Dietary flaxseed intake exacerbates acute colonic mucosal injury and inflammation induced by dextran sodium sulfate

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Abbreviated title: Flaxseed exacerbates acute colonic injury and inflammation

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ABSTRACT

Flaxseed (FS) is a dietary oilseed containing a variety of anti-inflammatory bioactives including fermentable fiber, phenolic compounds (lignans), and the n3-polyunsaturated fatty acid (n3-PUFA) \( \alpha \)-linolenic acid. The objective of this study was to determine the effects of FS, and its n3-PUFA-rich kernel or lignan- and soluble fibre-rich hull, on colitis severity in a mouse model of acute colonic inflammation. C57Bl/6 male mice were fed basal diet (NEG), or basal diet supplemented with 10% FS, 6% kernel, or 4% hull for 3 weeks prior to and during colitis induction via 5 day dextran sodium sulfate (DSS; 2% w/v) in their drinking water (n=12/group). Although consumption of all FS-based diets increased anti-inflammatory metabolites (hepatic n3-PUFAs, serum mammalian lignans, and cecal short chain fatty acids (SCFAs)), this was not associated with anti-inflammatory effects in DSS-exposed mice. In fact, dietary FS exacerbated DSS-induced acute colitis as indicated by a heightened disease activity index and increased colonic injury and inflammatory biomarkers (histological damage, apoptosis, myeloperoxidase, inflammatory cytokines (IL-6 and IL-1\( \beta \)), and NF-\( \kappa \)B signalling-related genes (Nfkb1, Ccl5, Bcl2a1a, Egfr, Relb, Birc3 and Atf1)). Additionally, the adverse effect of FS diet was extended systemically, as serum cytokines (IL-6, IFN-\( \gamma \), and IL-1\( \beta \)) and hepatic cholesterol levels were increased. The adverse effects of FS were not associated with alterations in fecal microbial load or systemic bacterial translocation (endotoxemia). Collectively, this study demonstrates that although consumption of a 10% FS diet enhanced the levels of n3-PUFAs, SCFAs, and lignans in mice, it exacerbated DSS-induced colonic injury and inflammation.

KEYWORDS: Flaxseed, experimental colitis, n3-PUFAs, SCFAs, lignans
INTRODUCTION

Colitis is a major contributor to a number of gastrointestinal disorders and diseases including inflammatory bowel disease (IBD) (i.e. ulcerative colitis), intestinal infections, ischemia, injury, and colon cancer (33, 41, 59, 76, 79, 90, 105). It is associated with numerous clinical symptoms (i.e. abdominal pain, diarrhea, and rectal bleeding), colonic histological changes (immune cell infiltration, necrosis, or ulceration), increased production of colonic and systemic inflammatory chemokines and cytokines (i.e. IL-6, IL-1β and TNF-α) (72, 99) and various extra-intestinal manifestations, including hepatic disorders (94). While some of the most common non-surgical treatments for colitis-associated diseases include anti-inflammatory agents, immunosuppressants, and antibiotics, patients often suffer from severe side effects, fail to respond, or become resistant to such therapies (23, 38, 60). Therefore, alternative or complementary therapies, including prebiotics, probiotics, and butyrate (29, 33, 65, 96, 98), phenolic compounds (e.g. curcumin, quercitrin, and rutin) (37, 69), and n3-polyunsaturated fatty acids (n3-PUFAs) (39, 48, 102), are being studied for their ability to improve gut health by attenuating colitis and its associated diseases.

Flaxseed (FS) is an oilseed rich in numerous anti-inflammatory gut health-promoting bioactives including fermentable dietary fibers (31, 57), phenolic compounds (e.g. lignans) (1, 26, 40), and the n3-PUFA α-linolenic acid (ALA) (27, 39). Gut microbial fermentation of dietary fibers (e.g. FS gum, inulin, pectin, and resistant starch), results in the production of short chain fatty acids (SCFAs), including acetate, propionate, and butyrate, which play a role in the gut health promoting effects of prebiotics (57, 77, 81). In particular, butyrate serves as an important energy source for colonocytes, enhances gut barrier integrity, and exerts anti-inflammatory effects, in part through modulation of inflammation-related cell signalling pathways, such as NF-κB (11, 21, 36, 56, 78, 82, 83, 87, 89). FS is also the richest dietary
source of plant lignans, predominantly secoisolariciresinol diglycoside (SDG), which are metabolized by the colonic microbiota to their more biologically active forms, the mammalian lignans, enterolactone (EL) and enterodiol (ED) (18, 54). Although mammalian lignans have not been previously investigated for their effects on colonic inflammation, lignans are associated with reduced colon cancer risk potentially through their anti-proliferative, pro-apoptotic, anti-oxidative, and anti-inflammatory activities (1, 8, 19, 20, 26, 74). Furthermore, FS is a rich source of ALA, which is the dietary precursor to the long chain n3-PUFAs (docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)). n3-PUFAs, in particular EPA and DHA, have long been studied for their role in suppressing inflammation and are currently being investigated as potential therapeutic agents in numerous inflammatory conditions, including colitis (4, 22, 46, 48, 92, 101, 102). Additionally, emerging data indicates that ALA can also reduce colonic inflammation through modulation of NF-κB signalling and reducing oxidative stress (39, 93).

Although dietary FS has demonstrated gut health promoting potential with regards to reducing colon cancer development (6, 7, 45), no study to date has determined its potential in attenuating colonic inflammation. Recently, studies have shown that dietary FS is a potent anti-inflammatory agent in injury-induced models of pulmonary disease in rodents (17, 55), and thus may also prove beneficial in attenuating inflammation in other tissues, including the colon. Therefore, the primary objective of this study was to determine if consumption of dietary FS, resulting in production of SCFAs, mammalian lignans, and n3-PUFAs, could attenuate the severity of colonic inflammation induced by mucosal injury in a mouse model of acute colitis. Additionally, since FS bioactives are unequally distributed throughout the seed compartments, with the hull enriched in fibre and lignans, and the kernel enriched in ALA (68), our secondary objective was to determine the potential use of FS hull and kernal as dietary modulators of
colonic inflammation. Although, FS kernel and hull are commercially available as natural health products or nutraceuticals, there is limited research on the efficacy of different FS fractions on human health and disease (14, 15).

MATERIALS AND METHODS

Purification and chemical analysis of FS, hull and kernel

Whole FS, hull (Natunola® Flax Hull Lignans) and kernel (Natunola® Omega Flax 70) were purchased from Natunola (Winchester, Canada). To reduce the amount of whole seeds and hull from the kernel product, the kernel product was mechanically separated with the Clipper Model 400 Office Tester & Cleaner (A.T. Ferrell Company Inc., Bluffton, IN) which increased the purity from 40 to 75%. FS, hull and kernel were further manually purified to obtain the FS ingredients with the purity of >98%. To determine the proportions of hull and kernel to the whole seed, randomly selected whole seeds were weighed and then separated into hull and kernel fractions and reweighed. All samples were then milled with a standard coffee grinder, sieved (mesh size 1000 µ) to obtain equivalent particle size, and analyzed for fat, protein, carbohydrates, lignan, and fibre content.

Total lipids were extracted from 10g sample with chloroform/methanol mixture (1:1) and the fat profile was determined as described by Glew et al. (2010) (34). Total carbohydrate in FS, hull and kernel were analysed as described by Dubois et al. (1956) (25). Available carbohydrates were measured based on method described by Mopper et al. (62). To determine protein content, whole seeds and hull, were first washed in hexane (1:2, w/v) to remove any oil and then air dried. All FS samples were then disintegrated to small particles using a laboratory mortar and pestle. Total nitrogen content of these ground samples were determined according to AOCS
method Ba 4f-00 (AOCS, 1997) by combustion analysis using a Flash EA®1112 N-analyzer (Thermo Fisher Scientific, Ottawa, Canada) and a conversion factor of 6.25 was used to calculate protein content.

