Decreased \( \omega_6/\omega_3 \) PUFA ratio attenuates ethanol-induced alterations in intestinal homeostasis, microbiota and liver injury

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Running title: Decreased \( \omega-6:\omega-3 \) PUFA ratio and intestinal and liver health

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ABBREVIATIONS

ALD, alcoholic liver disease; ALT, alanine aminotransferase; AMP, anti-microbial peptide; BA, bile acid; DSS, dextran sulfate sodium; CAE, chloroacetate esterase; C. elegans, Caenorhabditis elegans; fat-1, omega-3 fatty acid desaturase-1; EtOH, ethanol; H&E, hematoxylin and eosin; LPS, lipopolysaccharide; PF, pair fed; ISC, intestinal stem cell; RvD1, resolvin D1; SPM, specialized pro-resolving mediator; 5-HT, 5-hydroxytryptamine; I3S, 3-indoxyl sulphate; SEM, standard error of the mean; OTU, operational taxonomic unit; SCFA, short chain fatty acid; PCA, principal component analysis; PLS-DA, partial least squares-discriminant analysis; CA, cholic acid; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UC, ulcerative colitis; GEO, gene expression omnibus; KEGG, Kyoto encyclopedia of genes and genomes.
ABSTRACT
Ethanol-induced alterations in intestinal homeostasis lead to multi-system pathologies, including liver injury. \( \omega_6 \)-PUFAs exert pro-inflammatory activity, while \( \omega_3 \)-PUFAs promote anti-inflammatory activity that is mediated, in part, through specialized pro-resolving mediators (e.g. RvD1). We tested the hypothesis that a decrease in \( \omega_6/\omega_3 \) PUFA ratio would attenuate ethanol-mediated alterations in the gut-liver axis. \textit{fat-1} mice, which endogenously increase \( \omega_3 \)-PUFA levels, were protected against ethanol-mediated down-regulation of intestinal tight junction proteins in organoid cultures and \textit{in vivo}. Ethanol and LPS-induced expression of INF-\( \gamma \), Il-6, and Cxcl1 was attenuated in \textit{fat-1} and WT RvD1-treated mice. RNA-seq of ileum tissue revealed up-regulation of several genes involved in cell proliferation and stem cell renewal, and anti-microbial defense (including Alpi and Leap2) in \textit{fat-1} vs WT mice fed ethanol. \textit{fat-1} mice were also resistant to ethanol-mediated down-regulation of genes important for xenobiotic/bile acid detoxification. Further, gut microbiome and plasma metabolomics revealed several changes in \textit{fat-1} vs. WT mice that may contribute to a reduced inflammatory response. Finally, these data correlated with a significant reduction in liver injury. Our study suggests that \( \omega_3 \)-PUFA enrichment or treatment with resolvins can attenuate the disruption in alcohol-induced intestinal homeostasis caused by ethanol consumption and systemic inflammation with a concomitant reduction in liver injury.

KEY WORDS
Intestine, inflammation, omega-3 fatty acids, diet and dietary lipids, gut microbiome, bile acid metabolism, alcoholic liver disease.

INTRODUCTION
The gastrointestinal tract is the tissue of first contact with ingested ethanol (EtOH), and excessive consumption can lead to numerous deleterious effects, including loss of gut barrier integrity, inflammation, and pathological changes in the gut microbiota composition and function (1). These intestinal alterations induced by EtOH as well as bidirectional communication between the gut and other organs, for example, the liver, play important roles in the pathogenesis of alcohol-induced multi-organ pathology, including alcoholic liver disease (ALD). A large body of both pre-clinical animal and human studies indicates that in the case of EtOH-mediated intestinal barrier dysfunction and increased permeability, the liver is exposed to a plethora of gut microbiota-derived products that contribute to hepatic inflammation and to the development of alcohol-related liver injury (2). The gut microbiota is also an integral part of the bile acid (BA) enterohepatic circulation, and EtOH-mediated changes in BA metabolism may be a mechanistic link between EtOH-induced alterations in the gut microbiota and impaired host phenotype, including EtOH-induced alterations in the gut microbiota and impaired host phenotype, including intestinal and liver damage (3).

