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RESEARCH PAPER

Diet alters the effects of lipopolysaccharide on intestinal health and cecal microbiota composition in C57Bl/6 male mice

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Abstract

Lipopolysaccharide (LPS), a component of the gram-negative bacteria, induces an inflammatory cascade in mice, negatively impacting aspects of the microbiota gut-brain axis (mGBA). Flaxseed (FS), an oilseed enriched in dietary fiber and n3 poly-unsaturated fatty acids, has been shown to partially attenuate LPS-induced systemic and neuroinflammation. In this study, we investigated the impact of FS and FS oil (FO) diets on microbial dysbiosis, biomarkers of intestinal health, hepatic inflammation and oxidative stress, and metabolic homeostasis in male mice, 24-hours post LPS-exposure. Compared to saline-treated mice, LPS mice showed diet-dependent shifts in the cecal microbiome. Most notably, LPS-treated basal diet (BD)-fed mice had reduced *Muribaculaceae* and *Lachnospiraceae*, FS-LPS mice had elevated *Akkermansia* and *Enterobacteriaceae*, and both the FS-LPS and FO-LPS mice had increased *Bacteroides*. LPS increased cecal short-chain fatty acid concentrations, the highest of which were found in FS-fed mice. Intestinal health biomarkers were modulated by LPS in a diet-specific manner such that ileal mucous content was elevated in FS- and FO-fed mice, while LPS-induced inflammation (IL-1β) was attenuated in FS-fed mice. On the other hand, LPS-induced hepatic inflammation and oxidative stress, which were not attenuated by FS or FO diets. *Bacteroides* abundance and serum Il-10 levels, and cecal butyrate concentrations and hippocampal IL-6 mRNA, were negatively correlated in FS-fed mice only, suggesting a potential role of the microbiome in the anti-inflammatory effects of FS post-LPS treatment. Collectively, LPS exposure negatively impacted the cecal microbiome and markers of intestinal, hepatic, and metabolic health, the former being beneficially altered by FS diet.
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Keywords: LPS; Gut-brain axis; Microbiota; Anorexia; Flaxseed; Short-chain fatty acids; Intestinal homeostasis.

1. Introduction

Lipopolysaccharide (LPS) is an endotoxin found on the outer membrane of gram-negative bacteria that can trigger an acute inflammatory and anorexic response in the host organism [1,2]. In the GI tract, LPS has been shown to induce physiological changes in the ileum and proximal colon, including increased mucous production [3,4], elevated intestinal permeability [5,6], and inflammation [7]. Further, in recent years, studies have highlighted that an exogenous LPS-challenge results in a dysbiotic gut microbiota [8–11], collectively indicating widespread negative impacts of LPS on gut health. In addition to previous research highlighting inflammatory impacts of LPS both systemically (i.e. serum, liver) [12] and in the brain [13], we previously reported that an LPS challenge induces negative effects throughout the microbiota gut brain axis (mGBA), in a background-diet dependant manner [8]. More specifically, we reported that dietary supplementation with flaxseed (FS), but not FS oil (FO), partially attenuated the LPS-induced effects across the mGBA (i.e. improvements to systemic- and neuro-inflammation), which may be linked to diet-induced alterations of the fecal microbiota composition and function [8].

Flaxseed (FS) is an accessible source of dietary fiber, n3 polyunsaturated fatty acid (n3-PUFA) (alpha-linolenic acid (ALA)), and a key lignan (secoisolariciresinol diglucoside [SDG]). These components, or their downstream metabolites, have been shown to induce anti-inflammatory and/or antioxidant effects across the mGBA

Abbreviations: LPS, lipopolysaccharide; SAL, saline; mGBA, microbiota-gut-brain axis; SCFA, short-chain fatty acid; FS, flaxseed; FO, flaxseed oil; BD, basal diet.

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both *in vivo* [14–16], and *in vitro* [16,17]. In the gut, supplementation with dietary FS results in morphological changes in the intestinal wall architecture, including increased colon crypt height, goblet cell density, and epithelial mucous content [18]. In line with these structural changes, a human study has shown that dietary supplementation with FS improves intestinal barrier integrity [19]. Dietary FS has also been shown to result in potentially beneficial changes to the fecal and cecal microbiota composition and function in both human (fecal only) and rodents, including elevations in the short-chain fatty acid (SCFA) producing bacteria *Lachnospiraceae* [8,18,20], *Muribaculaceae* (formerly *S24-7*) [8,18], and *Roseburia* [18,20,21]. In female mice, dietary FS has been shown to induce site-specific (i.e. cecal vs. fecal) impacts on the microbiota composition [20], which is relevant given that the cecal microbiota may be a more optimal sampling site for determining environmental/dietary effects on the microbiota [22]. Dietary supplementation with FS increases the production of SCFAs [8,18], which have established beneficial roles across the mGBA [23]. Of note, SCFAs have been shown to attenuate intestinal inflammation [24], permeability [25] and neuroinflammation [26,27]. Collectively, there is a breadth of literature indicating that dietary FS can significantly alter intestinal physiology, which may be coupled to alterations in the gut microbiota and its potential in attenuating inflammation across the mGBA.

Given the anti-inflammatory potential of dietary supplementation with FS, we wanted to further investigate potential mechanistic targets. The liver is a critical component of communication across the mGBA [28,29], especially related to its role in clearing endotoxins (i.e. LPS) from circulation [30]. Exogenous LPS is well established to induce hepatic inflammation through interactions with the LPS receptor, toll-like receptor 4 (TLR4) [12,30–32]. Dietary supplementation with FS is shown to induce anti-inflammatory responses in mice [33] and human [34] liver in response to injury. This may be explained by the FS-induced elevated production of SCFAs, especially since SCFAs (primarily butyrate) have been shown to attenuate LPS-induced hepatic inflammation [35]. Given these highlighted benefits, the partial attenuation of the LPS-induced effects across the mGBA in FS-fed mice [8], may be related to the anti-inflammatory/anti-oxidant impacts on the liver. Taken together, we hypothesized that dietary supplementation with FS will result in improved intestinal and hepatic health through modulation of the intestinal structure, the cecal microbiota composition and SCFA production, which will result in diet-dependent attenuation of the LPS-induced inflammatory response across the mGBA.

2. Materials and methods

2.1. Study design

To address our hypothesis, we expanded the analysis from our previous research [8]. Seventy-two male C57Bl/6 mice (5-weeks-old) (Charles River, Kingston, NY, USA) were single-caged and acclimatized for 1-week on a purified basal diet (BD) (AIN-93G with 7% corn oil) (Envigo, Madison, WI, USA), after which they continued to feed on either, BD, a BD supplemented with 10% whole ground FS (FS), or a BD supplemented with 4% FO (FO) (n=24/diet). After 3 weeks of dietary supplementation, the mice were randomized by BW, and were subjected to an intraperitoneal (i.p) injection of 1 mg/kg BW LPS or an equal volume injection of saline (SAL) (n=12/treatment/diet). The mice were monitored for 24 hours and euthanized by decapitation. Tissues, serum and cecal content were collected and stored at −80°C until use. The mice were maintained on a 12-hour light:dark cycle, with room temperature set to 23°C and humidity at 40%. All experimental conditions were approved by the University of Ottawa Animal Care Committee (#HSe-2857-R3). For a graphical depiction of the study design, see Figure 1.

