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Altered mouse cecal microbiome-serum enterolignans relationships in response to dietary lignans ingested through whole flaxseed or flaxseed hull

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Flaxseed (FS) is rich in bioactive compounds, including fiber and lignans, which provide health benefits largely mediated by gut microbial metabolism. However, gut microbiota responses, including their relationship with fiber- and lignan-derived microbial metabolites (short chain fatty acids (SCFA) and enterolignans enterodiol and enterolactone), remain unclear. We addressed this through administration of an isocaloric flaxseed (FS) or flaxseed hull (FH) diet to female mice, where FH provided a higher amount of fiber and lignan secoisolariciresinol diglucoside compared to FS. Both diets increased cecal SCFA and serum enterolignans concentrations compared to the basal control diet (BD). Compared to FS, FH increased serum secoisolariciresinol, enterodiol, and total lignans, but not SCFA concentrations. FS and FH increased α- and β-diversity and altered microbiota composition and functional potential compared to BD, but no differences were observed between FS and FH, except for altered abundance of select taxa and a limited number of functions. However, the two diets altered the microbial network structure, including keystone species shifts from *Intestinimonas* in FS to *Carnobacterium* in FH, and taxa relationships with enterolignans and SCFA. Our findings suggest that while intestinal microbiota composition responses to whole flaxseed result in increased circulating enterolignans and intestinal SCFA production, FH can further elevate serum enterolignans *via* reorganization of interactions among taxa.

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1. Introduction

Flaxseed (*Linum usitatissimum*) is a functional food that has been consumed for medicinal purposes since the medieval period in Asia and Europe.[1] Today, it is used in various food products, especially in baked goods and cooking or baking alternatives,[2] with benefits for the prevention and treatment of chronic diseases, such as breast cancer and cardiovascular disease. For example, in preclinical studies, flaxseed provided at 10% of total diet is sufficient to reduce breast tumor growth.[3–5] In human trials, 25 g or 30 g of flaxseed per day resulted in significant reductions in tumor growth in postmenopausal breast cancer patients[6] and lower cholesterol in patients with peripheral artery disease,[7] respectively.

Flaxseed represents the richest dietary source of secoisolariciresinol diglucoside (SDG) (75–800 times higher than other plant foods), the main dietary lignan phytoestrogen. SDG is mainly localized in the flaxseed hull (2.68% of the hull), with a smaller proportion located in the flaxseed kernel (0.12% SDG in kernel).[8] Both the kernel and the hull also contain soluble fiber, accounting for 3.5% and 14.75% of their total fiber content, respectively. Thus, the ingestion of a flaxseed-equivalent amount of flaxseed hull brings about 22 and 4 times more lignan and soluble fiber, respectively, compared to FS.

SDG is a major determinant of flaxseed health properties, and its effects depend on its conversion by the gut microbiota into the enterolignans enterodiol (END) and enterolactone (ENL).[9] END and ENL share structural similarities with 17β-estradiol (E2), enabling them to bind to estrogen receptors and act as partial agonists or antagonists, competing with each other and with E2.[10,11] The generation of enterolignans

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in the intestine relies on a sequential set of reactions performed by a consortium of taxa with overlapping and specific activities. These include the initial deglycosylation of SDG to secoisolariciresinol (SECO) by genera *Bacteroides*, *Bifidobacterium*, *Clostridium*, and *Eggerthella*, followed by demethylation, dihydroxylation, and dehydrogenation steps performed by members of the family *Coriobacteriaceae*, and genera such as *Eubacterium*, *Ruminococcus*, *Bacteroides* and *Lactonifactor*,[12–17] with *Lactonifactor longoviformis*, *Ruminococcus* sp END-1, *Clostridiaceae bacterium* END-2 species ultimately dehydrogenating END into ENL.[18] The majority of humans are capable of producing enterolignans following ingestion of SDG.[19,20] The degree of the conversion of SDG to enterolignans likely depends on the individual composition and metabolic potential of the microbiota.[19–21] Interestingly, fiber intake may enhance microbial conversion of SDG. In humans, higher serum ENL concentration was found to be associated with increased intake of high-fibre foods such as whole-grain products, fruits, and vegetables.[22–25] Soluble fibre is fermented by the gut microbiota, leading to the production of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate. Besides serving as energy source for epithelial cells (butyrate) and being absorbed to reach the circulation, SCFA also take part in syntropic interactions within members of the gut microbiota, contributing to its phenotype.

We previously found that flaxseed-fed mice have greater microbial diversity in their cecum compared to mice fed an equivalent amount of purified SDG.[26] We also observed enrichment of fermentation pathways and of fibre, cholesterol, and polyphenol metabolizing taxa such as the *Coriobacteriaceae* family and *Bacteroides* genus in the flaxseed-fed, but not SDG-fed, mice, suggesting that flaxseed components, particularly fermentable soluble fiber, work together to drive intestinal microbial responses to flaxseed, especially SDG processing. Fiber provision may increase the production of SDG metabolic intermediates by fiber fermenting genera (*e.g.*, *Bacteroides*) thus providing increased substrate to the enterolignan producers. It is also possible that enterolignan producers are metabolically related to the fiber fermenting taxa and thus boosted by fiber intake. Comprehensively, this is aligned with our observation that isolated flaxseed components such as SDG, are not sufficient to recapitulate the microbiota effects that they have when provided at an equivalent amount within the whole flaxseed.[26] We also previously found that consumption of lignans and fiber through a 10% flaxseed diet results in an anti-oncogenic mammary gland phenotype in healthy female mice, which is not observed when the same amount of SDG is provided alone.[27] This is important for clinical recommendations and to support consumers choices. The effects of isolated SDG may be limited by the lack of fiber, either within the supplement or the diet of the individual. Dietary flaxseed hull represents an opportunity to deliver an increasing amount of both flaxseed fiber and SDG, within a food matrix. Thus, this study aimed to investigate the impact of flaxseed and flaxseed hull on the intestinal microbiota and metabolites networks.