Previous work from our group and others underscores the importance of different types of dietary fat in EtOH-associated pathologies. For example, a diet high in \( \omega_6 \) PUFAs exacerbated EtOH-induced intestinal permeability, endotoxemia and liver injury in mice (4). We also showed that a diet high in \( \omega_6 \) PUFAs combined with excessive alcohol intake resulted in intestinal inflammation (5) and gut microbiota alterations, specifically a prominent reduction in \textit{Bacteroidetes} and an increase in the gram-negative \textit{Proteobacteria} and the gram-positive \textit{Actinobacteria} phyla (6). However, the effects of dietary \( \omega_3 \) PUFAs on alcohol-mediated tissue and organ alterations are not clear and are somewhat controversial (7). Recent promising studies demonstrated that both dietary \( \omega_3 \) PUFA supplementation and an endogenous
increase in ω-3 PUFAs with a consequent decrease in the ω-6:ω-3 PUFA ratio in transgenic fat-1 mice (due to endogenous conversion of ω-6 to ω-3 PUFAs by the fat-1 gene, which encodes an ω-3 PUFA desaturase) attenuated acute alcohol-induced liver injury (8, 9). It has been also shown that fat-1 mice have reduced metabolic endotoxemia and systemic low-grade inflammation in response to a diet high in ω-6 PUFAs (10) and are protected from high fat/high sucrose diet-induced intestinal permeability and alterations in intestinal tight junctions (11). Further, endogenous conversion of ω-6 to ω-3 PUFAs in fat-1 mice can modulate the gut microbiota (10), and transplantation of feces from fat-1 mice to wild type littermates prevented obesity and associated metabolic disorders (12). Evidence suggests that ω-3 PUFA supplementation can alter gut microbial composition in humans (13) and may induce an increase in several beneficial taxa, including the butyrate-producing Roseburia, Bifidobacterium and Lactobacillus (14). Studies showing reduced intestinal inflammation by ω-3 PUFA supplementation, both in patients and in animal models, suggest anti-inflammatory and pro-resolving properties of ω-3 PUFAs and their derivatives in the gut (15). Supplementation with ω-3 PUFAs resulted in significant improvement of metabolic risk factors and liver steatosis in patients with nonalcoholic liver disease (16), and substitution of the ω-6 PUFA, linoleic acid, with ω-3 PUFAs prevented Western diet-induced nonalcoholic steatohepatitis (17). Many of the benefits of increased ω-3 PUFAs can be attributed to the class of bioactive metabolites collectively termed specialized pro-resolving mediators (SPMs), which include resolvins, protectins, and maresins and that have been shown to limit the inflammatory response in a number of models of disease [reviewed in (18)].

Despite promising studies suggesting beneficial effects of ω-3 PUFAs and their metabolites in a variety of pathological conditions (19-21), there is still limited evidence for their beneficial effects on alcohol-mediated tissue/organ damage. Given that the Western diet is heavily reliant on ω-6 PUFA-rich sources (e.g. corn oil and soybean oil) at the expense of ω-3 PUFA-rich food (e.g. oily fish), leading to an increase in the ratio of ω-6:ω-3 PUFA which contributes to numerous deleterious health effects (22), it is essential to investigate the role of modulation/decrease of the ω-6:ω-3 PUFA ratio as a potential dietary preventative/therapeutic approach in different pathological conditions, including alcohol-induced multi-organ pathology. Further compounding the problem of excessive ω-6 PUFA intake is the observation that the rise in obesity rates mirrors that of soybean oil consumption (23). Obesity has been linked to exacerbation of ALD (24). The objective of the current study was to investigate the impact of a decreased ω-6:ω-3 PUFA ratio due to endogenous ω-3 PUFA enrichment on EtOH-induced alterations in the intestinal homeostasis, gut microbiota, bile acid metabolism, and associated liver injury in transgenic fat-1 mice subjected to chronic EtOH administration.
MATERIALS AND METHODS