2.2. Assessment of intestinal health

2.2.1. Cecal DNA extraction and 16S rRNA gene sequencing

In this study, the cecal content was collected at euthanasia and immediately placed in a prechilled microcentrifuge tube on dry ice then was stored at −80°C. Cecal DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen #51604, Germany), with the recommended protocol. DNA concentration was determined using the Quant-iT PicoGreen dsDNA Assay kit (Thermo Fisher Scientific, #P11496) as per the manufacturer's instructions. The 16S rRNA library (V3-V4 regions) was prepared following Illumina guidelines.

2.2.2. Data processing and analyses

Data processing and bioinformatics were performed as highlighted in our previous study [8] with minor modifications. In brief, the data was processed and analysed using Qiime2 (Version 2023.9) [36], and was mapped to GreenGenes classifier 13.8 [37]. The microbial β-diversity (Bray-Curtis) was visualized using MicrobiomeAnalyst [38,39], and the font size and type were altered to improve visibility using InkScape (V1.2.1, Inkscape developers). Differences in taxa relative abundance (RA) were assessed using multiple Mann-Whitney U tests with a 10% FDR correction, while the differential abundance (DA) was assessed using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) [40]. The taxonomic bar plots and the α-diversity plots were generated using GraphPad Prism 10 (v10.1.2).

2.2.3. Quantification of cecal short chain fatty acids (SCFAs)

The cecal SCFAs were prepared and quantified as described in our previous study with slight modifications [8]. The cecal SCFAs in this study were diluted 20x, as opposed to the 10x diluted fecal SCFAs described previously.

2.2.4. Histomorphological assessment of the ileum and proximal colon

At euthanasia the proximal colon (PC) and ileum were excised, and a small (~1cm) piece of each section was snap-frozen in

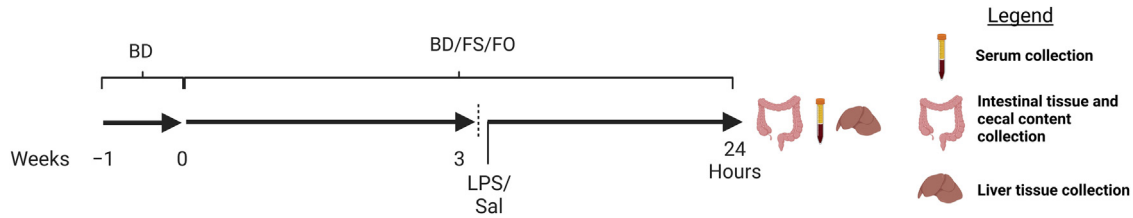


Fig. 1. Experimental design. Following 3 weeks of dietary supplementation, BD-, FS-, and FO-fed mice were randomized and subjected to an i.p injection of 1mg/kg LPS or an equal volume of SAL. Twenty-four hours post injection, mice were euthanized and intestinal tissue, cecal content, serum, and liver were collected. Created in BioRender. Livingston, D. (2025) https://BioRender.com/d87r098.

liquid nitrogen and stored at −80°C until later analysis. An additional section (~1cm) of both the PC and ileum were placed into histology cassettes, fixed in 10% formalin for 24 hours, and then transferred and stored in 70% ethanol at 4°C. The sections were then paraffin-embedded and sectioned (4μm) by the University of Ottawa Histology Core (Roger Guindon Hall, Ottawa, ON). PC and ileum cross-sections were stained with hematoxylin and eosin (H&E), and Alcian Blue (AB) for visualization and assessment of crypt length, villi height, goblet cell density and mucous content as described previously [18,41–45]. A Zeiss AXIO Imager M2 equipped with Axiocam 506 mono microscope was used to capture images at 10X magnification for analysis. Image J (NIH) was used to measure crypt length or villi height from the basal epithelial layer to the lumen from 20 to 25 fully elongated crypts/villi. Goblet cell density was determined by counting the number of goblet cells in 20–25 fully elongated crypts/villi, and calculating the average goblet cells per crypt/villi. Mucous content was calculated by tabulating the total area covered by alcian blue stain on all viable sections (excluding any mucous in the lumen) relative to the epithelium area (excluding the lumen), highlighting the density of mucous within the villi/crypts/goblet cells. The final presented values were the average of 2 fully trained independent scorers.

2.2.5. Quantitative PCR of intestinal and hepatic tissue

In addition to the PC and ileum, we similarly snap-froze sections of liver to use for qPCR analysis. From each tissue, RNA was extracted, and cDNA was synthesized as previously described [8]. Quantitative PCR was then used to measure the relative mRNA expression of several genes related to inflammation, intestinal permeability, mucous production and oxidative stress (Supplementary Table S1). Housekeeping genes were selected based on stability within each tissue type: PC (PPIA), IL (GAPDH), Liver (PPIA+EEF2). The relative gene expression was then calculated using the ΔΔCq method, using the housekeeping gene as a reference.

2.3. Serum analyses

Circulating markers of blood glucose homeostasis (insulin and glucagon), appetite (GLP-1, GIP, ghrelin and leptin), and metabolic syndrome (PAI-1, resistin) were assessed using the Bio-Plex Pro Mouse Diabetes 8-Plex Assay (Bio-Rad, #171F7001M) as per the manufacturer's instructions. The multiplex was read using the Luminex xPonent for Magpix (v4.2) and analyzed using Milliplex analyst (v5.1.0.0) (VigeneTech Inc., Carlisle, MA, USA).

2.4. Statistical analysis

All data was subjected outlier testing using the ROUT method (q=1%), followed by normality and homoscedasticity testing (D'Agostino–Pearson or Anderson-Darling, and Brown-Forsythe respectively). Two-way ANOVA with Tukey's multiple comparison's test were used to make comparisons across both diet (i.e. BD/FS/FO) and treatment (i.e. SAL/LPS) when assumptions were met. When normality and/or homoscedasticity were not achieved, the data was log-transformed prior to 2-way ANOVA. If data did still not meet assumptions, a one-way ANOVA was attempted. Non-normally distributed datasets were assessed by the nonparametric Kruskal-Wallis test. Spearman correlations were performed given the variable normality of our data. All figures show nontransformed data. Detailed statistics can be found in Supplementary Table S3.

3. Results

3.1. The impact of LPS on the cecal microbiota diversity and composition 24-hours post-injection are dependent on the background diet

In our previous study, we showed LPS and diet-specific impacts on the fecal microbiota composition [8]. The cecum may be a more relevant sampling location for determining mechanistic impacts of diet on the microbiota [22]. Therefore, we investigated the effects of LPS and diet on the diversity and composition of the cecal microbiota collected 24-hours post-LPS/SAL injection. A 2-way ANOVA revealed significant effects of diet on α-diversity metrics (Shannon [P=.0012], Observed features [P=.0403], Evenness [P=.020]), although there were no treatment or interaction effects (Fig. 2A, Supplementary Table S3). The effect of diet was driven primarily by a reduction in α-diversity in the FO-LPS mice compared to the FS-LPS mice (P=.0026). FS induced a significant shift in cecal β-diversity (Bray-Curtis) compared to BD or FO in our SAL-treated mice (q=0.0001), while no differences were found between BD and FO (q=0.198) (Fig. 2B). To determine if LPS altered the cecal microbiota community structure, PEMANOVAs were performed between SAL and LPS challenged mice (within diet), and we found that β-diversity was shifted significantly, regardless of background diet (Fig. 2C-E).