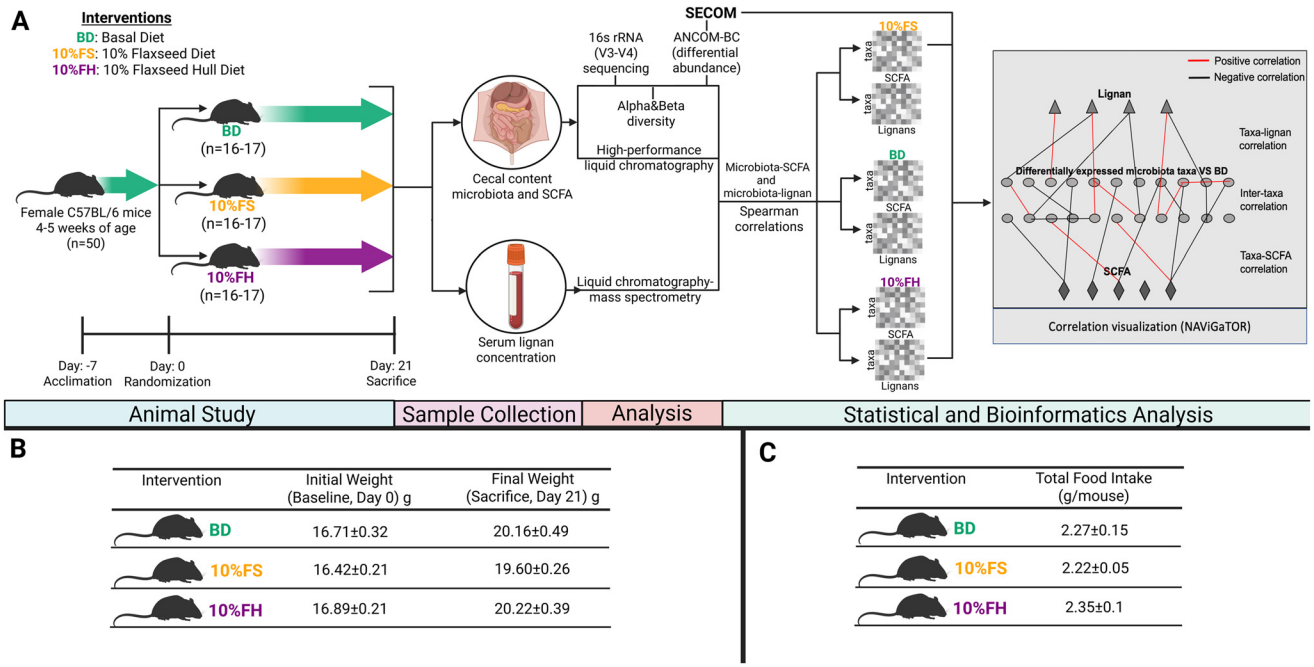
2. Materials and methods

Animal study and sample collection

Fifty C57BL/6 female mice were purchased from Charles River Laboratories (Senneville, QC, Canada) at 4–5 weeks of age, randomized into three groups (n = 16–17 per group) and maintained on a basal modified (see below) AIN93G diet (Basal Diet, BD) for 7 days acclimatization. The mice were housed three per cage at 21 °C and on a 14 : 10 light/dark cycle with high-efficiency particulate filtered air (HEPA). At the start of intervention (Day 0), all mice were inspected for vaginal opening to confirm the onset of puberty and then fed either (a) BD, (b) 10% flaxseed (FS; about 70 g FS day^{-1} for a 70 kg person), (c) 10% flaxseed hull (FH) diets *ad libitum* for 3 weeks (Day 0 to 21) when they were sacrificed (Fig. 1A). The milled FS and FH, prepared from the same batch of FS (cultivar: Bethune), were kindly provided by Natunola (Winchester, ON, Canada); their proximate and fatty acid composition were analyzed by Bureau Veritas (Mississauga, ON, Canada), while SDG content was determined using high-performance liquid chromatography (HPLC) (Table S1). These data were used to design the AIN93G-based study diets,[28] which were prepared by Dyets Inc. (Bethlehem, PA, USA) (Table S2). Specifically, the AIN93G diet was modified such that the fat percentage was increased from 7% to 20%, as in our previous studies,[3–5] and the amount of casein, cornstarch, corn oil, and cellulose were adjusted to compensate for the addition of FS and FH (Table S2). Due to its low n-3 polyunsaturated fatty acid (PUFA) and phytoestrogen content, corn oil was used instead of soybean oil to minimize its potential confounding effect. Diets were isocaloric, made with equivalent macronutrient levels, and stored at 4 °C until needed. We previously confirmed the stability of the diet under these conditions.[29] Diets in cages were replaced every 2 to 3 days. Food intake and body weight were measured twice a week. At Day 21, freshly passed feces were collected and mice were sacrificed *via* CO_2 followed by cervical dislocation and blood was collected *via* cardiac puncture in a serum vacutainer for serum lignan analysis. Cecum tissue and its contents were collected and stored at −80 °C for microbial analysis. All the animal procedures were performed in accordance with the Regulations of the Animals for Research Act in Ontario and the Guidelines of the Canadian Council on Animal Care and were approved by the animal ethics committee of the University of Toronto (Protocol #: 20012299).