Animals and Experimental Setup

Animal studies were approved by and performed in accordance with the guidelines of the University of Louisville Institutional Animal Care and Use Committee (protocol no. 15423 to IAK). Transgenic fat-1 mice were provided by Dr. J. Kang (Harvard Medical School, Boston, MA) and maintained as heterozygotes. Expression of the the fat-1 gene is driven by the chicken β-actin promotor and the cytomegalovirus enhancer (25). To produce mice for the experiments described herein, heterozygous fat-1 male mice were mated to female wild-type (WT) C57BL/6J mice that were bred in-house (Supplemental Figure S1a). Age and sex-matched fat-1+/− and WT littermates were used for all experiments. For simplicity, fat-1 will be used throughout to indicate heterozygous mice. Mice were genotyped by PCR (Supplemental Figure S1b) and primers to Gdf5 were used as a positive control (see Supplemental Table S1 for sequences). Mice were housed in a temperature-controlled room (23.9 °C) with a 12h light-dark cycle in a specific pathogen-free animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

All mice were maintained on autoclaved laboratory rodent chow diet (5010, LabDiet, St. Louis, MO) and had ad libitum access to food and water. At 8-10 weeks of age, male fat-1 mice and their WT littermates were placed on control (maltose dextrin) or EtOH-containing Lieber-DeCarli liquid diets (BioServ, Flemington, NJ, catalog numbers F1259SP and F1258SP, respectively). The mice were fed for 6 weeks with a step-wise increase in EtOH concentration (0%, 1%, and 2% for two days each, 4% and 5% for one week each and then 6% for 3 weeks). Lipopolysaccharide (LPS, LPS-EB Ultrapure from InvivoGen, San Diego, CA [p/n tlr1-3pelps]), was given to a subgroup of EtOH-treated mice 24h prior to euthanasia (i.p., 5 mg/kg) to mimic human alcoholic hepatitis in which systemic inflammation and infection are major contributors to morbidity and mortality (26). Additionally, a subset of EtOH+LPS-treated mice received resolvin D1 (RvD1, Cayman Chemical, Ann Arbor, MI) treatment (500 ng, i.p. on each of the final 5 days of the experiment). The experimental design is presented in the Supplemental Figure S1C. The control and EtOH-containing diets were prepared fresh each day, and food consumption monitored daily. There was no significant difference in food intake between WT and fat-1 mice. The control pair-fed (PF) groups received the same amount of isocaloric food (maltose dextrin containing diets) that EtOH-fed animals consumed in the previous day.

Intestinal tissue PUFA analysis

Fatty acid (FA) profile in intestinal samples were analyzed by gas chromatography in Dr. J. Kang’s laboratory as described previously (27). Briefly, tissue samples were ground to powder under liquid nitrogen and subjected to total lipid extraction and fatty acid methylation by 14% boron trifluoride-methanol reagent (Sigma-Aldrich) at 100 °C for 1 h. FA methyl esters were analyzed using a fully automated HP5890 gas chromatography system equipped with a flame-ionization detector (Agilent Technologies, Palo Alto, CA). The FA peaks were identified by comparing their relative retention times with the commercial mixed standards (NuChek Prep, Elysian, MN), and area percentages for all resolved peaks were analyzed by using a PerkinElmer M1 integrator.

Histopathological and immunohistochemical analysis of the intestinal tissue

Formalin-fixed, paraffin-embedded samples of ileum were sectioned, stained with hematoxylin and eosin (H&E), and evaluated by light microscopy. The ileum was chosen for analysis because it is the region that has the highest degree of gut barrier permeability in response to alcohol feeding (4). For morphometric analysis, complete villus-crypt structures were randomly selected from H&E stained sections, and villus height, width, and crypt depth were measured using ImageJ software (NIH). Villus length was measured from the tip to the base of villus, villus width was measured at the base of villus, and the crypt length was measured from the bottom of the crypt to the opening of the crypt (28).

