phylum-level. (F) Firmicutes to Bacteroidetes ratio. (G) Levels of fecal acetate, propionate, butyrate, and total SCFA at week 8 (before DSS addition). Data are expressed as means ± SEM (n = 10 or 12 mice per group). For (B), the significance level ($P < 0.05$) was determined by PERMANOVA analysis. For (C), (D), (F), the significance level ($P < 0.05$) was determined by Kruskal-Wallis test followed by Dunn's post-test. Values assigned to different letters are significantly different. For (G), the significance level ($P < 0.05$) was determined by one-way ANOVA followed by Fisher's LSD post-test.

3.3 Effect of flavonoid-rich diets on gut microbiota

Microbiota dysbiosis is one of the key characteristics of IBD. Thus, we next investigated whether flavonoid-rich maize diets were able to modulate gut microbiota and retrieve gut homeostasis. Endpoint cecal digesta from conventional mice groups were collected and subjected to 16S rRNA sequencing. Microbiota β-diversity calculated using Bray-Curtis distance matrix revealed differential clustering of treatment groups. Among all the treatment groups, DSS and A diet groups displayed similar clustering patterns and were different in microbiota composition compared to healthy controls whereas, B, C and D diet groups are still different but less distant from the control group and more similar to each other (Fig. 4A). Interestingly, PERMANOVA of Bray-Curtis

showed significance between B and C diet groups but did not show any significance between B and D diet groups or C and D diet groups (Fig. 4B). These results indicate that different flavonoids might modulate gut microbiota differently. Microbiota α-diversity indexes were evaluated using Faith's Phylogenetic Diversity and Pielou's Evenness. Flavonoid-rich diets did not restore bacterial richness (Fig. 4C); however, B and C diets were found to significantly increase bacterial evenness compared to the DSS group (Fig. 4D). DSS treatment led to alterations in bacterial composition at the phylum level, with the most differences observed in *Firmicutes* and *Bacteroidetes* proportions. Conversely, mice consuming C and D diets had comparable *Firmicutes* to *Bacteroidetes* ratios to healthy controls (Fig. 4E-F). Before the addition of DSS, we also found that fecal short-chain fatty acid (SCFA) levels in mice consuming B, C and D diets were higher than in mice consuming control and A diets (Fig. 4G), indicating that intake of maize anthocyanins and phlobaphenes up-regulated the abundance of SCFA-producing bacteria.

Table 2 Quantification of serum phenolic acids in conventional and antibiotics-treated mice groups

		Metabolites serum extraction (μg/ml)						
Treatment	Antibiotics	Trans-ferulic acid	Protocatechuic acid	Chlorogenic acid	4-OH-BALD	3-OH-PPA	3-Ome-PAA	4-OH-PAA
Control	-	0.076±0.031	0.050±0.004	0.018±0.003	7.63±0.97	6.42±1.72	15.11±2.12	24.67±2.56
	+	0.032±0.016*	0.050±0.003	ND	3.76±1.17*	10.06±1.47	17.75±2.50	34.58±6.13
DSS+A	-	0.116±0.032	0.041±0.001	0.025±0.009	9.13±2.44	6.74±0.57	13.70±3.81	30.93±6.88
	+	ND	0.035±0.011	ND	5.21±0.53	7.22±1.17	8.90±1.37	35.05±2.86
DSS+B	-	0.101±0.020	0.040±0.001	0.019±0.005	6.72±1.07	13.64±8.33	16.78±6.10	29.88±2.98
	+	0.021±0.016*	0.043±0.010	ND	4.56±0.98	4.63±0.81	10.97±1.88	31.82±5.36
DSS+C	-	0.125±0.037	0.034±0.007	ND	4.88±0.96	5.04±1.26	11.29±2.41	20.87±1.12
	+	0.017±0.011*	0.044±0.029	ND	4.31±0.51	5.04±0.62	8.89±1.76	26.46±1.47
DSS+D	-	0.246±0.029	0.047±0.001	0.020±0.005	7.24±1.17	6.45±1.39	18.08±4.16	29.76±3.92
	+	0.068±0.030*	0.047±0.002	ND	4.31±0.41*	7.87±2.39	9.43±2.11	30.19±3.10

-, Conventional group; +, antibiotic-treated group; ND, not detected.

Data expressed as mean ± SE (n = 6).

* Mean value was significantly different from that of the conventional group ($P < 0.05$, two-tailed Student's *t* test).