Cecal content DNA extraction and microbiota analysis (composition, inter taxa correlations and function)

Total DNA from cecum content (n = 7–8 per group, representing all cages within each group and selected based on body weight closer to the average cage body weight) was extracted using the E.Z.N.A.™ Stool DNA Isolation Kit (Omega Bio-Tek, Doraville, GA, USA) as we did previously,[30] and its quality and quantity were assessed using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Library preparations and sequencing of the 16S rRNA hypervariable region V3–V4 were performed as previously described.[26,31] Pair-



B

Intervention	Initial Weight (Baseline, Day 0) g	Final Weight (Sacrifice, Day 21) g
BD	16.71±0.32	20.16±0.49
10%FS	16.42±0.21	19.60±0.26
10%FH	16.89±0.21	20.22±0.39

C

Intervention	Total Food Intake (g/mouse)
BD	2.27±0.15
10%FS	2.22±0.05
10%FH	2.35±0.1

Fig. 1 Study design (A), initial and final body weight (B) and food intake (C) of mice in the three dietary groups. BD, Basal Diet (control); FS, flaxseed; FH, flaxseed hull; SCFA, short chain fatty acids; ANCOM-BC, Analysis of Compositions of Microbiomes with Bias Correction; SECOM, Sparse Estimation of Correlations among Microbiomes. Created with BioRender.com.

end reads were first quality-filtered and assembled, then clustered at 97% similarity with uclust.[32] Operational taxonomic units (OTUs) were picked using a closed-reference approach and assigned based on the GreenGenes reference database (gg_otus_13_8).[33] The data are deposited at the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra, ID: PRJNA 1365335). Alpha-diversity metrics were used to evaluate microbial richness (Chao 1) and diversity (Shannon index). Beta-diversity analyses (weighted UniFrac distance matrix and Bray–Curtis dissimilarities) were assessed by permutational multivariate analysis of variance (PERMANOVA), followed by principal coordinate analysis (PCoA) (QIIME V2.0). Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) version 2.2.0 was used to determine differentially abundant microbial species.[34] Within each diet, inter-taxa correlations were identified using Sparse Estimation of Correlations among Microbiomes (SECOM)[35] with Spearman correlations, a prevalence cutoff of 0.2, at the species taxonomic level. Correlations with a p-value <0.05 were considered significant. All other parameters were set to default. PICRUSt2 version 2.3.0[36] and MetaCyc database[37] were used to predict microbial pathway abundance in cecal bacterial communities. Differentially abundant MetaCyc pathways were identified with ALDEx2[38] using a generalized linear model and default parameters. Benjamini–Hochberg adjusted p-values below 0.05 were considered significant.

Quantification of *Bacteroides* and total bacteria in cecum contents

Bacteroides and total bacterial load in cecum content were determined by standard curve-based quantitative PCR, using 50 ng and 10 ng of total DNA, respectively, TaqMan™ Gene Expression Master Mix (Thermo Fisher Scientific, CA, U.S.), and primers (*Bacteroides*,[39] total bacteria[40]), as previously described.[39] The amplifications were completed in triplicates using an Applied Biosystems' 7900 HT Real-Time PCR machine equipped with a 384-wells block (Thermo Fisher Scientific), following the default amplification cycle. Data are expressed as log 10 copies per gram of sample.

Serum lignan analysis

Serum concentrations of the lignans enterodiol (END), enterolactone (ENL), and secoisolariciresinol (SECO) (n = 14–16 per group) were determined by liquid chromatography-mass spectrometry (LCMS/MS) as previously described.[8]

Cecal short and branched chain fatty acid analysis

SCFAs and BCFAs were extracted from cecal content and quantified by gas chromatography (GC) (Shimadzu, Kyoto, Japan; Nexis GC-2030) as previously described[41,42] (n = 7–8 per group). A standard curve was created using a serially diluted Volatile Free Fatty Acid Mix (Millipore Sigma, Burlington, MA, USA; 46975-U) to make the following concentrations: 0.078 mM, 0.156 mM, 0.313 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, and 10 mM. Total SCFA was calculated as the sum of acetic, butyric, propionic, and valeric acid concentrations.

Statistical analysis and networks construction

Statistical analyses for microbiota composition data are described above. Kruskal–Wallis test followed by Dunn's *post-hoc* multiple comparisons test (P < 0.05) was performed for

alpha-diversity metrics and qPCR data using GraphPad Prism 8.0.2 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as means ± standard error of mean (SEM). Spearman correlations ($p < 0.05$) were run in SciPy version 1.7.3 and performed between microbial species relative abundance and (1) SCFAs for the BD, FS, and FH diets, (2) BCFAs within the BD, FS, and FH diets, and (3) serum SECO, END, ENL, and total lignans within the FS and FH diets. Networks representing relationships between lignans (except for BD group, not detectable), taxa, and SCFAs were created in NAViGaTOR.[43]

3. Results

FS and FH modify serum lignans and cecal short chain fatty acids concentrations

Mice received a BD diet, or a BD diet modified to provide the same amount (10%) of either FS or FH at the same calories provision for 3 weeks (Fig. 1A). There were no significant differences in initial and final body weight (Fig. 1B), and total food intake (Fig. 1C) among groups. END, ENL, and SECO were detectable in the serum of mice receiving FS or FH but not BD (Table 1). Multiple comparison analysis showed significant differences in END, SECO, and total lignan concentrations, but not ENL, between the 10% FS- and 10% FH-fed groups, with the FH group exhibiting higher concentrations in all lignans. The relative levels of END, SECO, and ENL to total lignans also differed significantly between the two diet groups. While END had the highest proportion in both groups, 54.32% in FS-fed and 64.93% in FH-fed mice, SECO had the lowest, at 0.97% in FS-fed and 3.54% in FH-fed mice. SCFA (acetic acid, propionic acid, butyric acid, and valeric acid, Table 2), and BCFA (isobutyric acid and isovaleric acid, Table 3) were detected in all groups. The concentration of propionic acid, butyric acid, and total SCFA was higher in the 10% FS and in the 10% FH groups compared to BD. No significant differences were found in SCFA and BCFA concentrations between the 10% FS and 10% FH-fed mice.