For immunohistochemical analyses, intestinal sections were stained for the tight
junction protein, ZO1 (Invitrogen, Camarillo, CA, catalog No. 61-7300), Paneth cells (anti-lysosome, Abcam, Cambridge, MA, ab36362), and goblet cells (Alcian Blue 8GX, Sigma-Aldrich, St. Louis, MO). Neutrophil accumulation was assayed by chloroacetate esterase (CAE) staining using a commercially available kit (Sigma-Aldrich, St. Louis, MO). Quantification of CAE staining was performed by counting CAE-positive cells in a random series of 20-30 digital images per section (200X field) in a blinded approach; the number of CAE-positive cells were then summed and averaged to obtain an estimate for each mouse (n=3-8 animals/group).

Isolation of intestinal crypts, and crypt-villus organoid culture

Isolation of intestinal crypts and subsequent organoid culture were largely performed as previously described (29, 30) using commercially available reagents (StemCell Technologies, Vancouver, Canada). In brief, mice were humanely euthanized by CO2 asphyxiation and the entire length of the small intestine was removed and the lumen flushed thoroughly with cold phosphate-buffered saline (PBS). The intestine was then opened longitudinally and the mucosal layer was collected by gently scraping the luminal aspect with a glass microscope slide. The mucosa was washed and processed according to the manufacturer’s instructions (StemCell Technologies). Isolated crypts (200-400 per well) were suspended in Matrigel (BD Biosciences, San Jose, CA) and plated in 24-well plates pre-warmed to 37°C. The crypts were grown with crypt niche growth factors in IntestiCult Growth Media (StemCell Technologies). The organoid cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2.

For experiments evaluating organoid growth, crypts were isolated from the small intestines of naïve WT and fat-1 mice, and resulting organoids were cultured in triplicate and treated with 50 mM EtOH. Growth was analyzed by measuring the cross-sectional area of representative organoids every 24h (>10 in each condition). In a second series of experiments, intestinal organoids were established from the experimental WT and fat-1 mice fed control or EtOH-containing diets. Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA), and cDNA was synthesized and amplified using the Nugen Ovation PicoSL WTA System V2 (San Carlos, CA). Changes in gene expression were analyzed by RT-qPCR using PerfeCTa SYBR Green Fast Mix (Quanta Biosciences, Beverly, MA) on the Applied Biosystems 7900HT platform (Foster City, CA). Primers are presented in Supplemental Table S1.

Cytokine measurement in intestinal tissue

Ileum samples were homogenized in PBS + 0.05% Tween-20 supplemented with protease and phosphatase inhibitors (Halt™, Thermo Fisher [Waltham, MA]), in tubes filled with 1.5 mm zirconium beads (Benchmark Scientific, Edison, NJ). Following centrifugation at 20,000xg, supernatants were collected and protein concentrations were determined by the BCA method (Pierce™ BCA protein assay kit, Thermo Fisher). Six hundred µg was analyzed on the V-PLEX immunoassay proinflammatory panel 1 plate and data were collected on the MESO Sector S 600 instrument and analyzed with Discovery Workbench, v. 4.0 (MesoScale Discovery, Rockville, MD).

RNA Isolation and real time quantitative reverse transcription PCR (RT-qPCR)

Total RNA from intestinal and liver tissues was isolated with Trizol reagent (Thermo Fisher) as described by the manufacturer, and any contaminating genomic DNA was removed by digestion with DNase I (Thermo Fisher). cDNA was synthesized with qScript cDNA Supermix (Quanta Biosciences, Beverly, MA) and a cDNA equivalent of 10 ng RNA (or 0.1 ng for 18S ribosomal RNA) was analyzed in each qPCR reaction. RT-qPCR assays were performed with PerfeCTa SYBR Green Fast Mix (Quanta Biosciences, Beverly, MA) on the Applied Biosystems 7900HT platform (Foster City, CA). Primers are presented in Supplemental Table S1.

RNA-seq analysis and bioinformatics

RNA-seq analysis was performed in the ileal segments of the intestine by the University of Louisville Center for Genetics in Molecular Medicine Core facility, and bioinformatic
analysis was conducted by the NIH-funded Kentucky Biomedical Research Infrastructure Network Bioinformatics Core according to the protocols presented in the Supplemental Materials and Methods.