In SAL-treated mice, the FS-induced shift in β-diversity was driven by changes in the relative abundance (RA) of select microbial groups. At the phylum level, dietary supplementation with FS elevated Firmicutes, and reduced Deferribacteres and Tenericutes (Supplementary Table S2). At the genus level, we observed FS-induced increases in *Lachnospiraceae, Bacillaceae, Clostridium* (*Lachnospiraceae* family) and *Ruminococcus* (*Lachnospiraceae* family), and decreases in *Dorea, Defluviitalea, Parabacteroides, Anaeroplasma, Mucispirillum, Anaerotruncus, Oscillospira* and *Clostridium* (*Ruminococcus* family) compared to BD (Fig. 3A, Supplementary Table S2). Consistent with the lack of effects on β-diversity, there were no differences in the RA of microbes between the FO-SAL and BD-SAL groups (Fig. 3A, Supplementary Table S2). Notably, RA analyses assess proportional changes in the microbiota between groups, however this approach is highly dependent on the inter-sample sequencing depth [46]. Therefore, we performed additional comparisons using differential abundance (DA) analysis and tested our hypotheses using ANCOM-BC, which considers the estimated sampling fraction of the microbiota [40]. Using this approach, there were 10 significantly enriched taxa between the FS and BD mice induced by dietary supplementation with FS (FS-SAL vs BD-SAL), including elevated *Muribaculaceae* (formerly *S24-7* [47]), *Bacillaceae, Bifidobacterium,* Clostridiales, *Enterobacteriaceae* and *Bacteroides,* amongst others) (Fig. 3B). There were similarly 10 enriched taxa between the FS and FO mice, 8 of which overlapped, excluding the FS-induced elevations in *Enterobacteriaceae* and *Bifidobacterium* compared to the BD- fed mice (Fig. 3B). Notably, compared to the BD- fed mice, the FO-fed mice had one enriched taxon (*Roseburia*) (W=−11.58, q=0.026) (data not shown).

We then investigated the impact of LPS on the cecal microbiota composition within diet, twenty-four hours post-LPS-challenge. We highlighted both proportional (RA) and differential (DA) changes in the cecal microbiota across all diets. From an RA perspective, we saw diet-independent impacts of LPS on 2 highly prevalent taxa, specifically, a reduction of *Muribaculaceae*, and increased *Bacteroides* (Fig. 3A, Supplementary Table S2). Notably the RA of *Akkermansia* was significantly enhanced by LPS in the FS- and FO- mice, but not the BD mice (Fig. 3A, S1, Supplementary Table S2). There were diet-dependent impacts of LPS on the cecal microbiota including an increase in Proteobacteria, Verrucomicrobia, *Parabac-*

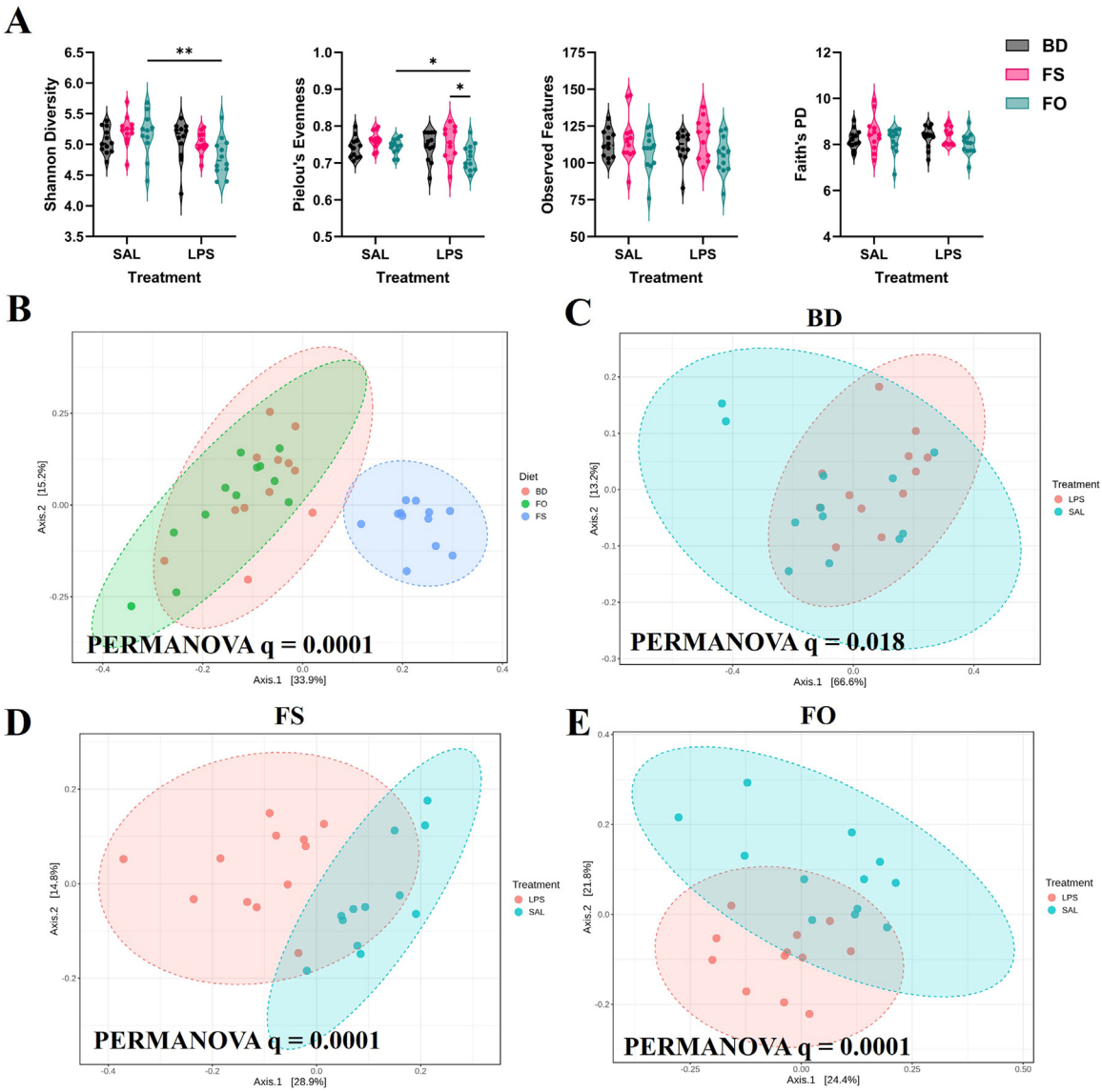


Fig. 2. The effects of dietary supplementation with and without an LPS challenge on the cecal microbiota diversity. (A) α-diversity metrics, including Shannon, Pielou's evenness, observed features and Faith's PD. (B) Bray-Curtis dissimilarity index (β-diversity) between diets within the SAL treated mice. (C-E) Bray-Curtis dissimilarity index between SAL- and LPS- treated mice within diets. α-diversity was assessed using 2-way ANOVAs with Tukey's multiple comparison's test. β-diversity was assessed using PERMANOVA. n=11–12/group. Detailed statistics can be found in Supplementary Table S3.

teroides and reductions in Bacteroidetes, *Bacillaceae* and *Dorea* in the FS-LPS mice (Supplementary Table S2). Furthermore, the FO-LPS mice uniquely showed a reduction in *rc4-4*, and collectively with the BD-LPS mice showed a lower RA of *Lachnospiraceae*. (Supplementary Table S2).