Table 1 Serum lignan metabolites concentrations and proportions

	BD	10% FS	10% FH	*P*-value
Lignan (nM)				
SECO	ND	15.95 ± 5.908[a]	92.26 ± 20.93[b]	0.0019**
END	ND	746.0 ± 88.17[a]	1480 ± 126.8[b]	0.0002***
ENL	ND	612.1 ± 80.48[a]	714.6 ± 69.40[a]	0.2572
Total	ND	1374.0 ± 145.3[a]	2287.0 ± 175.4[b]	0.0006***
Lignan (%)				
SECO	ND	0.9743 ± 0.3365[a]	3.542 ± 0.7717[b]	0.0057**
END	ND	54.32 ± 3.444[a]	64.93 ± 2.160[b]	0.0172*
ENL	ND	44.71 ± 3.569[a]	31.53 ± 2.323[b]	0.0033**
Total	—	100	100	—

Data are means ± SEM of n = 14–16 per group per metabolite measured. [a,b]Values with different letter within the same row are significantly different by Mann–Whitney comparisons test. ND, nondetectable; SECO = secoisolariciresinol; END = enterodiol; ENL = enterolactone; BD = basal diet; FS = flaxseed; FH = flaxseed hull. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

FS and FH affect cecal microbiota diversity in a similar manner

FS and FH significantly increased microbial richness (Chao 1) in the cecum compared to BD-fed mice ($P < 0.05$, Fig. 2A). The Shannon index (richness and evenness, Fig. 2B) was also higher in FS- and FH-fed mice compared to BD ($P = 0.0008$ and $P = 0.0050$, respectively). PCoA plots for β-diversity (Fig. 2D) showed a separation among the three groups (Bray Curtis, $P = 0.001$; weighted UniFrac, $P = 0.001$).

FS and FH modify cecal microbiota taxa-SCFA correlations in a different manner

Overall, there were 11, 25, and 11 significant microbiota-SCFA correlations in the BD, FS, and FH diets, respectively, and of these, 2, 14, and 5 were positively correlated with SCFAs (Fig. 3A–C; Table S3). Genus *Anaerotruncus* and order *Clostridiales* were positively correlated with propionic acid in the BD diet, with no positive correlations with acetic acid, butyric acid, and valeric acid. In the FH diet, *Clostridium lacta-*

Table 3 BCFA concentration

BCFA (μmol g^{-1} dry feces)	BD	10% FS	10% FH	*P*-value
Isobutyric acid	0.51 ± 0.17[a]	0.31 ± 0.15[a]	0.55 ± 0.09[a]	0.3036
Isovaleric acid	0.51 ± 0.17[a]	0.36 ± 0.16[a]	0.41 ± 0.12[a]	0.6945
Total	1.03 ± 0.34[a]	0.66 ± 0.29[a]	0.96 ± 0.17[a]	0.5186

Data are means ± SEM of n = 7–8 per group per metabolite measured. [a,b]Values with different letter within the same row are significantly different by Kruskal–Walis multiple comparisons test with Dunn's *post hoc* test. BD = basal diet; FS = flaxseed; FH = flaxseed hull. *, $p < 0.05$.

Table 2 SCFA concentration

SCFA (μmol g^{-1} dry feces)	BD	10% FS	10% FH	*P*-value
Acetic acid	31.73 ± 6.16[a]	58.69 ± 14.09[a]	60.11 ± 7.62[a]	0.0609
Propionic acid	3.59 ± 0.69[a]	6.64 ± 1.81[ab]	7.86 ± 0.83[b]	0.0210*
Butyric acid	4.54 ± 0.88[a]	10.33 ± 2.57[ab]	10.75 ± 1.61[b]	0.0236*
Valeric acid	0.50 ± 0.12[a]	0.54 ± 0.12[a]	0.50 ± 0.06[a]	0.9801
Total	40.34 ± 7.72[a]	76.24 ± 18.48[ab]	79.20 ± 9.74[b]	0.0337*

Data are means ± SEM of n = 7–8 per group per metabolite measured. [a,b]Values with different letter within the same row are significantly different by Kruskal–Walis multiple comparisons test with Dunn's *post hoc* test. BD = basal diet; FS = flaxseed; FH = flaxseed hull. *, $p < 0.05$.