Dietary fibre was determined according the Megazyme Dietary Fibre method (Megazyme International, Bray, Ireland) with some modifications. Defatted sample was dissolved into 50ml MES buffer. α-amylase, proteinase and amyloglucosidase were added into the solution according to the Megazyme instructions. The digest was centrifuged at 9500g for 15 min followed by decanting. The residue (R-I) was oven dried at 103 °C overnight. The supernatant was mixed with 4 volumes of 95% ethanol and then centrifuged at 9500g for 15 min. The residue (R-II) was also oven dried at 103°C overnight. Both residues (R-I and R-II) were weighed, analysed for protein content by using NA2100 Nitrogen and Protein Analyzer (Thermo Quest, Milan, Italy), and ash content by using 550°C muffler oven. The results were recorded as Protein I & II, and Ash I & II, respectively. The content of insoluble dietary fibre was calculated by equation I:

\[
\text{Insoluble Dietary Fibre \%} = \frac{(R-I \text{ (g) } - \text{Protein I (g) } - \text{ Ash I (g)}) \times 100}{\text{Sample (g)});
\]

The content of soluble dietary fibre was calculated by equation II:

\[
\text{Soluble Dietary Fibre \%} = \frac{(R-II \text{ (g) } - \text{Protein II (g) } - \text{ Ash II (g)}) \times 100}{\text{Sample (g)});
\]

The content of total dietary fibre was the sum of soluble and insoluble dietary fibre. All the results were recorded on a dry weight basis.

Under the alkaline hydrolysis regime, a HPLC method was used to measure SDG, as the main lignan in FS. Milled FS samples were hydrolyzed with 80% ethanol containing 0.3 M NaOH at 60 °C for 2 hr under constant stirring. The samples were centrifuged and the supernatant were transferred. The residues were washed twice with 80% ethanol and centrifuged. The supernatant was combined for each sample and the final volume was set to 25 ml by adding 80% ethanol. Triplicate extractions were performed on each sample. The extracts were filtered
through 0.2 mm filter and analysed by HPLC system (Agilent Technology 1100 Series, Palo Alto, CA) equipped with a quaternary pump, an inline degasser, a thermostatic autosampler, and a diode array detector (detects signals from 190 nm to 600 nm). A Phenomenex Kinetex 2.6 m C18 column (100 X 4.0 mm) was used for the separation. Nutritional composition of FS, hull and kernel is demonstrated in Table 1.

**Study design and experimental diets**

Sixty 3-wk-old male C57Bl/6 mice were purchased from Charles River Laboratories (Portage, MI), maintained in a 12-hr light/dark cycle at 23 ±2°C and given food and tap water ad libitum. Mice were housed 3-4 mice/cage, acclimatized for 1 week on AIN-93G basal diet (BD) containing 7% corn oil instead of soybean oil (to eliminate the influence of anti-inflammatory bioactives in soybean oil such as tocopherol, isoflavones, and ALA) (30, 47, 63)). Animals were divided into experimental groups such that initial body weight (BW) between groups did not differ. The experimental groups included a BD-fed negative control group (NEG) (n=24 mice/group), 10% FS-fed group, 6% kernel-fed group, and 4% hull-fed group (n=12 mice/group). The 4% hull and 6% kernel diets contained the same amount of hull or kernel present in the 10% FS diet. Diets were prepared by Harlan laboratories (Madison, WI) and protein, fat, and fibre levels were adjusted to equalize macronutrients across diets, and cornstarch was adjusted to equalize caloric content (Table 2). One week prior to DSS administration, BW and diet intake (DI) were assessed twice weekly and fresh fecal samples were collected, snap-frozen and stored at -80°C to assess microbial content. After 16 days of diet, all the animals except half of NEG group received 2% DSS (MW: 36,000-50,000, MP Biomedicals, Solon, OH) in their drinking water for 5 days. During the DSS cycle, DI and water intake (WI) of each cage was measured, and disease activity index (DAI) (summation of BW loss, stool blood and
consistency scores) was assessed for each mouse daily. The stool consistency and stool blood were assessed on fresh fecal samples smeared onto Hemoccult Sensa slides which were processed according to the manufacturer’s instructions (Beckman coulter, Brea, CA). Each individual score had a scale of 0-3: BW loss score: 0=0-1%, 1=1-5%, 2=5-10% and 3=10-20% BW loss; stool consistency score: 0=normal, 1=soft but formed, 2=very soft, and 3=diarrhea; stool blood score: 0= negative hemoccult, 1= positive hemoccult, 2=visible bloody stool, 3=rectal bleeding. At the end of DSS cycle, mice were euthanized by cervical dislocation, blood was collected by cardiac puncture, and serum separated and stored at -80°C for later cytokine, lignan, and endotoxin analyses. Mouse colons and cecums were removed intact, and the colon length and cecum weight (including content) were measured. Cecum content was snap frozen and stored at -80°C for later SCFA analyses. Livers were excised, weighed, and snap frozen and stored at -80°C for later analyses of cholesterol levels and fatty acid profiles.

After removing feces, colons were weighed and 1 cm of the distal colon was formalin-fixed and sent to the veterinary pathologist (Dr. Geoffrey Wood) for assessment of histological damage. A piece of the proximal and distal colon (0.5 cm) were snap frozen and stored at -80°C for myeloperoxidase (MPO) and cytokine analyses (distal colon) and mRNA expression analyses (proximal colon). The remaining colon was Swiss-rolled and formalin-fixed for immunohistochemistry analyses. All experimental protocols involving animals were submitted and approved by the Animal Care Committee, University of Guelph (Animal Utilization Protocol # 10R067).

**Cecal SCFA concentrations**
Cecal SCFA concentrations, including acetate, propionate, and butyrate, were measured by GC as previously described with slight modifications (88, 104). Cecum content was freeze-dried, homogenized in milliQ H₂O, and the resultant supernatant was filtered through a 0.2µm PVDF filter (Chromatographic Specialties Inc., Brockville, Canada). 1µl of the filtrate was used for injection into the GC (Hewlett Packard 5890 Series II, Canada) using J & W GC columns, CP WAX 52 CB 30 X 0.53 (1.0) (Agilent Technologies, Amstelveen, Netherlands). Injector and detector temperatures were maintained at 240°C and 280°C, respectively. The oven temperature, after an initial period of 1 min at 75°C, was increased to 180°C at a rate of 6°C/min; then increased by 10°C/min, and held at 230°C for 6 min. The final oven temperature was increased by 2°C/min, and kept at 240°C for 5 min. The peaks were identified by comparing their retention times with acetate, propionate, and butyrate GC standards (Sigma-Aldrich, Oakville, Canada). Data collection was managed using HPCHEM software developed by Agilent Technologies, Canada. The SCFA concentrations were corrected for cecum content dry weight and expressed as mg SCFA/cecum.

**Serum lignans**

Serum samples were analysed for plant lignan secoisolariciresinol (SECO) and mammalian lignans (EL and ED). Mouse serum samples (50 µl) were mixed with 100 µl freshly prepared hydrolysis reagent (contained 2 mg/ml β-glucuronidase/sulfatase in 50 mM NaAC buffer, pH 4) and incubated at 37 °C for 19 hr. 600 µl MeOH was added to each sample (80% final MeOH concentration) after hydrolysis. The samples were vigorously vortexed and placed on a shaker for 1 hr, then centrifuged at 20,000 g for 10 min at 4 °C. The supernatant was transferred to a new microfuge tube, and the pellet was washed with 400 µl of 80% MeOH and
then centrifuged. The supernatant was combined and evaporated to dryness using Speed-Vac with solvent compatible ultra-cold trap and vacuum pump. The dried samples were re-dissolved into 50 µl of 80% MeOH by vertexing, sonication for 10 min and centrifuged at 20,000 g for 10 min at 4°C. The supernatant was transferred into HPLC vial inserts and analyzed using LC-MS/MS.