Following DA analysis within the BD-fed mice, we found that LPS-treated mice had lower *Muribaculaceae* (W=3.057, q=0.063), *Lachnospiraceae* (W=3.465, q=0.016) and *Streptococcaceae* (W=3.097, q=0.056) compared to SAL-treated mice, but contrary to RA analysis (Fig. 3C), no significant change in *Bacteroides* (W=−2.47, q=0.35). In the FS-fed groups, DA analysis shows no change in *Muribaculaceae* between the treatments (W=−0.116, q=1), however significant LPS-induced effects were noted with elevated *Akkermansia* (W=−4.261, q<0.001), *Bacteroides* (W=−4.589, q<0.001), and *Enterobacteriaceae* (W=−6.52, q<0.0001), amongst several other taxonomic differences (Fig. 3C). In the FO-fed groups, differential abundance showed that LPS resulted in increased *Bacteroides* (W=−3.455, q=0.0138) and *Clostridiales* (W=4.436, q=0.0002), although no differences in *Muribaculaceae* (W=2.062, q=0.706), or *Akkermansia* (W=−0.414, q=1) (Fig. 3C).

3.2. The effects of exogenous LPS on the microbiota may be in-part related to the LPS-induced anorexia

We showed previously that LPS-challenged mice experienced the highest sickness behaviours (high sickness scores, reduced nest building behaviours) throughout the first 8-hours post-LPS injection [8]. In fact, throughout the 24-hours, the BD and FS mice lost most of their body weight (BW) within these first 8-hours (Supplementary Fig. S2). Contrastingly, a 2-way ANOVA showed that the FO-LPS mice continued to lose BW between 8- and 24- hours post-injection (Supplementary Fig. S2, Supplementary Table S3). Furthermore, there were time-point variations in DI, as the BD-fed mice ate significantly more food after the first 8-hours compared to the FS- and FO-fed mice (Supplementary Fig. S2, Supplementary

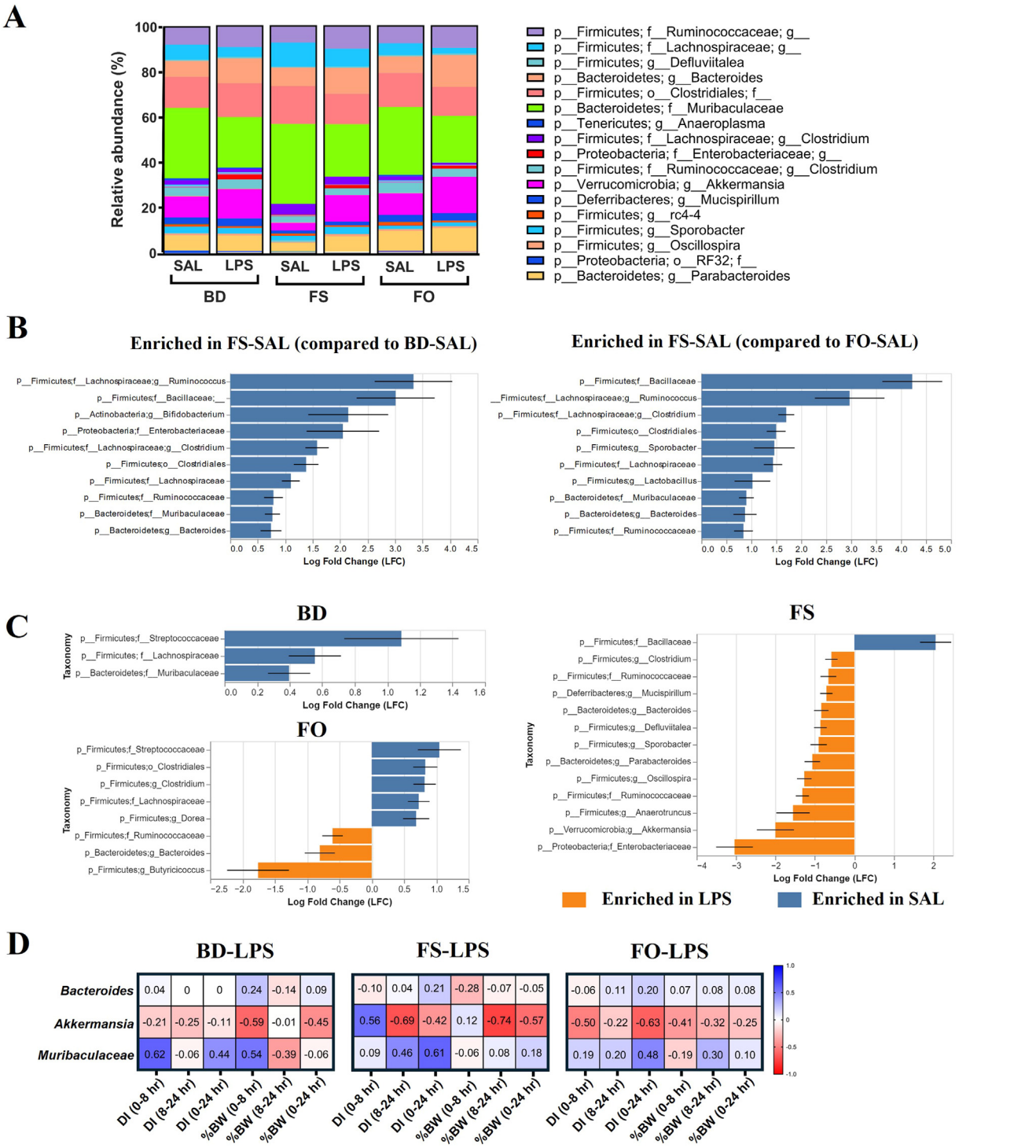


Fig. 3. Impacts of diet and LPS on the relative and differential abundance of the cecal microbiota composition and relationships to diet intake and BW loss (A) Relative abundance plot of the cecal microbiota 24-hours post-injection at the genus level. (B) B. DA analysis between diets (within SAL groups), comparing BD and FS (left) and FS and FO (right) (C) DA analysis (ANCOM-BC) within each diet (between treatment) (D) Spearman correlation matrices between the RA of *Bacteroides, Akkermansia,* and *Muribaculaceae* and DI and %BW change at different time frames. The legend (right) indicates the spearman rank correlation coefficient values. n=12/group.

Table S3). Collectively, given the timepoint- and diet- dependent impacts on BW and DI in our LPS-challenged mice, alongside the variability in BW loss between mice [8], we sought to determine if the key changes in taxonomic RA in the LPS-challenged mice were correlated to the severity of the LPS response (assessed by %BW and DI). In this analysis, we found diet-dependent relationships between BW and DI and the microbiota. In the BD-LPS mice, the RA of *Muribaculaceae* positively correlated to the 0–8-hour DI (P=.035) and %BW change (P=.046). In the FS-LPS mice, we found that the DI between 0- and 24-hours post-injection significantly positively correlated to *Muribaculaceae* (P=.038), but did not reach the threshold for significance in the BD-LPS (P=.179) nor the FO-LPS mice (P=.119) (Fig. 3D). The relationship between *Akkermansia* and DI/BW was intriguing, as it negatively correlated to DI (P=.017)