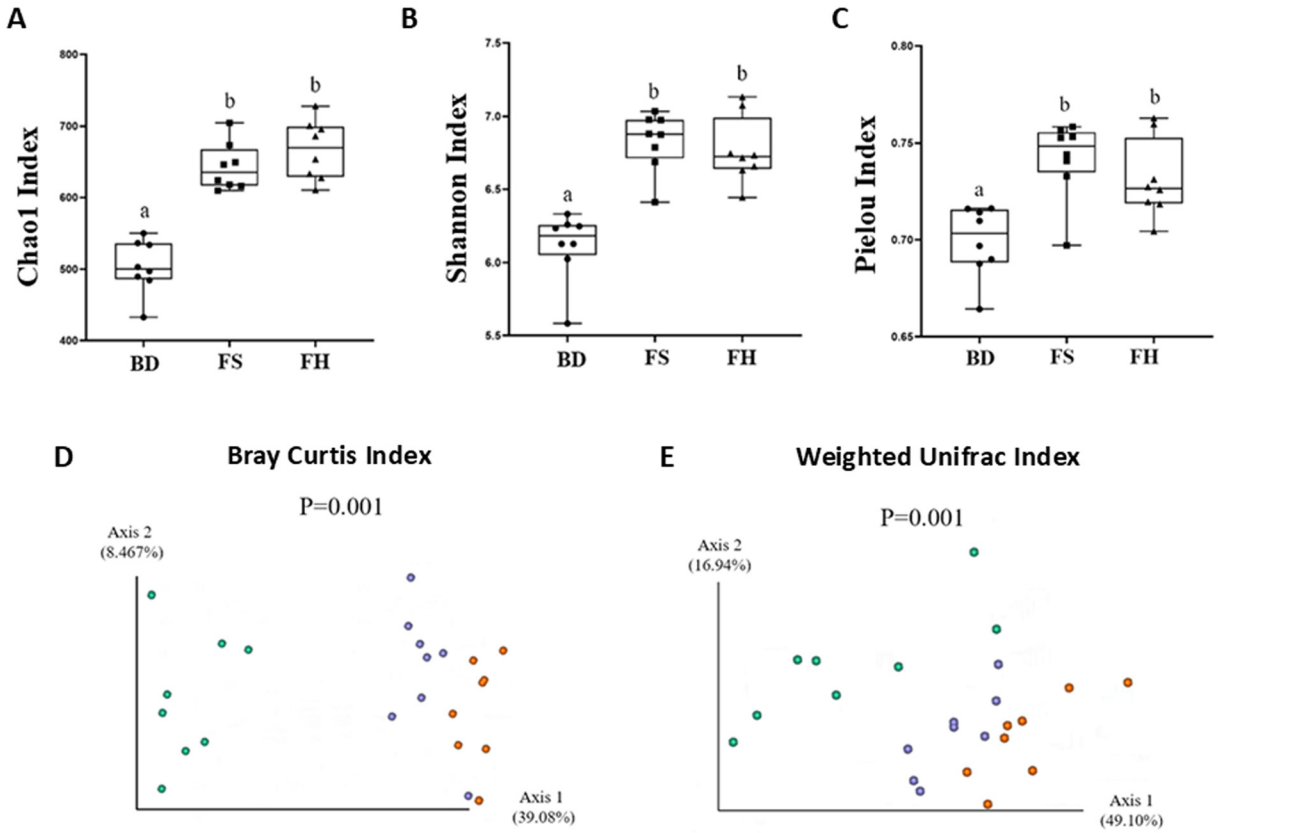


Fig. 2 Flaxseed (FS) and flaxseed hull (FH) affect cecal microbiota alpha- and beta-diversity. Box-plots illustrate Chao 1 (A; richness), Shannon (B; richness and evenness) and Pielou (C; evenness) alpha-diversity indices (n = 8 per group; Kruskal–Wallis followed by Dunn's *post hoc* tests for multiple comparisons, different letters on the top of each box show significantly different groups at $p < 0.05$). Variation in microbial community structure is represented by principal coordinates analysis (PCoA) of the Bray–Curtis dissimilarity matrix (D) and weighted UniFrac (E) indices (N = 8 per group; PERMANOVA). Each dot represents an individual mouse, and the colors represent different dietary groups (blue: FS, green: BD and orange: FH).

tifermentans and *Paraprevotella clara* were positively correlated with butyric acid, *Clostridiales* were positively correlated with propionic acid and *Eubacterium coprostanoligenes* was positively correlated with acetic acid and total SCFA. In the FS diet, genus *Lactobacillus* was positively correlated with acetic acid, butyric acid, and total SCFA; in addition, *Lactobacillus intestinalis* was also correlated positively with propionic and valeric acid. In the FS diet, *Eubacterium coprostanoligenes* was negatively correlated with acetic acid, butyric acid, valeric acid, propionic acid, and total SCFA. In summary, there were more taxa correlating with SCFA in the FS group compared to BD and FH. No correlations were shared across all three groups; however, one correlation was shared between FH and BD (*Clostridiales* and propionic acid, positive in both) and two were shared between FS and FH (*Eubacterium coprostanoligenes* with either acetic acid or total SCFA, positive in FH and negative in FS) (Fig. S1).

Cecal microbiota taxa have more correlations with serum lignans in response to FH than to FS

In the FS diet, there were 10 significant correlations between taxa and SECO, END, ENL, or total lignans, with 9 of these positively correlated (Fig. 3B and Fig. S2; Table S3). Of these, *Lactococcus chungangensis* and *Oscillibacter valericigenes* were positively correlated with ENL and total lignans. *Paraprevotella clara* was positively correlated with END. *Alistipes finegoldii* and *Streptococcus agalactiae* were positively correlated with SECO. Family *Lachnospiraceae* was negatively correlated with SECO. In the FH diet, there were 25 significant taxa-lignan correlations, 15 of which were positive (Fig. 3C and S2). Among these, members of the *Bacteroides* genus were positively correlated with SECO and ENL. Similar to the FS diet, family *Lachnospiraceae* was negatively correlated with SECO, END, and total lignan. *Alistipes finegoldii* was positively correlated with END, while *Alistipes shahii* was positively correlated with ENL. No taxa involved in lignan correlations were in common with those in SCFA correlations in both FH and FS diets.

FH and FS have shared and distinct effects on the cecal microbiota

Compared to BD, there were 32 and 29 significantly differentially abundant taxa in response to the FS and FH diets, respectively (Fig. 4A, B and Fig. S3). Of the differentially abundant taxa between BD and FH, 12 taxa were also found to be