3.4 Effect of antibiotics on serum metabolic profile in the acute colitis model

Metabolite fluctuations are often mirrored in microbiota composition. A decrease in gut microbiota could impact the bacterial-dependent metabolism of flavonoids and thus will shift the metabolic profile of the host. Phenolic acids commonly found in edible plants are the main metabolites

generated through flavonoid metabolism. To further investigate the effect of antibiotics on flavonoid biotransformation in the context of DSS, endpoint serum extracts were used for specific phenolic acid quantification. Major phenolic acids detected in conventional mice groups were trans-ferulic acid, protocatechuic acid, chlorogenic acid, 4-hydroxybenzaldehyle (4-OH-BALD), 3-(3-hydroxyphenyl) propionic acid (3-OH-PPA), 3-(3-methoxyphenyl)acetic acid (3-Ome-PAA) and 4-hydroxyphenylacetic acid (4-OH-PAA) (Table 2). Mice consuming the D diet had the highest abundance of trans-ferulic acid in serum compared to the other three maize diets, however, serum trans-ferulic levels in antibiotic-treated mice were all shown to be significantly reduced compared to their corresponding conventional group. Microbiota depletion did not affect serum protocatechuic acid, 3-OH-PPA and 4-OH-PAA levels but resulted in decreased serum 3-Ome-PPA levels in mice fed NILs diets though no statistically significant differences were determined. Nonetheless, microbiota depletion resulted in reductions of serum chlorogenic acid and 4-OH-BALD levels, with the former compound decreased to a non-detectable level. Taken together, these data suggest that antibiotic treatment will affect flavonoid metabolism, which, could explain at least partially the loss of protective effects of the flavonoid-rich diets in the acute colitis model.

4. Discussion

The present study reported the use of NILs enriched in different flavonoid classes to investigate the protective effects of anthocyanins and/or phlobaphenes against acute colitis within a whole-food matrix in mice. This study could serve as a model to understand how diets rich in these compounds could provide benefits when consumed by patients who suffer from colitis. Anthocyanins have been intensively studied for their anti-glycation, anti-oxidant and anti-inflammatory effects. Also, there is other supporting evidence for the prebiotic activity that collectively demonstrated their health benefits [6, 27-29]. Conversely, the health-promoting effects of phlobaphenes *in vivo* are poorly understood even though they serve as potential UV-B filters in plants and several studies have revealed the high anti-oxidant capacity of their monomer flavan-4-ols [30-32].

We utilized the well-known DSS model to investigate the anti-colitis effects provided by long-term intake of dietary anthocyanins and/or phlobaphenes. Overall, we found that intake of anthocyanins and phlobaphenes (alone or in combination) containing maize diets alleviated DSS-induced colitis in mice, as evidenced by the fact that mice consuming these diets had reduced weight loss, restored colon length, and less colonic damage. These observations suggest that the higher anthocyanins and phlobaphenes in food matrices *per se* are likely to provide some beneficial effects. Although maize line A lacks both pigments, the intact food matrix still provides bound antioxidants such as dietary fiber and phenolic acids (DF-PC) to exert health benefits [33-35]. However, apart from the whole-food matrix effect, our data showed that anthocyanins, compared to phlobaphenes, were more effective in lowering the expression level of pro-inflammatory cytokine IL-6 and may play a role in stimulating mucus secretion in the context of colitis compared to phlobaphenes. Similar to our study, tomato NILs with enhanced anthocyanins protected against

DSS-induced colitis in mice and reduced the release of IL-6 and TNF-α [36, 37]. Previously we reported that intake of flavan-4-ols enriched maize diet was able to protect the intestinal barrier function in mice by restoring mucus thickness under a low-grade inflammation condition [32]. In the same study, we also concluded that the protective effects of flavan-4-ols were dose-dependent. However, in the DSS model, we noticed that mice consuming lines A, B and D had similar MUC2 protein levels, indicating that mucus restoration by phlobaphenes could be context-dependent. A higher intake of phlobaphenes may be needed to generate a pronounced effect under the acute colitis condition.

From our data, the total anthocyanin content in line C was ~7-fold higher than that in line D, whereas the phlobaphene content in line D was ~2-fold higher than that in line B. Mice consuming C diet received ~8 μg/kcal/d anthocyanins, which is a physiologically relevant level comparable to the 6 μg/kcal/d anthocyanin intake in people following a standard Western diet [38]. Nevertheless, the average anthocyanin intake in mice consuming D diet was estimated to be only ~1.2 μg/kcal/d, roughly 5-fold lower than the average anthocyanins intake. Considering the evident alleviation of colitis symptoms observed in mice consuming D diet, we speculate an additive or synergistic interaction between phlobaphenes and anthocyanins, but further investigation is needed to verify this.