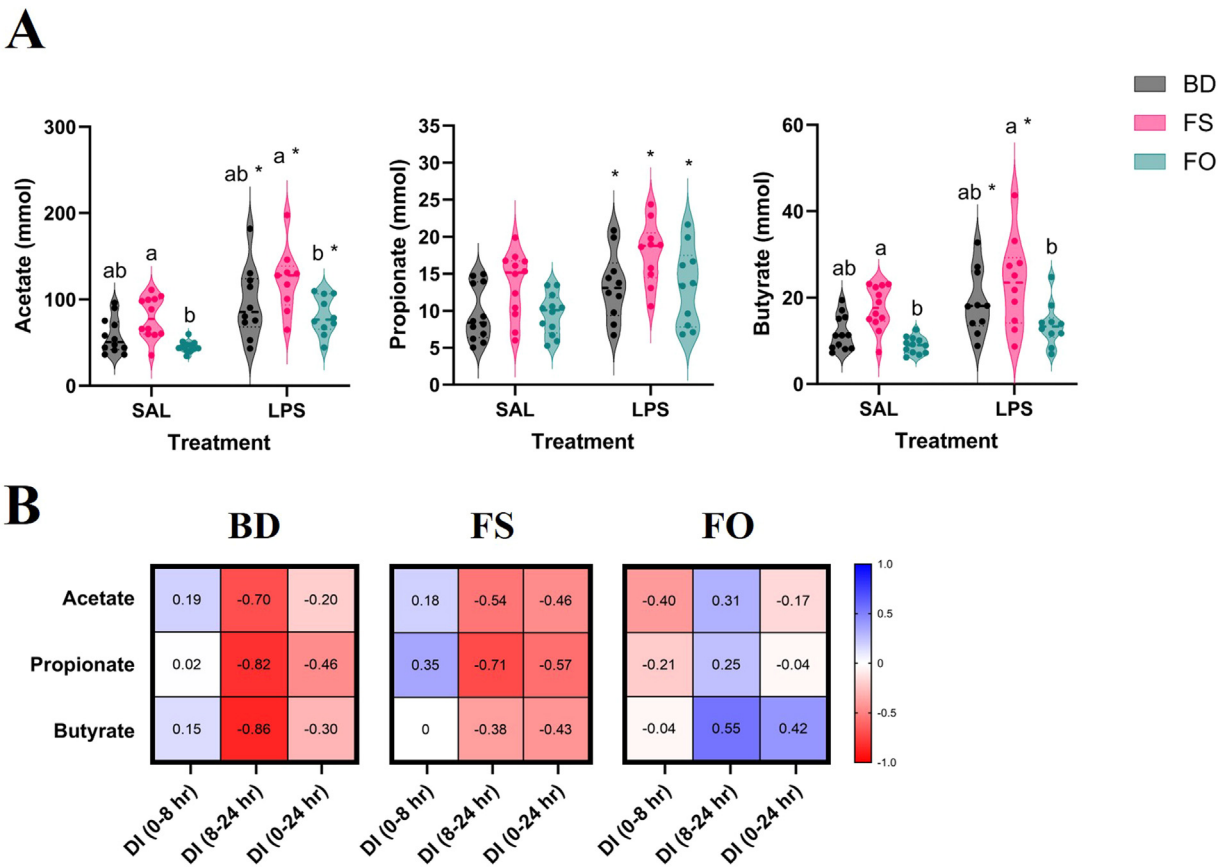


Fig. 4. Cecal SCFAs and their relationships to diet intake 24-hours post-injection in LPS-challenged mice. (A) The effects of LPS and diet on the cecal SCFAs (acetate, propionate, butyrate) 24-hours post-injection. (B) Spearman correlation matrices between each SCFA and DI at different intervals post-injection. SCFA concentrations were analyzed by 2-way ANOVA followed by Tukey's multiple comparison's test. Violin plots within treatment (SAL or LPS) that do not share a letter are significantly different ($P<.05$); An asterisk (*) indicates significant difference ($P<.05$) across treatments within each dietary group; n=11–12/group. For detailed statistics, see Supplementary Table S3.

and %BW change ($P=.008$) between 8 and 24 hours postinjection in the FS-LPS mice, while in the FO-LPS mice, it negatively correlated to the 24-hr DI ($P=.033$), but not %BW change (Fig. 3D). There were no significant correlations between DI and the RA of *Bacteroides* (Fig. 3D).

3.3. Cecal SCFA concentrations are elevated post-LPS injection

In our previous paper, we reported that fecal SCFAs were impacted by diet, such that FS-fed mice had elevated levels compared to BD and FO-fed mice, but were not impacted by treatment (i.e. LPS) [8]. Since ~95% of SCFAs are absorbed from the colon, with feces containing the remaining unabsorbed 5%, we measured their concentrations in the cecum as a major site of SCFA production. A 2-way ANOVA revealed significant effects of diet and treatment across the 3 main SCFAs (acetate, propionate, and butyrate) (Fig. 4A, Supplementary Table S3). Following multiple comparison testing, FS-fed mice displayed elevated cecal acetate and butyrate concentrations compared to the FO- fed mice (Fig. 4A). With regards to the treatment effects, LPS-treated mice had increased acetate and propionate compared to SAL-treated mice, regardless of diet group (Fig. 4A, Supplementary Table S3). Butyrate was significantly increased in the LPS-challenged groups fed BD or FS, but not FO when compared to their respective SAL-treated group (Fig. 4A).

Since we found associations between DI and SCFA-producing bacteria (*Muribaculaceae, Akkermansia*) (Fig. 3D), we wanted to determine if the cecal SCFA concentrations may be similarly related to DI. Spearman correlations suggested that the cecal concentrations of acetate, propionate and butyrate (Fig. 4B) negatively correlated to diet intake 8–24 hours post-injection in the BD-LPS mice, which was not seen at either to 0–8- or 0–24-hour intervals (Fig. 4B). In the FS-LPS mice, there was a similar correlation between diet intake (8–24 hour) and propionate, but not acetate or butyrate (Fig. 4B). In the FO-LPS mice there were no significant correlations between SCFA concentrations and DI (Fig. 4B).

3.4. Exogenous LPS induces intestinal inflammation with minimal impacts on the structural integrity of the intestine

In line with the changes in microbiota composition and function 24-hours post-injection, we then examined the effects of diet and LPS on the intestinal architecture. In the proximal colon (PC) and ileum, we observed no effect of diet or treatment on crypt depth or villi height, respectively, or goblet cell density (Table 1). Further, there was no significant effect of LPS on the PC mucous content, however there was a significant treatment effect in the ileum ($P=.0001$), such that LPS significantly increased mucous content in the FS- and FO- fed mice (Table 1, Supplementary Table S3). Provided the diet-dependent effects of LPS on intestinal physiology, we then wanted to determine if there were changes in gene expression of inflammatory and barrier integrity mediators in both the ileum and PC. In the ileum, using a 2-way ANOVA to assess relative mRNA expression of IL-1β, we found significant treatment ($P=.0424$), but no diet effects. After multiple comparison testing, there was an increase in IL-1β in the BD- ($P=.0327$)

Table 1
The effects of LPS on the intestinal structure 24-hours post-injection

	BD		FS		FO	
	SAL	LPS	SAL	LPS	SAL	LPS
Ileum villi height (μm)	187.0±24.64	177.7±20.69	190.9±17.06	174.4±14.78	170.1±19.83	174.7±12.49
Ileum goblet cell density (cells/villi)	12.42±2.809	12.74±2.621	12.60±2.545	11.58±1.582	11.43±2.486	11.99±1.522
Ileum mucous content (%)	3.365±0.8323	4.227±1.596	3.110±0.8097	4.074±1.255*	2.896±1.322	4.614±1.058*
Prox. colon crypt depth (μm)	177.0±9.505	183.9±13.45	177.0±9.290	178.5±9.502	177.9±13.24	173.8±7.280
Prox. colon goblet cell density (cells/villi)	18.83±2.939	20.04±4.594	20.78±3.871	17.93±0.9320	19.97±4.938	19.65±1.985
Prox. Colon mucous content (%)	33.28±2.242	33.59±4.011	32.99±3.881	34.81±1.898	33.23±3.143	34.22±2.269

Values show mean±SD; n=10–12/group. Data was analyzed by 2-way ANOVA. An asterisk denotes a significant difference between SAL and LPS within diet. For detailed statistics see Supplementary Table S3. Representative images are found in Supplementary Figure S3.