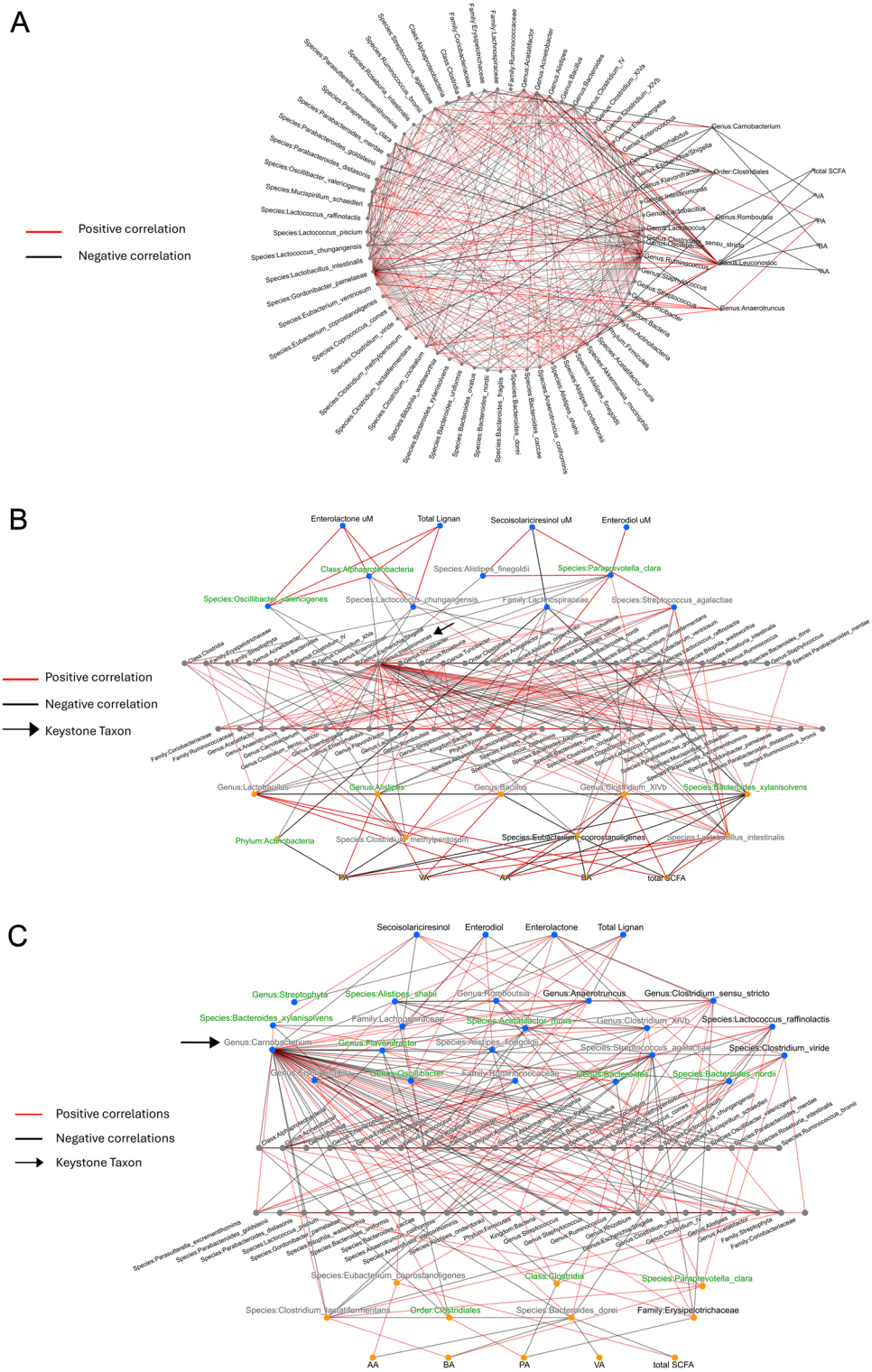


Fig. 3 Flaxseed (FS) and flaxseed hull (FH) modify the relationship among cecal taxa and between taxa and lignan and shorth chain fatty acids (SCFA) in a different manner. Networks show the cecal inter-taxa and taxa-SCFA correlations (assessed *via* Sparse Estimation of Correlations among Microbiomes) in the BD-fed mice (A) and the cecal inter-taxa, taxa-SCFA and taxa-lignan correlations in the FS- (B) and FH-(C) fed mice (significance: $P < 0.05$). Data for SCFA (acetic acid, propionic acid, butyric acid, valeric acid, and total SCFAs) and lignans are from Tables 2 and 3, respectively. Each line within the figures represents a significant correlation (red lines, positive; black lines, negative). Green text indicates higher, black indicates lower, and grey indicates no change in taxa abundance compared to BD. Blue indicates significant lignan-taxa correlations and yellow indicates significant SCFA-taxa correlations (no overlap in species between the two).

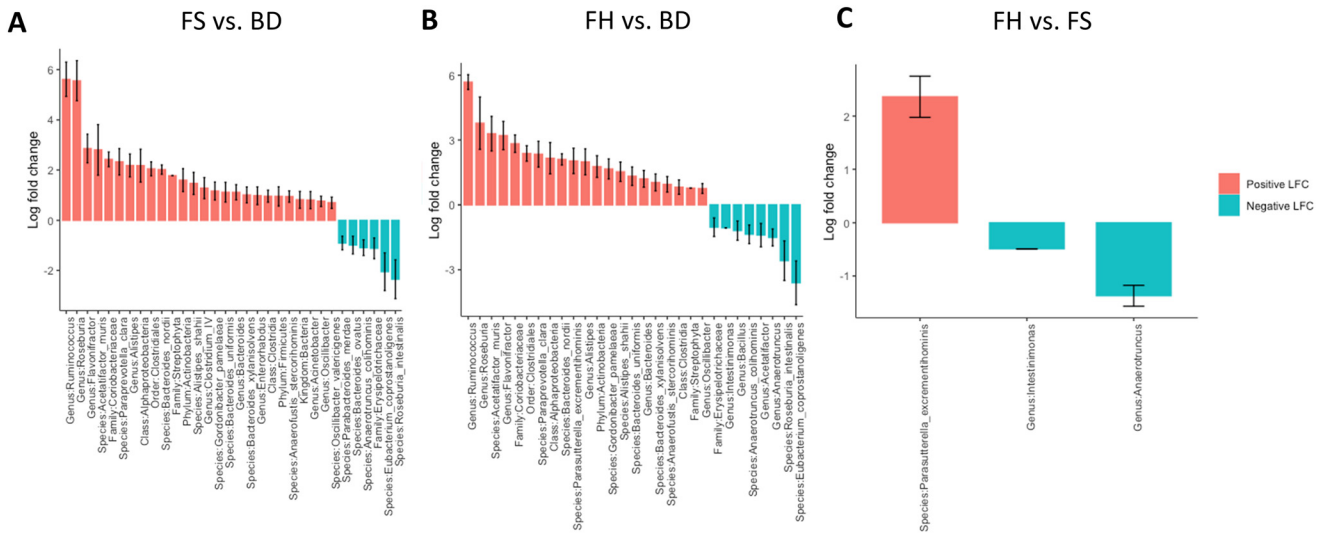


Fig. 4 Flaxseed (FS) and flaxseed hull (FH) modify the cecal microbiota composition with shared and distinct effects *versus* basal diet (BD). Panels A, B and C show the waterfall plots for the FS *vs.* BD (relative abundance of 26 taxa increased, 6 taxa decreased), FH *vs.* BD (relative abundance of 21 taxa increased, 8 taxa decreased) and FH *vs.* FS (relative abundance of 1 taxon increased, 2 taxa decreased) comparisons, respectively (ANCOM-BC analysis).