To analyze the dependency of flavonoids on gut microbiota to exert protective effects, antibiotics were used to decrease endogenous bacteria in mice. We lowered the concentration of DSS due to the increased risk of epithelial injury in the absence of gut microbiota [20] and still found profound effects of DSS but found no protective effects of anthocyanins or phlobaphenes in antibiotics-treated mice groups. In contrast with conventional groups, body weight loss, colon length, and colon histology scores were not significantly different between the DSS group and four maize diet groups in mice treated with antibiotics, suggesting that the health benefits of flavonoid rich maize lines were dependent on gut microbiota or their microbial metabolites.

16S rRNA sequencing was employed to investigate the impact of maize NIL diets on gut microbiota. Our data suggested that the intake of flavonoid-rich diets affected the β-diversity of the gut microbiota by shaping the community towards a more similar status to the control group to retrieve intestinal homeostasis. This contribution can be further attributed to matrix-bound anthocyanins and phlobaphenes, as more distant bacterial communities between the control group and A diet group were observed. The balancing effects of these flavonoids were also mirrored in the increased Firmicutes/Bacteroidetes ratios in mice fed B, C, and D diets. A significantly decreased Firmicutes/Bacteroidetes ratio was found in DSS group mice. This observation is consistent with other reports where this ratio was found to be lower in active IBD patients and associated with increased disease severity [39, 40]. Collectively, our data suggest that matrix-bound maize anthocyanins and phlobaphenes possess prebiotic potential, and exert their gut microbiota-modulating capacity to protect against colitis.

We next investigated whether the impact of gut microbiota on flavonoid metabolism could explain the ineffectiveness of flavonoid-rich diets in antibiotic-treated mice. Considering that

flavonoids could be metabolized into a wide range of low-molecular-weight phenolic acids [39], we selected various intermediate phenolic acids as targets for flavonoid metabolite detection. Out of the seven phenolic acids detected, serum levels of trans-ferulic acid, chlorogenic acid, 4-OH-BALD and 3-Ome-PAA were found to be decreased in antibiotic-treated mice, whereas serum levels of protocatechuic acid, 3-OH-PPA, and 4-OH-PAA remained comparable between the two groups. Our results lined up with previous studies, suggesting that the absence of gut microbiota influenced flavonoid metabolism [21, 41]. Lin et al. reported lower serum phenolic metabolite concentrations and accelerated fecal flavonoid excretion in antibiotic-treated mice group after the ingestion of purified flavonoid mix. Orrego-Lagarón et al. detected comparable levels of several phenolic acids in plasma between conventional and rifaximin-treated mice after oral gavage of naringenin. This phenomenon could be due to the existence of metabolic pathways where phenolic acids were produced independently of the ingested flavonoids.

5. Conclusions

Utilization of isogenic food materials allows for the dissection of contributions of a specific subclass of phytochemicals in a complex nutrient background and facilitates the investigation of whole-food matrices to promote health. Taken together, our data show that anthocyanins and the combination of anthocyanins and phlobaphenes from whole-foods protected intestinal barrier function, mucosal integrity and modulated gut microbiota to help retain intestinal homeostasis under DSS exposure. However, the maize lines containing anthocyanins and/or phlobaphenes did not show any protective effects in gut microbiota-ablated mice suggesting the essential role of intestinal microbiota in the anti-colitic activity of these compounds. Given that the ulcerative colitis is associated with gut bacterial dysbiosis, currently, we are in the process of assessing the role of UC-associated dysbiosis in the anti-colitic activity of anthocyanins and/or phlobaphenes.

Authors' contributions

Conceptualization, L.R. and S. C.; Methodology, B.W., S.L. and L.R.; Formal analysis, B.W.; Investigation, B.W., S.L., A.D.C., H.C and L.R; Writing – Original Draft, B.W; Writing – Review & Editing, S.L., M.K., C.R., S.C. and L.R; Visualization, B.W., S.L.and L.R.; Funding acquisition, L.R.

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Data Availability Statement

The original contributions generated for this study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Conflicts of interest

There are no conflicts to declare.

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