Table 2
Relative gene expression of the ileum and proximal colon

	IL-1β		ZO-1		Occludin		MUC2	
Diet	SAL	LPS	SAL	LPS	SAL	LPS	SAL	LPS
Ileum								
BD	5.296±3.071	7.967±4.077*	29.86±14.73	30.44±11.77	30.38±9.944	28.78±8.813	61.22±26.82	55.18±36.57
FS	6.673±2.382	6.098±2.684	32.93±18.67	28.90±15.93	32.02±12.56	27.37±8.980	61.39±27.35	51.44±30.09
FO	5.141±2.648	7.523±2.825*	31.39±14.29	30.39±13.29	27.83±5.073	28.97±9.919	53.26±13.50	43.60±20.08
Proximal Colon								
BD	1.872±1.388	2.780±1.167*	13.17±3.982	14.27±4.932	8.989±3.948	9.291±4.528	5.598±2.570	5.398±2.834
FS	2.153±1.661	4.327±2.188*	13.51±3.894	15.00±3.915	6.720±4.660	9.343±6.321	5.026±3.395	3.896±2.580
FO	1.667±0.995	5.353±1.388*	13.72±7.178	16.61±6.291	8.986±7.141	6.073±7.046	4.461±3.123	5.204±3.608

Values show mean±SD. n=11–12/group. Data was analyzed by 2-way ANOVA with Tukey's multiple comparison's test.
An asterisk denotes a significant difference within diet. Expression of Proximal colon IL-1β, ZO-1, and Occludin were log-transformed to achieve normality prior to 2-way analysis. For detailed statistics see Supplementary Table S3.

and FO- (P=.0559) fed mice following an LPS challenge, although no significant change in the FS- fed mice (P=.5889) (Table 2). In the PC, there was a strong treatment (P<.0001) effect on IL-1β expression, as indicated by a significant elevation in IL-1β expression in the LPS-challenged mice across all diets. There were no treatment nor diet effects on intestinal permeability markers (ZO-1, Occludin), nor MUC2 expression in either intestinal region (Table 2, Supplementary Table S3). Overall, these results indicate that an acute exogenous challenge with LPS induces intestinal inflammation (IL-1β) without impacting the structural integrity of the intestine, in a diet-dependent manner.

3.5. Dietary FS or FO do not attenuate LPS-induced hepatic inflammation or oxidative stress

In our previous study, we observed an increase in markers of systemic- and neuro- inflammation in LPS-treated mice compared to SAL-treated mice, which was partially attenuated in mice fed FS diets [8]. Given the role of the liver in clearance of LPS, and the anti-inflammatory potential of SCFAs, we wanted to determine if LPS-induced hepatic inflammation was partially attenuated in the FS-fed mice in a diet-dependent manner. In the liver, 24-hours post-injection, 2-way ANOVA analysis showed the expected treatment effect (P<.0001) of elevated IL-1β and TLR-4 in the LPS-challenged mice, and following multiple comparison testing showed diet-independent increases in the LPS-challenged mice, with a further elevation of IL-1β in the FS-LPS mice (compared to the BD-LPS mice) (Table 3). There was a significant treatment effect of NF-κB gene expression, which translated to elevated expression in the LPS-challenged mice fed FS- or FO, but not BD (P=.9494). Alternatively, there was no treatment effect on the relative expression of TNF-α (Table 3). Next, we examined markers of oxidative stress, specifically catalase and super oxide dismutase 1 (SOD1), and showed diet-independent reductions in the LPS-challenged mice (Table 3). While we found no significant associations between cecal SCFA concentrations and liver inflammation, we found a negative correlation between the total SCFAs and relative expression of hepatic SOD1 in the FS-LPS mice, which was not seen across the other 2 diets (Supplementary Fig. S4).

3.6. Blood markers of metabolic homeostasis are significantly impacted by LPS, largely independent of background diet

Given that DI may have been a contributing factor in influencing the cecal microbiota composition, and SCFA production, we sought to examine the potential diet-dependent effects of LPS on systemic markers of appetite (leptin, ghrelin, GLP-1, GIP) and blood glucose homeostasis (insulin, glucagon). We collected serum from the mice 24-hours post-injection, and using a 2-way ANOVA, we found a significant treatment effects, such that LPS induced reductions in circulating leptin, GLP-1 and insulin, and an increase in ghrelin and GIP in diet dependent manners (Supplementary Fig. S5). Alongside the significant overall treatment effects, leptin (reduced) and GIP (increased) were significantly impacted by LPS in the FS- and FO- fed mice, but not the BD-fed mice. There was no significant treatment effect (P=.0607) on glucagon in the serum (Supplementary Fig. S5). We also measured PAI-1 and resistin to address the potential diet-dependant responses on these markers of metabolic syndrome. Both of these markers were assessed by nonparametric Kruskal-Wallis test, with LPS inducing a diet-independent increase in PAI-1 concentration, while resistin was significantly reduced by LPS in the FS- and FO-fed mice, despite the clear reductions in the BD-fed mice as well (Supplementary Fig. S5)

Table 3
Relative gene expression in the liver

Gene	Relative expression (mean±SD)					
	BD-SAL	BD-LPS	FS-SAL	FS-LPS	FO-SAL	FO-LPS
IL-1β	0.884±0.425	8.237±2.051*	0.800±0.264	12.47±3.259*,#	0.658±0.206	11.18±3.275*
TNF-α	6.683±3.156	5.374±2.678	7.279±2.350	5.677±3.757	5.685±3.014	5.160±2.891
TLR-4	0.6748±0.1656	0.8594±0.1738*	0.5566±0.1614	1.129±0.3966*	0.5431±0.1352	0.9277±0.2420*
NF-κB	1.022±0.1747	1.018±0.1399	0.8331±0.1393	1.113±0.2172*	0.9702±0.1293δ	1.131±0.0.1511*
SOD1	0.990±0.197	0.791±0.085*	1.100±0.195	0.888±0.093*	1.085±0.245	0.916±0.102*
Catalase	1.041±0.161	0.794±0.128*	1.139±0.167	0.856±0.125*	1.168±0.109	0.845±0.113*

Values show mean±SD. n=11–12/group. Data was analyzed by 2-way ANOVA with Tukey's multiple comparison's test.
An asterisk (*) denotes a significant difference within diet (between SAL and LPS). A # denotes a significant difference to BD-LPS. A δ indicates a significant difference to BD-SAL. IL-1β, TLR-4, and TNF-α expression levels were log-transformed prior to statistical analysis. For detailed statistics see Supplementary Table S3.