LC-MS/MS analysis of serum samples was carried out by using a SHIMADZU UPLC system (Kyoto, Japan) connected with a triple quadrupole IONICS 3Q Molecular Analyzer (IONICS, Bolton, Canada). The UPLC system consists of two dual-plunger parallel-flow pumps, a membrane degasser, a column oven, an autosampler, and an UV/VIS photodiode array detector. A Kinetex Phenyl-Hexyl 10Å column (100 x 4.6 mm, 2.6 um, Phenomenex, Torrance, CA) with a KrudKatcher ultra in–line filter as guard column was used for separation. Two mobile phases, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in Methanol) were used. The chromatographic elution condition was as following: 0 – 10 min, isocratic 58% B; 10 – 12 min, gradient 58% to 80% B; 12 – 13 min gradient 80% to 100% B; 13 – 15 min, isocratic 100% B; 15 – 16 min, gradient 100% to 58% B; 16 – 23 min, isocratic 58% B. The column oven was controlled at 30 ºC. The flow rate was set at 0.4 ml/min and the injection amount was 5 µl. ESI negative mode was used for the data collection. The optimized ESI conditions using standards of SECO, EL and ED were as follows: the drying gas, 125; the nebulizer gas, 350; the heating gas, 350; the hot source induced desolvation (HSID), 250 degree; the ESI probe temperatures; 300 degree; and the ion spray voltage; – 5800 V. Quantification was accomplished at multiple reaction monitoring (MRM) mode by monitoring a transition pair of m/z 360.66 (molecular ion) / 164.76 (fragment ion) for SECO), m/z 296.68 / 252.80 for EL and m/z 300.67 / 252.78 for ED. The dwell time for MRM data collection was 100 ms. Peak areas
obtained from MRM mode were used to make calibration curves by using least-squares regression with all the values of $R^2$ greater than 0.99. To verify the accuracy of the sample preparation procedure, recovering was determined by calculating the percent recovery of known amounts of lignan standards added to serum from mouse fed basal diet. Serum was spiked with 10 μl of 5 μg/ml of lignan standard mixture (SECO, ED and EL) and hydrolyzed and extracted the same way as unspiked samples. The average recovery rate was over 99%.

**Hepatic cholesterol and fatty acid profiles**

Total cholesterol and free fatty acids (FFAs) were sequentially extracted from snap frozen liver samples (n=12/group). Briefly, pre-weighed liver samples were homogenized in 15 ml methanol using a PowerGene 125 homogenizer (Fisher scientific, Ottawa, Canada) and incubated at 55°C for 15 min. Total lipid was extracted using 15 ml of hexane/chloroform (4:1 v/v) for 15 min, 4 ml of 0.89 % NaCl/water (w/v) was added and the samples vortexed. After centrifugation (1500 rpm for 15 min), the supernatant was collected, 5 ml hexane added and centrifuged again. The top layer was collected, dried under nitrogen gas, saponified using 3 ml of methanol/100 mM KOH solution (94:6 v/v), and heated at 95°C for 2 hr. After adding 1 ml of double-distilled water and 3 ml hexane, samples were shaken and centrifuged. The top layer was collected, dried, reconstituted in 2 ml hexane and used for cholesterol analysis by GC (Agilent 6890, Hewlett-Packard, USA). To each 150 μl of sample, 25 μl of 2 mg/ml 5α-cholestane was added as internal standard. The GC was equipped with capillary flow technology, enabling the detection by both a flame ionization detector (FID) and an electron impact MS detector. Peaks were identified by comparison of their mass spectra with a National Institute of Standards and Technology (NIST) database. Cholesterol separations were conducted
using a HP-5MS column (30 m X 0.25 mm ID, 0.25 µm coating) with constant flow of 1 ml/min.

Corrected areas obtained from the FID signal were normalized to the signal for the internal standard $5\alpha$-cholestane and converted to amount of cholesterol per sample.

The bottom layer was acidified to pH 1.0-1.5 and FFA extraction achieved with 4 ml hexane, shaking and centrifugation. The top layer was collected, dried, methylated into fatty acid methyl esters (FAMES) using 3 ml of methanolic-HCl at 95°C for 1 hr, and 1 ml 0.89 % NaCl/water (w/v) and 4 ml hexane were added and vortexed. The top layer was collected, dried and reconstituted in 1 ml hexane. To each 150 µl of sample, 18.75 µl of 1 mg/ml methyl tricosanoate was added as internal standard for FAMES analysis by GC. Peaks were identified by comparison of their mass spectra with a NIST database. FAMES separations were conducted using a DB-23 column (60 m X 0.25 mm ID, 0.15 µm coating). Corrected areas obtained from the FID signal were presented as relative % of the total FAMES signal.

**Histological and Immunohistochemical Analyses**

For histological evaluation, formalin-fixed distal colon segments were paraffin embedded and 5 µm sections were stained with haematoxylin and eosin and evaluated in a blind manner by a veterinary pathologist (Dr. Geoffrey Wood) using an Olympus BX41 light microscope (Olympus America, Melville, NY). Histological scoring was based on inflammation and crypt dilation. Inflammation severity was graded from 0 (no inflammation) to 3 (severe inflammation with erosions or ulcers), and crypt dilation was graded from 0 to 3, where grade 0 was intact crypts, grade 1 was 20% to 50%, grade 2 was 50%-90% and grade 3 was almost all crypts dilated. The average of the inflammation and crypt dilation scores was used as the total histology score.
The number of apoptotic cells within the colon epithelium of DSS-exposed mice (n=9-11/group) was determined by immunohistochemistry using the active caspase-3 antibody (cleaved caspase -3 (ASPF175) (#9661) (Cell Signaling Technology, Danvers, MA) as previously described (104). Briefly, formalin-fixed, paraffin embedded sections of colon Swiss-rolls were deparaffinised in xylene, rehydrated in graded ethanol, and antigens retrieved by microwave in 10 mM citrate buffer (pH 6.0) 15 min. Sections were incubated in a solution of 3% H$_2$O$_2$ for 10 min to inhibit endogenous peroxidase activity. Sections were then blocked with 5% normal goat serum in TBST for 1 hr at room temperature. Primary antibody (1:300) was prepared in SignalStain antibody diluent (#8112) (Cell Signaling Technology, Danvers, MA) and added to sections overnight at 4°C in a humidified chamber. Washed sections were then treated with SignalStain Boost IHC detection Reagent (HRP, Rabbit) #8114 (Cell Signaling Technology, Danvers, MA) for 30 min in a humidified chamber, followed by liquid DAB+ substrate chromogen system (Dako, Burlington, Canada) for 3.5 min. Sections were counterstained with haematoxylin, dehydrated, and coverslipped using Permount (Fisher Scientific, Ottawa, Canada). Negative control sections were treated the same as above, except primary antibody was replaced with antibody diluent. Sections were viewed blindly (400X magnification) using a BX51 microscope (Olympus America, Melville, NY) equipped with an Olympus DP72 Digital Camera System. The number of positive cells (brown staining) within crypts were counted throughout the colon length (measured using Olympus DP72 imaging), which was further divided into the proximal and mid-distal colon sections, and expressed as number of positive cells/mm colon.