significantly correlated with serum lignan correlations which included taxa *Streptophyta*, *Oscillibacter*, *Flavonifactor*, *Bacteroides*, *Alistipes shahii*, *Bacteroides xylanisolvens*, *Acetatifactor muris*, and *Bacteroides nordii*. Four taxa were found to be significantly correlated with SCFA concentrations, with significantly increased taxa including class Clostridia, order Clostridiales, *Paraprevotella clara*, and decreased taxa in family Erysipelotrichaceae. In the FS diet, 3 of the differentially expressed taxa were found to be significantly correlated with serum lignans and 4 differentially expressed taxa were found to be significantly correlated with SCFA concentrations (Fig. 3B). Significantly increased taxa correlated with serum lignans in the FS diet included class Alphaproteobacteria, *Paraprevotella clara*, *Oscillibacter valericigenes*. Significantly increased taxa correlated with SCFA concentrations included genus Alistipes, *Bacteroides xylanisolvens*, and phylum Actinobacteria. *Eubacterium coprostanoligenes* was significantly decreased and correlated with all SCFAs. When comparing to FS, three taxa were significantly changed in the FH diet, including decreases in genera *Anaerotruncus* and *Intestinimonas*, and an increase in *Parasutterella excrementhominis* (Fig. 4C).

FH and FS modify inter-taxa correlations in a different manner

In the FS and FH diet, there were 232 and 244 taxa-taxa correlations, respectively (Fig. 3B and C). In the FS diet, genus *Intestinimonas*, which includes butyrate-producing taxa[44,45] had the highest prevalence among intra-diet taxa correlations, with 38/70 negatively correlated with other taxa and 32/70 positively correlated. This taxon was found to be significantly reduced in the FH diet compared to the BD and FS diets. No change in this taxon was detected between the BD and FS diets. In the FH diet, carbohydrate and arginine metabolizing, lactic acid producing genus *Carnobacterium*[46] was the most prevalent among FH-taxa correlations, with 31/55 negatively correlated and 24/55 positively correlated. However, *Carnobacterium* was not found to be significantly different between any two diet groups.

FS and FH have similar effects on cecal microbial functions

A total of 10 and 18 significantly differentially represented inferred metabolic pathways were identified in mice fed FS and FH diets, respectively, compared to BD (Table S4). All the significantly differentially expressed pathways identified in the FS diet were also found to be significant in the FH diet. However, when comparing FS and FH diets, no microbial metabolic pathways showed significant differential representation, resulting in a perfect separation between FS and FH *vs.* BD following unsupervised hierarchical clustering (Fig. S4). Among the significant pathways identified, 5 are superpathways related to the biosynthesis of menaquinol or demethylmenaquinol. Other metabolic pathways, including glycolysis, Bifidobacterium shunt, allantoin degradation to glyoxylate, peptidoglycan biosynthesis, fructuronate degradation, and superpathways for O-antigen building block synthesis, were only found to be significant when comparing FH diet to BD, but not in the FS diet.

4. Discussion

The gut microbiota is necessary to unlock FS benefits, largely through metabolism of its fiber and lignan components. However, its concerted response to these components as provided in a food matrix and altered relationship with the metabolites produced remain unclear. Here we used a clinically

translatable approach, *i.e. via* two functional foods, and studied microbial responses to FS and FH, where FH provides a higher amount of dietary fiber and SDG compared to FS; the diets were isocaloric and used FS and purified FH from the same batch.

As expected, increasing provision of dietary lignans *via* the hull resulted in increased concentrations of most circulating enterolignans. We notice that the mean serum SECO concentration observed in the FS group was lower than reported previously,[47,48] which may reflect differences in FS SDG content across studies (1.5% *versus* 0.93% in the FS here (Table S1)). The relatively lower serum SECO concentration compared to other enterolignans may also indicate its rapid conversion in the intestine, resulting in higher END amounts (as observed). Though, ENL, which is generated from END, was not affected in response to FH. A diverse consortium of bacteria is responsible for lignan degradation. Species such as *Clostridium saccharogumia*, *Eggerthella lenta*, *Blautia producta*, and *Lactonifactor longoviformis* participate in the deglycosylation, demethylation, and dehydroxylation steps required to convert SDG to END and ENL.[18] A greater number of species catalyzing the earlier steps, such as multiple *Bacteroides* species involved in initial transformations and *Eubacterium* species in downstream steps, appears to enhance overall conversion rate. In contrast, the final step from END to ENL is primarily attributed to *L. longoviformis*, suggesting it may be a rate-limiting step, yet an efficient one.[18] Our results detect many species involved in the initial steps of lignan transformation. Interestingly, however, the family Lachnospiraceae, of which *L. longoviformis* is a member, showed significant negative correlations with SECO, END, and total enterolignans in both FS and FH diets. This may explain the lack of significant differences in ENL concentrations between the two diets, potentially due to limited microbial species for the final conversion step. This may lead to bacteria being overwhelmed by the large influx of SDG and END from the FH-diet, suggesting that bacterial metabolic limitations could constrain further increases in SDG intake. Urine clearance can also play a role. Although not assessed here, several human studies have reported dose-dependent increases in urinary END, ENL, and total lignan excretion in pre- and post-menopausal women consuming FS-supplemented diets.[49,50] While END was found to be produced at a higher concentration than ENL after FS consumption,[49] a trend previously seen by Cunnane and colleagues,[51] higher urinary ENL excretion levels than END levels after consumption of FS[52] and SDG extract from FS[53] have been reported. These findings suggest that the relative proportions of serum END and ENL in response to increased lignan intake are variable and may depend on the individuals' microbiota and excretion rates.[54] Assessing concentrations of enterolignans in intestinal content and of conjugated enterolignans in serum may have helped clarifying our findings. Individual differences in enterolignan metabolism, influenced by gut microbiota composition, have shown that key enterolignan producers are often found within the Ruminococcaceae and Lachnospiraceae families. Rikenellaceae (notably *Alistipes*) have been identified as one of the most abundant and diverse contributors to ENL production, alongside Ruminococcaceae.[55] Specifically, *Alistipes shahii* was significantly associated with plasma ENL concentrations following lignan intake in men.[56] This relationship aligns with our findings, where *A. shahii* was positively correlated with ENL in the FH diet, supporting the cross-species relevance of this association. It will be important to conduct FH studies in male mice, to further investigate conserved and distinct responses across sexes, and to better understand implications for disease prevention and treatment.