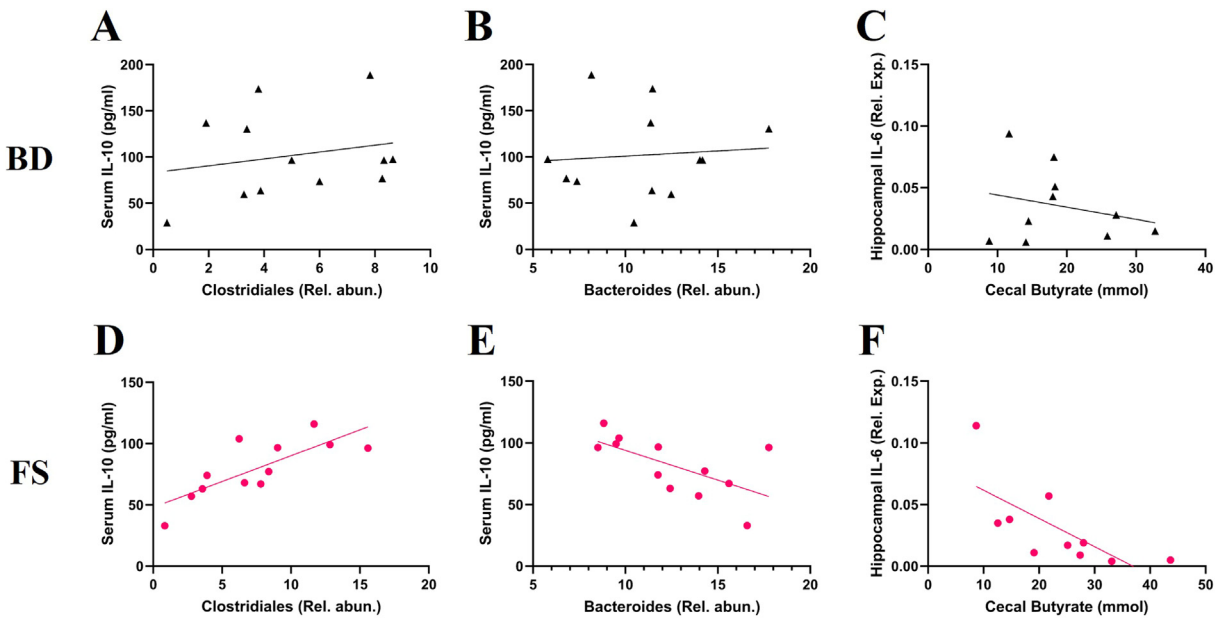


Fig. 5. Correlations outlining potential connections across the mGBA in the BD-LPS and FS-LPS mice. (A-B) Correlations between the relative abundance of an unknown genus in the Clostridiales order (A) and *Bacteroides* (B) to serum IL-10 concentrations in the BD-LPS mice. (C) Correlation between cecal butyrate concentrations and relative expression of hippocampal IL-6 in the BD-LPS mice. (D-E) Correlations between the relative abundance of an unknown genus in the Clostridiales order (D) and *Bacteroides* (E) to serum IL-10 concentrations in the FS-LPS mice. (F) Correlation between cecal butyrate concentrations and relative expression of hippocampal IL-6 in the FS-LPS mice. n=11–12/group.

3.7. The microbiota and the cecal SCFAs appear to modulate the inflammatory response in FS-LPS mice

As there were minimal diet-dependent differences in hepatic inflammation, oxidative stress, or serum markers of appetite or hunger, we wanted to perform a secondary analysis from our previous publication [8], where we performed spearman correlations to determine other targets which may be related to the FS-induced partial attenuation of systemic- and neuro-inflammation, and which could be explored more in depth in future studies. There were few significant correlations between markers of systemic/neuroinflammation and the cecal microbiota in the FS-LPS mice, however there were correlations between the relative abundances of an unknown genus in the Clostridiales order (P=.0070, r=0.7483) and *Bacteroides* (P=.0302, r=−0.6340), and serum IL-10 (Fig. 5D,E), which were not seen in the BD-LPS mice ([P=.4808, r=0.2242], [P=.8743, r=0.0542]) (Fig. 5A,B). Examining the potential link between neuro-inflammation and SCFA concentrations showed us that the cecal concentrations of butyrate in the FS-LPS mice significantly correlated to hippocampal expression of IL-6 (P=.0088, r=−0.7939) (Fig. 5F), which was similarly not seen in the BD-LPS mice (P=.8651, r=0.0667) (Fig. 5C). These significant correlations may outline key links across the mGBA in the FS-fed mice.

4. Discussion

In this study, we had hypothesized that the LPS-induced response on gut health and hepatic inflammation would be improved in a diet-dependent manner, by dietary supplementation with FS, through modulation of the intestinal structure, the cecal microbiota composition and SCFA production. Collectively, we show here that LPS induced a shift in the cecal microbiota composition and function, differentially driven by dietary supplementation with FS or FO. While neither diet could attenuate the hepatic inflammatory/oxidative stress cascade induced by LPS, we highlighted potential FS-induced impacts on intestinal health, alongside potential microbial targets in the mGBA.

In the previous study from our lab [8], we highlighted that dietary supplementation with FS partially attenuated the negative LPS-induced effects across the mGBA, including altered fecal microbiota composition, improved neuro- and systemic- inflammation. Notably, in our previous study, the fecal microbiota α-diversity was strongly elevated in our FS-fed mice regardless of SAL/LPS, whereas in the cecum we did not mirror these changes, emphasizing the intestinal site-specific effects of our diet interventions. In this current study, by assessing the cecal microbiota we validate some of the taxonomic changes induced by dietary supplementation with FS in the fecal microbiota [8], including the FS-induced increases in *Lachnospiraceae, Bifidobacterium, Clostridium (Lachnospiraceae), Bacillaceae, Muribaculaceae* and *Enterobacteriaceae* (Fig. 3B) despite the differences in sampling location. The elevated *Enterobacteriaceae* is interesting, as this family is generally associated with a dysbiotic microbiota (Fig. 3A, Supplementary Table S2) [48], in which facultative anaerobes (such as *Enterobacteriaceae*) can bloom [49]. Our FS-SAL mice exhibit marginally elevated IL-1β expression in the ileum and proximal colon (compared to BD-SAL), which may be related to this elevation in *Enterobacteriaceae*, although this would have to be investigated further. Collectively, we show that the fecal [8] and cecal (Fig. 3C) microbiota show similar taxonomic changes in response to dietary supplementation with FS, however there are site-specific impacts including a FS-induced increase in *Coriobacteriales* and decrease in *rc4-4* only in the fecal microbiota [8]. We showed that the FO-fed mice had minimal impacts on the cecal microbiota, which we similarly showed in the feces previously [8], emphasizing that the oil component of FS does not signficantly alter the microbiota in our mice.

Exogenous administration of LPS is well established to induce neuroinflammation, which is coupled to an anorexic phenotype (reduced DI, significant BW loss) [50–52]. We speculate that the LPS-induced reduction in food intake is in-part responsible for the taxonomic changes following an LPS injection. In this study, we again show diet-dependent impacts of LPS on the cecal microbiota, determined by both RA and DA analyses (Fig. 3A,C, Supplementary Table S2). Of these taxonomic changes, in our BD-LPS mice, we show that a decrease in *Muribaculaceae* may be in relation to the degree of fasting within the first 8-hours post-injection (Fig. 3D). The abundance of the *Muribaculaceae* family is established to be elevated when provided substrate for nutrition (i.e. soluble fiber, resistant starch) [53], so logically in the absence of nutrition, there would be a reduction. We suggest that this may be an LPS-specific response on the microbiota that is only partially regulated by the reduction in overall diet intake.