**Distal Colon Homogenization and Protein Extraction**
Snap frozen distal colon samples were homogenized (Powerlyser; Mo Bio Laboratories, Carlsbad, CA) in tubes containing lysis buffer and 5x2.8 mm ceramic beads at 3500 rpm for 20 sec. The lysis buffer contained 200 mM NaCl, 500 mM EDTA, 10mM Trisma HCl, 10% glycerin, 28µg/ml aprotinin, 1 µg/ml leupeptin and 1mM phenylmethylsulfonyl fluoride. Homogenization was followed by 3 rounds of centrifugation (at 4°C and 1,500 g for 15 min) to eliminate cellular debris. The protein concentration was assessed by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and the absorbance was read at 595nm using Bio-Tek microplate reader (Bio-Tek, Winooski, VT).

**Colonic MPO and cytokine levels**

MPO levels, as a biomarker of neutrophil infiltration, were measured in distal colon protein lysates by an MPO ELISA kit according to the manufacturer's protocol (Hycult Biotech, Plymouth Meeting, PA) and expressed as ng/mg protein. IL-1β and IL-6 concentrations were measured in distal colon protein lysates using an ELISA kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN) and were expressed as pg/mg protein.

**Serum cytokines**

Serum cytokines were measured using the Bio-Plex Protein Array System with the Bio-Plex Pro™ mouse cytokine group (6-plex) Th17 Panel A (Bio-Rad, Hercules, CA). Samples were analyzed using a standard range of 0–13000 pg/ml and a sample dilution of 1:4, as recommended by the manufacturer.

**RT2 PCR array for NF-κB pathway**
RNA was isolated from snap frozen proximal colon segments by Trizol/chloroform extraction and purified using the RNeasy kit (Qiagen, Valencia, CA). Samples were DNase treated using the RNase-Free DNase kit (Qiagen, Valencia, CA) and RNA quality was assessed using Experion RNA Analysis kits (Bio-Rad, Hercules, CA). cDNA was prepared using RT first strand kit (Qiagen, Valencia, CA) with 1µg RNA. RT-PCR was performed using SABiosciences RT profiler PCR array kit specific for mice NF-κB pathway (PAMM-025ZA; Qiagen, Valencia, CA) and a viiA 7 real-time PCR system (Life Technologies, Grand Island, NY). Briefly, cDNA was amplified by 40 cycles of 95°C for 15 sec and 60°C for 1 min, using the RT Syber Green/Rox PCR master mix (Qiagen, Valencia, CA). Actb, B2m, Gusb and Hsp90ab1 were used as housekeeping genes to normalize the expression levels. The results were reported as fold change up or down-regulation in gene expression compared to the DSS group.

Analysis of Fecal Microbial Load

Fecal samples were collected from BD- and FS-fed mice (n=8/group) prior to DSS administration and used to determine if FS diet modulated the fecal microbial load. Total DNA was isolated from mouse stool samples (~200mg) using the QiaAmp DNA stool mini kit (Qiagen, Valencia, CA). Real-time quantitative PCR (qPCR) was performed using 16S rRNA gene-specific universal primers Uni331F (5’-TCCTACGGGAGGCAGCAGT-3’) and Uni797R (5’-GGACTACCAGGGTGATCTATCCTGTT-3’) (64) in a 20 µl reaction consisting of 10 µl SYBR Select Mastermix (Life Technologies, Grand Island, NY), 5µl primer mix (1.2 µM each) and 5 µl target DNA. The 16S rRNA genes from bacterial strains *Escherichia coli* K12 and *Clostridium perfringens* Str 13 were amplified and cloned into the pCR4-TOPO vector with the TOPO-TA cloning kit (Life Technologies, Grand Island, NY) for use as standards. Serial ten-fold dilutions of each standard (30 to 300,000 copies) were prepared to generate a standard curve.
for copy number determination. DNA from stool samples (5ul of a 1/1000 dilution) were run
along with the standard curve samples on a Viia7 instrument using the following cycling
conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C
for 20 sec, and 72°C for 45 sec. The default melting curve was run at the end of the reaction.

**Serum Endotoxemia**

The effect of FS diet on the level of serum endotoxins in DSS-exposed mice, as an
indirect biomarker of gut permeability, was assessed using a commercially available Limulus
Amebocyte Lysate Endotoxin detection kit (QCL-1000 LAL, Lonza, Walkersville, MD). All
materials in contact with serum samples and test reagents were endotoxin-free, and the endotoxin
levels were measured in 5-fold diluted serum samples based on manufacturer’s protocol (Lonza,
Walkersville, MD).

**Statistical analysis**

Data are expressed as means ± SEMs. Multiple comparisons were performed by one-way
ANOVA, followed by LSMean post-hoc. For data that were not normally distributed (i.e. colonic
MPO, serum and colon IL6, serum IFNγ), the data was Log transformed to resemble a normal
distribution before running ANOVA test. For nonparametric data (e.g. DAI, histological scores,
serum lignans), Kruskal Wallis analysis was used followed by Dunns post-hoc test. Comparison
between two treatment groups, was performed by two-tailed Student’s t test (serum endotoxins
and PCR data). The level of significance was set at $P<0.05$. Statistical analyses and/or graphical
presentation were performed using SAS Enterprise guide 4.3 (SAS Institute Inc., Cary, NC),
GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA), or Sigma Plot 12.0 (Systat
Software Inc., San Jose, CA).
RESULTS

Seed composition, proximate analyses, dietary fibre, and lignan concentration

After manual separation of whole seed compartments, it was determined that the hull and kernel represent 40% and 60% of the total seed weight, respectively. Nutrient composition and distribution in 100g FS, as well as 60g kernel and 40g hull (which are equivalent to the kernel and hull amount in 100g whole seed) is available in Table 1. The AIN-93G animal diet (basal diet; BD) was formulated to adjust for the oil, protein, available carbohydrate, and total fibre found in the FS, hull, and kernel (Table 2).

FS-based diets increase cecal SCFAs in DSS-exposed mice

Consumption of FS, kernel, and hull diets resulted in enhanced cecal microbial fermentation in DSS-exposed mice as demonstrated by increased SCFA concentrations; acetate, propionate, and butyrate ($P < 0.05$) (Figure 1). Increased cecal SCFA production may have been stimulated by the increased fermentable dietary components (e.g. soluble fiber) within the FS-based diets (Table 1). Although FS contained the highest level of soluble fiber (Table 1), cecal SCFA concentrations were similar between FS, hull, and kernel dietary groups. On the other hand, cecal weight was increased in the FS group only, compared to BD-fed mice (Figure 1).

FS-based diets increase serum mammalian lignans in DSS-exposed mice

Plant and mammalian lignans were measured in serum from BD-, FS-, kernel-, and hull-fed mice in order to demonstrate that the DSS-exposed mice harboured microbiota capable of converting plant lignan (SECO) to the microbial metabolites, EL and ED, as well as to determine
if there were differences between FS diets with regards to lignan bioavailability. Serum lignan concentrations from mice fed BD were relatively low, with a mean total lignan concentration of 0.07±0.04µM. Although highly variable, all FS-based diets similarly increased serum mammalian lignan levels, while only FS and hull diet increased serum levels of SECO compared to DSS group (P<0.05) (Table 3).

**FS-based diets differently modulate hepatic fatty acid profiles in DSS-exposed mice**

Liver fatty acid profiles were measured to determine the potential for FS-based diet to modulate systemic n6- and n3-PUFA levels in DSS-exposed mice. Exposure to DSS in BD-fed mice altered the liver fatty acid profile by increasing the n6-PUFA, linoleic acid (LA) (P<0.05) (Table 4). Compared to the NEG and DSS groups, consumption of FS and kernel diets induced significant effects on the liver fatty acid profile as indicated by a reduction in LA and arachidonic acid (AA), and an increase in ALA, EPA, and DHA; thereby decreasing the n6:n3 ratio (P <0.05). Changes in hepatic fatty acid profiles were less affected by hull diets, however there was a significant increase in DHA and a reduction in AA and n6:n3 ratio (P <0.05), compared to the DSS and NEG groups (Table 4).