Select genera, such as *Ruminococcus* and *Bacteroides*, may contribute to both lignan and fiber processing. Although we lack metagenomics data and can thus not investigate species and their specific metabolic functions, the relative abundance of *Ruminococcus* and *Bacteroides* spp, associated with the largest increase in response to FS and FH *vs.* BD, did not differ between FH- and FS-fed mice. Aligned with this, SCFA cecal concentrations increased in response to FH and FS *vs.* BD, but not between FH and FS. Although SCFA concentrations do not always positively correlate with dietary fiber intake,[57] these data may indicate a plateau in fiber fermentation capacity, and reflect the minimal difference in microbiota composition. Taken together with the observation made above about SDG conversion, this suggests that higher daily intake of FS may not necessarily result in increased lignans and SCFA production.

To better understand the response of lignans and SCFA to FS and FH diets, we examined the microbiota structure, inter-taxa relationships, and relationships between taxa and metabolites. Microbiota α-diversity increased in response to FS, aligned with previous studies,[26,48] and to FH. Beta-diversity analysis demonstrated distinct separation of the microbial community present in the FS- and FH-fed mice. Differences in the functional potential were related to FH substrate input (*i.e.*, fructuronate degradation). Although neither α- nor β-diversity significantly differed between FS and FH, and only three taxa were differentially abundant, these two diets altered the way taxa interact in a different manner. When comparing the FS and FH diet groups, there is a shift in keystone species (involved in the highest numbers of intra-diet taxa correlations) from the genus *Intestinimonas* in FS to *Carnobacterium* in FH. The differently altered microbial networks were accompanied by different relationships between taxa and metabolites (lignans and SCFA) concentrations. Within the FS diet, seven taxa were significantly correlated with serum lignans. Only one of these correlations was shared with the 25 taxa-lignan correlations in the FH diet. This suggests that the increased proportions of fibre and SDG in FH may promote the recruitment of a unique set of taxa to increase symbiotic SDG conversion to enterolignans.

Our results also demonstrate overlapping taxa that are shared across diets but exhibit different significant correlations. *Eubacterium coprostanoligenes* showed opposite correlations with SCFAs in the FH and FS diets. While *E. coprostanoligenes* plays an important role in cholesterol

reduction by converting cholesterol to coprostanol and thereby limiting its reabsorption,[58,59] the genus *Eubacterium* also includes members known to produce SCFAs, particularly butyrate. It is possible that the shift from butyrate to acetate-dominant fermentation between FH and FS diets influences the metabolic role of *E. coprostanoligenes*, leading it to participate more actively in fiber fermentation under the FH diet. There is a substantial body of literature supporting flaxseed benefits in the context of cardiovascular health. This study did not assess characteristics of cardiometabolic health (*i.e.* lipid, glucose metabolism). However, because high doses of FS (30–40 g day^{-1}) were found to lower cholesterol clinically,[7,60] these findings suggest that FH could be an important option in this context; future studies are required. Additionally, compared to the BD diet, *Paraprevotella clara* is positively associated with END in the FS diet and with butyric acid in the FH diet, which may reflect an indirect role in butyrate production. In the FH diet, *P. clara* is also positively correlated with *Lactococcus raffinolactis*, which produces lactate, and *Clostridium lactatifermentans*, which ferments lactate into butyrate. Notably, *L. raffinolactis* also shows a significant positive correlation with ENL. These correlations suggest that *P. clara* may contribute to cross-feeding interactions that support the growth or activity of butyrate-producing species.

Taken together, our findings suggest that both FS and FH modify the intestinal microbiota composition and diversity resulting in enterolignans and SCFA production. However, FH can further elevate serum enterolignans concentrations *vs.* FS without affecting microbiota diversity or taxa abundance but rather *via* reorganization of syntropic interactions among taxa. Therefore, FH may represent a viable opportunity to enhance FS-derived benefits *via* gut microbiota modulation.

Author contributions

Conceptualization: LUT, EMC. Data curation: MK, AT, DW, YTC, LLD, FL, SC. Formal analysis: MK, AT, DW, LLD, YTC, SC, FL, RL, RT, YY. Funding acquisition: EMC, LUT, KAP. Investigation: MK, AT, YY. Methodology: MK, AT, DW, RL, RT, KAP, LUT, EMC. Project administration: AT, RL, RT, KAP, LUT, EMC. Resources: RL, RT, KAP, LUT, EMC. Software: DW, LLD. Supervision: RL, RT, KAP, LUT, EMC. Validation: FL, SC, DW, LLD, YTC. Visualization: MK, DW, YTC, LLD, YY, SC, FL. Writing – original draft: MK, AT, DW, YTC, FL, SC, EMC. Writing – review and editing: all coauthors.

Conflicts of interest

The authors declare no conflict of interest.

Abbreviations

BD Basal diet
END Enterodiol
ENL Enterolactone
FS Flaxseed
FH Flaxseed hull
SDG Secoisolariciresinol diglucoside

Data availability

The 16S sequencing data generated here are available at the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra, ID: PRJNA1365335).

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d5fo03558a.

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