In our FS-LPS mice, we saw that the RA of *Muribaculaceae* positively correlated to 0–24-hour DI (Fig. 3D). We speculate that this delay (compared to BD-LPS mice) may be explained by the reduced DI (and %BW) in the FS-LPS mice throughout the 24-hours post-injection (albeit statistically insignificant) (Supplementary Fig. S2). This positive correlation may be indicative of recovery, and thus, our BD-LPS mice are recovering earlier (corroborated by the 24hr BW and DI changes [Supplementary Fig. S2]). In line with this hypothesis, the hepatic IL-1β expression is further elevated in the FS-LPS mice compared to their BD-LPS counterparts, despite the improvements in systemic and neuroinflammation shown in our previous study [8]. In a mouse model of colitis, dietary FS was shown to worsen colonic injury and inflammation [54], collectively indicating that despite the highlighted benefits of dietary FS across the mGBA [8,18,55,56], there remains some unknown mechanisms wherein the opposite may be true.

In previous studies, fasting has been shown to increase abundance of *Akkermansia*, despite the heterogeneity of study designs [57–60]. In our current study we saw elevated *Akkermansia* in our FS-LPS mice using DA analysis, and we also identified an increase in the FS-LPS and FO-LPS mice following RA approaches (the method used in the previous studies). Elevated *Akkermansia* in a fasted state is expected given its role as a mucin degrader [61]. Mucins play an active role in defence against pathogens and toxins, and the production (via MUC2), and ultimately secretion of mucins by goblet cells, is elevated downstream in response to TLR-4 activation [62]. Of note, in our BD-fed mice, 24-hours post LPS-injection, we did not see elevated mucus content in the PC or IL (Table 2) although we did see elevated ileal mucous in the FS-LPS and FO-LPS mice. This would match the relative shift of *Akkermansia,* as it was only significantly increased by LPS in the FS- and FO- fed mice (Supplementary Fig. S1). Furthermore, we did not find any impact of LPS on MUC2 gene expression, which is validated by a previous study showing no change in ileal MUC2 expression following 16 or 32 days of 1 mg/kg i.p LPS [4]. This collectively may stress the importance of *Akkermansia* and its mucin-degrading role in the recovery post-LPS injection.

Mucin degradation is a vital source of energy for the microbiota, but also for the host, through production of SCFAs. In fact, SCFAs can account for ~1%–10% of a human's energy requirements [63]. Individuals undergoing intermittent fasting have shown elevated concentrations of fecal SCFAs [64]. In high fat diet (HFD)-fed mice, short term food restriction (8–11 hours) resulted in elevated cecal butyrate [65], however in a short-term (24 hours) fast, chow-fed mice showed reductions in cecal acetate and butyrate, but no impact on propionate [59]. In Syrian hamsters fasted for 96 hours, the cecal SCFAs were elevated compared to nonfasted animals (n=6/group) [66]. In our study, we see elevated SCFAs in our LPS challenged mice (Fig. 4A), which we hypothesize is a result of the elevated RA of mucin degrading bacteria, regardless of DA analysis. This was an unexpected finding, as in our previous study, LPS had no impact on fecal SCFA concentrations [8], highlighting a site-specific intestinal effect of LPS, where this increase in cecal SCFAs may indicate an increase in bacterial energy scavenge.

In addition to their role in energy metabolism, SCFAs also have important roles in regulating inflammation and oxidative stress [23]. We predicted that the FS-induced elevation of the cecal SCFAs (acetate/butyrate), alongside the evidence of increased fecal SCFAs in the same FS-fed mice [8], would result in improvements in these markers. We show here that despite the elevated SCFAs, there remained significantly elevated hepatic inflammation and oxidative stress (Table 3). A previous study showed that high dose i.p sodium butyrate (500 mg/kg) significantly improved LPS-induced hepatic inflammation through the NF-κB pathway [35]. While this dose of butyrate is not physiologically relevant, it does point to its anti-inflammatory abilities. Inflammation (assessed by IL-1β gene expression) in the PC and ileum was elevated 24-hours post-injection, although not significantly elevated in the ileum of the FS-fed mice (Table 2), which may be in part due to the altered physiological defences (i.e. elevated mucin production in the ileum of FS-LPS mice and/or the FS-induced increase in cecal SCFAs). Taken together, we showed that the FS-induced increases in SCFAs may have been implicated in attenuating the LPS-induced ileal inflammation, but in the liver, the LPS-induced inflammatory cascade may have been too strong for attenuation by FS.

As a continuation of our previous research, we performed correlational analysis between the cecal microbiota of our FS-LPS mice and markers of systemic- and neuro-inflammation at 24-hours post-injection to highlight potential links across the mGBA. We were unable to find any significant correlations between taxonomic RA and markers of neuroinflammation, although we did find significant correlations between the RA of an unknown genus in the Clostridiales order and *Bacteroides* to serum IL-10 concentrations in the FS-LPS (but not the BD-LPS) mice (Fig. 5A,B). Notably, both

clades were increased in the FS-SAL mice compared to both the BD-SAL and FO-SAL mice (Fig. 3B). These findings may be in relation to the inhibitory effects of butyrate on specific *Bacteroides* strains [67], as the RA of the order Clostridiales is significantly correlated to butyrate concentrations in our FS-LPS mice (data not shown). Furthermore, the anti-inflammatory benefits of butyrate have been well established [26,27], even in the context of LPS-induced inflammation, both *in vitro* [68] and *in vivo* [69], which corroborates our findings where mice with greater cecal butyrate have lower hippocampal IL-6 expression (Fig. 5C). Collectively in this study, we outline key correlations across the mGBA within the first 24-hours post-injection, which may explain the partially attenuated systemic- and neural- response to LPS administration in the FS-fed mice [8].

We acknowledge our study has several limitations, for example, we are aware of the sex-dependent effects on the microbiota, and we recognize that the response to i.p LPS may be different in female mice [70], which will be addressed in a separate study. We used both RA and DA approaches to examine the cecal microbiota composition, although we recognise that metagenomics would be highly valuable to further our understanding of the implications of fasting and diet-restriction on the microbiota. Furthermore, investigating the effects of fasting on carbohydrate active enzymes (CAZymes) through proteomic approaches would be informative to determine the role of mucin-degrading enzymes in this LPS-induced anorexic state. Lastly, we cannot ultimately discern from this study design whether the taxonomic changes were induced by diet-restriction or were mediated by the LPS-response, which could be investigated using a pair-feeding approach.

5. Conclusion

In conclusion, we show here that an i.p challenge with LPS introduces alters the cecal microbiota composition and function, which may be in an effort to meet homeostatic energy demands. While the hepatic physiological response to the LPS-challenge was not mitigated by dietary supplementation with FS or FO, dietary supplementation with FS attenuated the LPS-induced ileal inflammation. This anti-inflammatory outcome could be corroborated by the potential relationships between the microbiota and systemic inflammation we show in this study. The oil component of FS (FO) does not appear to have any beneficial impacts across the mGBA, likely related to the minimal impact on the microbiota. LPS is often used in research to induce a severe inflammatory state, across a variety of experimental models, from our findings here, we suggest caution in using this model without addressing the impacts on the microbiota, especially when sampling within the first 24-hours post-injection.

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Declaration of competing interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2025.109951.

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