**FS-based diets modulate biomarkers of acute colitis in DSS-exposed mice**

Prior to DSS exposure, there were no differences in BW or DI between groups, indicating that the diets were well tolerated by mice (Table 5). 5 day DSS exposure reduced DI and WI, but the differences between groups were not significant (P>0.05) (Table 5). To determine the effect of FS-based diets on the severity of colonic injury and inflammation, DAI was calculated from the daily measurements of mice BW, stool consistency, and stool blood scores. While the DAI
within all DSS-exposed groups increased over the DSS cycle, within FS-based dietary groups, only the FS-fed mice differed from DSS controls. As shown in Figure 2a, at days 4 and 5 of the DSS cycle, FS-fed mice had an increased DAI, which was driven by an increase in the stool consistency score (Figure 2c) and BW loss (Figure 2b), as opposed to changes in stool blood score (Figure 2d), compared to DSS controls. Although the hull diet improved BW at DSS days 4 and 5, and the kernel diet aggravated the stool consistency score in the last day of the DSS cycle, there was no significant effect on the overall DAI induced by the hull and kernel diets (Figure 2a). Interestingly, consumption of FS, kernel, and hull diets prior to exposure to DSS (day 0) significantly increased the stool consistency score (Figure 2b), which may be attributed to the presence of soluble fiber in FS (Table 1), and its water holding capacity leading to softer stools.

Additionally, the colon weight/length ratio (w:l), an established biomarker of DSS-induced colonic inflammation, was increased in all DSS-treated groups (Figure 3). Furthermore, FS worsened DSS-induced changes in the colon weight and length, resulting in a significant increase in the w:l (Figure 3). FS diet also aggravated the colonic histological damage induced by DSS, including inflammation, crypt dilation, and overall histological scores, while kernel and hull diets did not modulate the colon histological damage induced by DSS (Figure 3).

In addition to direct effects in the colon, DSS exposure also induces adverse effects systemically, including the liver (51). In this study, DSS caused a reduction in liver weight which was significantly attenuated in mice consuming hull diet (P<0.05), but not affected by FS and kernel diets (Figure 3). Furthermore, while hepatic cholesterol levels were not significantly modulated in DSS control mice, hepatic cholesterol concentrations were significantly increased in FS-fed mice, compared to both DSS and healthy (NEG) controls (P<0.05) (Figure 3).
FS diet increases colonic and systemic biomarkers of DSS-induced injury and inflammation

Apoptosis

To further determine the extent to which dietary FS exacerbates DSS-induced mucosal injury, the level of epithelial apoptosis was quantified within colon Swiss-roll sections by immunohistochemistry. As shown in Figure 4, apoptotic cells were detected along the luminal surface of the crypts in DSS-exposed colons, which was significantly increased in mice fed FS diet. Furthermore, consumption of FS diet increased the number of apoptotic cells in both the proximal and mid-distal colon indicating that the adverse effects of FS may span throughout the colon and not limited to distinct areas (Figure 4).

Colonic and systemic biomarkers of inflammation

The level of MPO, as a biomarker of neutrophil infiltration, was measured in distal colon protein lysates from DSS-exposed and healthy control mice. As shown in Figure 5, DSS-exposed mice fed BD had elevated colon MPO levels compared to healthy controls (NEG), however, this was further aggravated by FS diet ($P < 0.05$) (Figure 5), indicating an increase in immune cell infiltration in FS-fed mice. An increased inflammatory response in FS-fed DSS-exposed mice was also evident by the increased protein levels of inflammatory cytokines in the colon (IL-1β and IL-6) and serum (IL-1β, IL-6, and IFNγ), while only colonic IL-6 concentrations were elevated in BD-fed DSS-exposed mice (Figure 5). Serum levels of IL-10, IL-17 and TNFα were not significantly affected by DSS or FS+DSS treatment (data not shown).

NF-κB PCR array
As a key mediator of inflammation, the colonic expression of 84 genes involved in NF-κB signalling pathway were compared between the DSS and FS+DSS groups. As shown in Table 6, FS up-regulated the expression of 7 genes in the NF-κB signalling pathway array, including Nfkβ1, Ccl5 and RelB, which have major roles in development of the innate and adaptive immune responses, and polarization, activation, and chemotaxis of a variety of immune cells (80, 103); Bcl2a1a and Birc3, which are involved in regulation of cell survival and apoptosis (50, 100); and activating transcription factor 1 (Atf1) and epidermal growth factor receptor (Egfr), which are involved in colonic epithelial response to injury and DNA damage (24, 42).

**FS diet does not modulate fecal microbial load or gut permeability in DSS-exposed mice**

Next we tested whether the adverse colonic and systemic effects induced by FS diet may be due to modulation of the gut microbiota and/or through enhanced gut permeability leading to microbial translocation or endotoxemia. To measure the bacterial load in BD- or FS-fed mice, DNA was isolated from eight stool samples from each group and total bacterial load was quantified by qPCR using universal primers for the 16S rRNA gene. There was no significant difference observed between the two groups in terms of the number of 16S gene copies/gram of feces (Figure 6). Furthermore, measurement of serum endotoxins did not demonstrate significant differences between BD and FS-fed DSS exposed mice (P>0.05) (Figure 6).

Collectively, these results suggest that FS diet did not exacerbate DSS-induced colonic injury and inflammation through modulation of microbial load or gut permeability.

**DISCUSSION**

The objective of this study was to determine if dietary FS, and its purified hull and kernel seed components, could attenuate the severity of DSS-induced colonic mucosal injury and
inflammation in mice. FS is an oilseed rich in fermentable fiber, phenolic compounds, and n3-PUFA ALA; dietary components with demonstrated anti-inflammatory bioactivities in animal models of colitis (39, 65, 69, 93, 98). Further, FS consumption has previously been associated with reducing colon carcinogenesis (6, 7, 45, 67, 84) and exhibiting potent anti-inflammatory effects in animal models of injury-induced pulmonary disease (17, 55), however, its role as an anti-inflammatory mediator in the colon had not been studied.

Collectively our results show that DSS exposure in male C57Bl/6 mice resulted in colonic mucosal injury and inflammation, as indicated by increased colonic histological damage, neutrophil infiltration, and levels of pro-inflammatory cytokine, IL-6. However, in mice fed 10% FS diet the severity of DSS-induced mucosal injury and inflammation was worsened. Compared to BD-fed DSS-exposed mice, FS-fed mice had increased DAI (Figure 2), colon histological damage scores (Figure 3), number of apoptotic cells (Figure 4), immune cell infiltration and inflammatory cytokines levels (IL-1β and IL-6) (Figure 5), and expression of NF-κB signalling-related genes (Table 6). FS diet also exacerbated systemic indicators of colonic mucosal injury and inflammation by increasing serum inflammatory cytokines (IL-1β, IL-6 and IFN-γ) (Figure 5) and impairing liver metabolic function (Figure 3).

It was initially hypothesized that consumption of FS-based diets would beneficially attenuate DSS-induced colitis severity due to its anti-inflammatory bioactives, in particular n3-PUFA ALA, plant lignans, and soluble fiber. Conversely, although consumption of FS-based diet resulted in an increase in the bioavailability of anti-inflammatory metabolites (DHA and EPA (Table 4), mammalian lignans EL and ED (Table 3), and SCFAs (Figure 1)), this was not associated with anti-inflammatory effects in DSS-exposed mice. In fact, the 10% FS diet, which contained the highest levels of n3-PUFA and soluble fiber, exacerbated colitis severity. Since the
water and diet intake did not differ between groups (Table 5) and thus no differences in the level of DSS exposure, it is likely that dietary component(s) within FS were responsible for driving the adverse effects.

Plant and mammalian lignans have not been previously studied in rodent models of intestinal inflammation, however these compounds have only demonstrated anti-inflammatory effects in a number of in vitro and in vivo studies (1, 16, 26, 71). Furthermore, the hull component of FS contains the majority of FS lignans (Table 1), and thus if the lignans were responsible for the adverse effects of FS, it would be expected that the hull group would also experience similar aggravation of DSS-induced colitis as the 10% FS group. On the contrary, although not reflected in histological scores, mice fed hull diets had improved body weight gain (Figure 2b) and liver weight (Figure 3a) compared to the DSS controls suggesting a slight attenuation of colitis. Thus, the anti-inflammatory potential of lignans may have been overwhelmed by the adverse effects induced by other FS components. Similarly, cecal SCFA concentrations (acetate, propionate, and butyrate) were increased by all FS-based diets (Figure 1). SCFAs, in particular butyrate, provide an important energy source for the maintenance and growth of colonocytes, enhance the integrity of the gut barrier, and exert direct effects within the colonic epithelium through modulation of inflammation-related signalling pathways, in part, through the inhibition of histone deacetylase activity (36, 56, 78). It is thus unlikely that SCFAs would exacerbate colitis severity in our DSS-exposed mice.

On the other hand, since FS diet enhanced SCFA concentrations, cecum weight, and mammalian lignan production, indicative of increased microbial activity, we hypothesized that FS diet may have also modulated the intestinal microbiota; an effect caused by consumption of other prebiotics (3, 95). It is well known that the colonic microbiota plays an important role in
colitis severity in a number of animal models (10, 35, 52, 75), and thus a FS-induced modulation
of the microbiota may have led to enhanced colitis severity in our DSS-exposed mice. In this
study, the fecal microbial load was not affected following FS diet consumption (Figure 6),
suggesting that an increase in total colonic bacteria was not involved in the adverse effects of FS.
Further examination of the fecal microbiota using a metagenomic approach is still required to
determine if the FS diet induced changes to the intestinal microbiota profile.

Without feeding purified FS components (e.g. FS oil (ALA), SDG, or FS mucilage
(soluble fiber)) it is difficult to conclude which FS component(s) were driving the adverse effects
of 10% FS diet, however, there is some evidence from the literature that may help explain our
unexpected findings. For example, although it is generally accepted that n3-PUFAs mediate anti-
inflammatory effects, studies have demonstrated that they can also impair intestinal wound
healing *in vitro* and exacerbate chemical- or pathogen-induced colitis severity in mice (46, 91,
101). The adverse effects of n3-PUFAs in response to colonic injury may be through their
incorporation into colonocyte and mitochondrial membranes, which may increase their
susceptibility to oxidative stress, induce apoptosis, and impede cell signalling pathways
necessary for colonocyte repair (13, 28, 46, 66, 91). In our current study, mice consuming FS-
based diets had increased hepatic n3-PUFAs, including ALA, EPA, and DHA (Table 4).
Furthermore, apoptosis was significantly increased throughout the colon of FS-fed mice (Figure
4), which may thus be mediated through the n3-PUFAs. While all FS-based diets increased
hepatic n-3 PUFAs, mice consuming the 10% FS diet ingested a higher level of ALA (Table 1)
and had the lowest hepatic n6:n3 (Table 4), which may have contributed to the enhanced colitis
severity in this group (Figure 3). In addition, synergist effects of combined FS bioactives within
10% FS diet may have contributed to the enhanced colitis severity in this group. In this regard,
Kolar et al. found that the combination of DHA and butyrate, synergistically increased colonocyte apoptosis by activation of mitochondrial apoptotic pathways, suggesting that combined food bioactives may have a stronger effect than individual bioactives alone (53). While the pro-apoptotic effects of n3-PUFAs is one of the major mechanisms involved in their anti-cancer effects (28), it can potentially impair wound healing and exacerbate mucosal damage in DSS-exposed colons (2, 44, 97).

In addition to having direct effects on the colon tissue, consumption of FS diet was also associated with increased hepatic cholesterol levels in DSS-exposed mice (Figure 3). Previous studies have shown that DSS-induced colonic injury is associated with bacterial translocation to the liver, which leads to alteration in hepatic function and increases hepatic cholesterol levels (32, 43, 61). Considering that consumption of FS is known to reduce cholesterol in mice that are not exposed to DSS (70), the adverse effect of FS on cholesterol metabolism may be related to its adverse effect on colonic injury. FS induced an increase in colonic histological damage (Figure 3), which may result in increased gut permeability and elevated bacterial translocation to the liver, leading to alteration in hepatic function and cholesterol metabolism. Conversely, our results indicate no difference in serum endotoxin concentrations (Figure 6), indicating that the FS diets did not enhance gut permeability in DSS-exposed mice. This may indicate that other mechanisms are responsible for FS-induced aggravation in hepatic cholesterol metabolism.

In conclusion, for the first time we have shown that consumption of a FS-supplemented diet, prior to and during DSS exposure, exacerbates colonic mucosal injury and inflammation in mice. The amount of ground FS supplemented into mouse diets in the current study (10%) has previously demonstrated anti-inflammatory (16), anti-cancer (6, 14, 73), and cardio-protective effects (5), and thus, in addition to the lack of effects on DI and BW in our current study (Table
5), indicates animal tolerance and safety of a 10% FS-supplemented diet. Although FS is known to contain cytotoxic materials such as cadmium and cyanogenic glycosides (9, 12, 85, 86), further studies are required to determine if levels achieved through consumption of a 10% FS diet could exacerbate DSS-induced colonic mucosal injury and inflammation. On the other hand, similar to previous studies utilizing dietary fish oil and its n3-PUFAs (28, 46, 91), FS diet may have aggravated the colitis phenotype in DSS-exposed mice through n3-PUFA mediated impairments in mucosal repair responses and enhanced apoptosis, which would led to increased colonic damage and inflammatory responses.

The relevance of these findings to colitis in humans and its associated diseases cannot be concluded based on the use of a single chemically-induced model of murine colitis. In fact, it has been previously shown that dietary components can have different, opposing effects on colonic inflammation depending on the model used. In this regard, dietary supplementation of tomato lycopene extract reduced LPS-induced inflammation in IL-10-/- NF-κBEGFP mice by blocking NF-κB signaling, while exacerbates DSS-induced acute colitis by increasing epithelial cell apoptosis (49). This finding highlights that the adverse effects of FS in our study may be related to specific DSS-induced mechanisms of intestinal injury. Therefore, additional experiments utilizing other models of colitis (58) are warranted to help better understand the implications of FS consumption on colonic inflammation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.Z. and K.A.P: conception and design of the research, experimental and statistical analyses, interpretation of the results, and manuscript preparation; L.Z., K.A.P, J.T.L, C.Z., and W.W: animal trial; W.W: SCFA analyses; D.L: Analyses of fecal microbial content; G.A.W: histological assessments; J.W: protein analysis in flaxseed components; S.C: dietary fibre analysis in flaxseed components; S.V: purification of flaxseed components; B.F: hepatic fatty acid and cholesterol analyses; R.T: flaxseed and serum lignan analyses; L.R: cytokine analysis by multiplex; All authors approved the final manuscript version.
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100. Werner AB, de Vries E, Tait SW, Bontjer I, and Borst J. Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit its collaboration with pro-apoptotic Bak or Bax. J Biol Chem 277: 22781-22788, 2002.


Figure legends:

Figure 1. The effect of FS-based diets on cecum size and SCFA levels in DSS-exposed mice
Cecum size and SCFA concentrations were measured from DSS-exposed mice fed BD, 10% FS, 6% kernel or 4% hull diets. All FS-based diets increased cecal acetate (b), propionate (c), and butyrate (d), while only 10%FS increased cecal weight (a). Values are means ± SE. *P<0.05 vs. DSS group, n=8/group for cecum weights, and n=10-12/group for SCFAs analysis.

Figure 2. The effect of FS-based diets on DAI in DSS-exposed mice
DAI (summation of BW loss, stool consistency, and stool blood scores) was determined in 5-day DSS-exposed mice fed BD (DSS: △), 10% FS (FS+DSS; ▼), 6% kernel (K+DSS; ♦), or 4% hull (H+DSS; ●) diet for 3 weeks, as well as healthy control mice fed BD (NEG; ■). While DAI (a) increased in all DSS-exposed mice throughout the DSS cycle, consumption of FS diet increased DAI compared to DSS group. Enhanced DAI by the FS group was caused by increased BW loss at day 5 (b) and stool consistency scores at days 4 and 5 (c), without changes in stool blood scores (d). Hull diet improved BW loss score at day 4 and 5, and kernel aggravated stool consistency score at day 5, without affecting DSS-induced DAI (a). Values are means ± SE, *P<0.05 vs. DSS group, n=12/group.

Figure 3. The effect of FS-based diets on colon morphology, histology, and biomarkers of hepatic function in DSS-exposed mice
a) The extent of colonic injury and inflammation caused by DSS was determined by measuring the colon weight to length ratio (w:l), histological damage, and liver weight and cholesterol
concentration. Colon w:l was increased in all DSS-exposed mice, however FS-fed mice had the greatest increase. Colonic histological damage was also increased in mice fed FS diet compared to DSS controls. Relative liver weight was reduced in DSS, FS+DSS, and K+DSS groups, but not in mice fed hull diet (H+DSS). FS diet (FS+DSS) increased hepatic cholesterol levels compared to DSS group and healthy controls (NEG). Values are means ± SE; *P<0.05 vs. DSS group, #P<0.05 vs. NEG control. b) Representative H&E stained distal colon cross sections demonstrate the DSS exposure caused increased histological damage (crypt dilation and inflammation), which was aggravated by FS diet (scale bar = 100 µm).

**Figure 4. The effect of FS diet on DSS-induced colonic mucosal apoptosis**

Cleaved caspase-3 expression was analyzed by immunohistochemistry in colon Swiss-roll sections of DSS-exposed mice fed basal diet (DSS) or FS diet (FS+DSS). a) Total number of positive cells/mm colon within crypt-containing areas quantified along the entire length of the colon and sub-divided into proximal and mid-distal halves. FS diet aggravated DSS-induced apoptosis in proximal, mid-distal and total colon. Values are means ± SE. *P<0.05 vs. DSS group. b) Representative images of mid-distal colon sections of DSS and FS+DSS groups demonstrating positive cleaved caspase-3 immunostaining (brown color) (scale bar = 50µm).

**Figure 5. The effect of FS diet on colonic and systemic inflammatory biomarkers**

Colonic MPO (a) and IL-6 (b) and IL-1β (c), as well as serum cytokines IL-1β (d), IL-6 (e), and IFN-γ (f) were measured from healthy mice (NEG), and BD-fed (DSS) and FS-fed (FS+DSS) DSS-exposed mice. Compared to healthy controls, DSS group had increased colonic MPO and IL-6, while FS-fed mice had increased colonic MPO, colonic and serum IL-1β, colonic and serum IL-
Figure 6. The effect of FS diet on fecal microbial load and serum endotoxins in DSS-exposed mice

Fecal samples were collected from BD- and FS-fed mice prior to DSS exposure to determine if consumption of 10% FS diet modulated fecal microbial load (a). Serum endotoxins (b) were also measured following DSS exposure from mice fed BD or FS diet. FS diet did not modulate fecal microbial load or serum endotoxins ($P>0.05$). Values are means ± SE, n=11/group.
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**Figure 3.** The effect of FS-based diets on colon morphology, histology, and biomarkers of hepatic function in DSS-exposed mice

**Figure 4.** The effect of FS diet on DSS-induced colonic mucosal apoptosis

**Figure 5.** The effect of FS diet on colonic and systemic inflammatory biomarkers

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<table>
<thead>
<tr>
<th></th>
<th>NEG</th>
<th>DSS</th>
<th>FS + DSS</th>
<th>K+DSS</th>
<th>H+DSS</th>
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<tr>
<td>Colon w:l (mg/cm)</td>
<td>16.85±1.27</td>
<td>23.10±1.49#</td>
<td>27.29±2.0#*</td>
<td>22.20±1.0#</td>
<td>21.09±1.18#</td>
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<tr>
<td>Colon histological damage</td>
<td>0</td>
<td>1.15±0.11</td>
<td>2.25±0.17*</td>
<td>1.55±0.09</td>
<td>1.48±0.18</td>
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<tr>
<td>Liver weight (g/Kg BW)</td>
<td>55.07±1.53</td>
<td>47.21±2.06#</td>
<td>49.76±1.19#</td>
<td>50.95±0.9#</td>
<td>52.40±1.27*</td>
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<tr>
<td>Cholesterol (mM/g liver)</td>
<td>1.88±0.08</td>
<td>1.87±0.13</td>
<td>2.33±0.16*#</td>
<td>1.65±0.13</td>
<td>1.87±0.12</td>
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</table>
Figure 4. The effect of FS diet on DSS-induced colonic mucosal apoptosis

a)

![Bar graph showing the effect of FS diet on DSS-induced colonic mucosal apoptosis.](image)

b)
**Figure 5.** The effect of FS diet on colonic and systemic inflammatory biomarkers
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Table 6. The effect of FS diet on the colonic expression of NF-κB related genes
Table 1. Nutrient composition and distribution in FS, kernel and hull

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>FS (g/100 g)</th>
<th>K (g/60 g)</th>
<th>H (g/40 g)</th>
</tr>
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<tr>
<td>Total oil</td>
<td>40.8</td>
<td>31.6</td>
<td>10.8</td>
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<tr>
<td>n6-PUFAs</td>
<td>6.5</td>
<td>4.9</td>
<td>1.7</td>
</tr>
<tr>
<td>n3-PUFAs</td>
<td>22.3</td>
<td>17.4</td>
<td>5.8</td>
</tr>
<tr>
<td>Total dietary fibre</td>
<td>40.4</td>
<td>21.5</td>
<td>19.6</td>
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<tr>
<td>Soluble fibre</td>
<td>9.1</td>
<td>2.1</td>
<td>5.9</td>
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<tr>
<td>Insoluble fibre</td>
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<td>Protein</td>
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<td>Available carbohydrates</td>
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<td>1.3</td>
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<tr>
<td>SDG</td>
<td>1.1</td>
<td>0.07</td>
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Distribution of oil, dietary fibre, protein, available carbohydrates, and secoisolariciresinol diglycoside (SDG) in 100g of flaxseed (FS), 60g of kernel (K), or 40g of hull (H)
### Table 2. Diet composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>BD (g/Kg)</th>
<th>10% FS (g/Kg)</th>
<th>6% K (g/Kg)</th>
<th>4% H (g/Kg)</th>
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<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>173</td>
<td>183.52</td>
<td>191.1</td>
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<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<td>Corn Starch</td>
<td>397.48</td>
<td>408.58</td>
<td>408.31</td>
<td>397.94</td>
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<td>Maltodextrin</td>
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<td>Sucrose</td>
<td>100</td>
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<td>98.68</td>
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<td>70</td>
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<td>Vitamin†</td>
<td>10</td>
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<tr>
<td>Choline Bitartrate</td>
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<tr>
<td>TBHQ‡</td>
<td>0.014</td>
<td>0.014</td>
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<tr>
<td>FS, kernel or hull</td>
<td>0</td>
<td>100</td>
<td>60.07</td>
<td>39.92</td>
</tr>
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</table>

BD, basal diet; FS, flaxseed; K, kernel; H, hull.
All diets contained 20% protein, 7% fat, 5% fiber, and 3.8 kcal/g calorie content. *Mineral mix (AIN-93G-MX), †Vitamin mix (AIN-93-VX), ‡TBHQ, tertiarybutylhydroquinone
Table 3. The effects of FS diets on serum lignan concentrations in DSS-exposed mice

<table>
<thead>
<tr>
<th></th>
<th>DSS</th>
<th>FS + DSS</th>
<th>K+DSS</th>
<th>H+DSS</th>
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<tbody>
<tr>
<td>ED (μM)</td>
<td>0.002±0.002</td>
<td>0.86±0.31***</td>
<td>0.40±0.23*</td>
<td>23.40±13.26***</td>
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<tr>
<td>EL (μM)</td>
<td>0.06±0.04</td>
<td>4.94±2.94***</td>
<td>2.37±1.04*</td>
<td>8.73±4.18***</td>
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<tr>
<td>SECO (μM)</td>
<td>0.003±0.002</td>
<td>0.53±0.39**</td>
<td>0.078±0.046</td>
<td>0.81±0.60*</td>
</tr>
<tr>
<td>Total lignans (μM)</td>
<td>0.07±0.04</td>
<td>6.33±3.6***</td>
<td>2.86±1.26*</td>
<td>35.47±19.60***</td>
</tr>
</tbody>
</table>

Enterodiol (ED), enterolactone (EL), and total lignan concentrations were significantly increased by FS, kernel (K), and hull (H) diets compared to the DSS group, while only FS and hull diet increased serum levels of secoisolariciresinol (SECO). Values are means ± SE, n=9-12. *P<0.05, **P<0.01, ***P<0.001 vs. DSS group.
Table 4. The effect of FS diets on hepatic fatty acid profiles in DSS-exposed mice

<table>
<thead>
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<th>FS + DSS</th>
<th>K+DSS</th>
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<tr>
<td>LA %</td>
<td>17.82±0.86</td>
<td>24.18±0.61</td>
<td>20.11±0.36</td>
<td>20.05±0.37</td>
<td>23.29±0.48</td>
</tr>
<tr>
<td>AA %</td>
<td>8.97±0.8</td>
<td>9.14±0.65</td>
<td>6.43±0.47</td>
<td>5.94±0.58</td>
<td>6.84±0.49</td>
</tr>
<tr>
<td>ALA %</td>
<td>0.14±0.03</td>
<td>0.22±0.04</td>
<td>4.97±0.37</td>
<td>3.79±0.31</td>
<td>1.12±0.05</td>
</tr>
<tr>
<td>EPA %</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td>2.01±0.13</td>
<td>1.22±0.12</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td>DHA %</td>
<td>1.69±0.16</td>
<td>2.15±0.21</td>
<td>7.10±0.33</td>
<td>6.02±0.51</td>
<td>4.23±0.42</td>
</tr>
<tr>
<td>Total n6-PUFAs</td>
<td>26.79±1.54</td>
<td>33.32±0.94</td>
<td>26.54±0.72</td>
<td>25.99±0.76</td>
<td>30.14±0.69</td>
</tr>
<tr>
<td>Total n3-PUFAs</td>
<td>1.84±0.16</td>
<td>2.37±0.24</td>
<td>14.08±0.25</td>
<td>11.03±0.47</td>
<td>5.63±0.42</td>
</tr>
<tr>
<td>n6:n3</td>
<td>15.08±0.59</td>
<td>15.37±1.25</td>
<td>1.89±0.05</td>
<td>2.38±0.05</td>
<td>5.73±0.53</td>
</tr>
</tbody>
</table>

Hepatic fatty acid profiles were measured in healthy (NEG) and DSS-exposed mice fed BD (DSS), 10% FS (FS+DSS), 6% kernel (K+DSS) or 4% hull (H+DSS). Linoleic acid (LA)% and total n6-PUFAs were increased by DSS, while FS and K increased α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)% and reduced LA and arachidonic acid (AA)% and n6:n3 compared to the DSS group. Hull diet increased DHA, and reduced AA and n6:n3 compared to DSS group. Values are means ± SE. *P<0.05 vs. DSS group, #P<0.05 vs. NEG group; n=12/group.
Table 5. The effect of FS diets on body weight, and diet and water intake

<table>
<thead>
<tr>
<th></th>
<th>NEG</th>
<th>DSS</th>
<th>10%FS</th>
<th>6%Kernel</th>
<th>4%Hull</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-DSS Exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial BW (g)</td>
<td>19.68±0.29</td>
<td>20.78±0.22</td>
<td>19.59±0.34</td>
<td>20.52±0.41</td>
<td></td>
</tr>
<tr>
<td>Final BW (g)</td>
<td>22.1±0.45</td>
<td>23.39±0.34</td>
<td>21.89±0.41</td>
<td>22.34±0.42</td>
<td></td>
</tr>
<tr>
<td>DI (g/mouse/day)</td>
<td>2.7±0.05</td>
<td>2.67±0.03</td>
<td>2.49±0.09</td>
<td>2.65±0.11</td>
<td></td>
</tr>
<tr>
<td><strong>DSS Exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DI (g/mouse/day)</td>
<td>2.73±0.06</td>
<td>2.18±0.46</td>
<td>2.2±0.41</td>
<td>2.18±0.42</td>
<td>2.4±0.56</td>
</tr>
<tr>
<td>WI (ml/mouse/day)</td>
<td>3.81±0.61</td>
<td>3.12±0.18</td>
<td>3.09±0.14</td>
<td>3.27±0.31</td>
<td>3.51±0.15</td>
</tr>
</tbody>
</table>

Prior to DSS exposure, consumption of FS-based diets did not modulate body weight (BW) or diet intake (DI) compared to BD-fed NEG controls. During DSS exposure, water intake (WI) and DI did not differ between groups (P>0.05).
Table 6. The effect of FS on the colonic expression of NF-κB related genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>FS+DSS vs. DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl2a1a</td>
<td>2.11***</td>
<td></td>
</tr>
<tr>
<td>Nfkb1</td>
<td>1.19**</td>
<td></td>
</tr>
<tr>
<td>Ccl5</td>
<td>2.05**</td>
<td></td>
</tr>
<tr>
<td>Relb</td>
<td>2.12*</td>
<td></td>
</tr>
<tr>
<td>Birc3</td>
<td>2.16*</td>
<td></td>
</tr>
<tr>
<td>Egfr</td>
<td>1.97*</td>
<td></td>
</tr>
<tr>
<td>Atf1</td>
<td>1.44*</td>
<td></td>
</tr>
</tbody>
</table>

Mice were fed BD or 10% FS diet for 3 weeks. After 5 days of 2% DSS exposure, the expression of NF-κB related genes in proximal colons were analysed. Actb, B2m, Gusb and Hsp90ab1 were used as housekeeping genes to normalize the expression levels. Values indicate the fold change up-regulation in gene expression compared to DSS group. *P<0.05, **P<0.01, ***P<0.001 vs. DSS group. n=5-6/group.