**Gut Microbiota Analysis**

DNA extraction of fecal samples, bacterial 16S rRNA gene amplification, and sequence analysis was performed as previously described (31, 32) with modifications shown in the Supplemental Materials and Methods.

**Fecal bile acid analysis**

Bile acid analysis from fecal samples was performed by the Wayne State University Lipidomic Core Facility (supported by NIH grant S10RR027926) according to the protocol presented in the Supplemental Materials and Methods Section.

**Serum bile acid analysis**

Total serum bile acid concentrations were determined using the Mouse Total Bile Acids Assay Kit from Crystal Chem (Elk Grove Village, IL).

**Blood plasma metabolomic analysis**

Blood metabolomic analysis was performed by the University of Louisville Metabolomic Core Facility according to the protocol presented in the Supplemental Materials and Methods Section.

**Measurement of blood alcohol levels**

Plasma blood alcohol levels were measured using the EnzyChrom™ ethanol assay kit (BioAssay Systems, USA, Hayward, CA).

**Assessment of Liver Injury**

Plasma levels of alanine aminotransferase (ALT, a marker of liver injury) were determined using the ALT/GPT Reagent (Thermo Fisher) according to the manufacturer’s instructions and using normal and abnormal serum for controls (Data Trol™, Thermo Fisher). Hepatic triglycerides were measured as previously described (33). For histological evaluation of liver injury, 5 µm sections were prepared from formalin-fixed paraffin-embedded liver tissue and were stained with hematoxylin and eosin (H&E).

**Statistical Analysis**

The data were expressed as mean value ± standard error of the mean (SEM). Differences among multiple groups were analyzed by Two-Way Analysis of Variance (ANOVA) followed by post hoc multiple comparisons tests. Differences between two groups were tested by the unpaired two-tailed Student’s t-test. A P-value of < 0.05 was considered statistically significant. The correlation between various characteristics was assessed using Pearson correlation analysis. Statistical analysis was performed using the package GraphPad Prism 5.01 software for Windows (GraphPad Software, Inc., La Jolla, CA).

Additional protocols and procedures are described in Supplementary Materials and Methods.
RESULTS

Intestinal ω-6:ω-3 PUFA ratio and EtOH-mediated alterations in the fatty acid composition in WT and fat-1 mice

Analysis of intestinal FA composition revealed that fat-1 mice compared to WT littermates showed a markedly decreased ω-6:ω-3 PUFA ratio in the ileal segments with a ratio of ~ 8:1 and 7:1 in fat-1 PF and EtOH-fed, respectively, and ~ 14:1 and 20:1 in WT PF and EtOH-fed, respectively (Fig. 1A). Interestingly, while total ω-3 PUFA levels were elevated in fat-1 as compared to WT in both PF and EtOH-fed animals (Fig. 1B), ω-6 PUFA levels were similar between genotypes in PF animals. However, EtOH consumption markedly increased total ω-6 PUFA levels in WT mice resulting in significantly higher ω-6 PUFA levels in WT-EtOH vs. WT-PF and fat-1-EtOH animals (Fig. 1C). Further analysis revealed elevated levels of linoleic acid (an ω-6 PUFA) in WT-EtOH fed mice, while eicosapentaenoic acid (EPA, an ω-3 PUFA) was below detectable levels in these mice (Supplemental Table S2). There were no noticeable effects of EtOH on the ω-3 PUFA docosahexaenoic acid (DHA) in either WT or fat-1 mice, although fat-1 mice had significantly higher DHA levels compared to WT-PF littermates.

Effects of a decreased ω-6:ω-3 PUFA ratio on the intestinal morphology and homeostasis

Histological evaluation of intestinal sections indicated that varying content of essential fatty acids in WT and fat-1 mice had no effects on the overall structure of the ileum tissue morphology in PF-fed mice (Fig. 1D). EtOH feeding resulted in increased villus length in WT mice compared to WT-PF and fat1-EtOH littermates, while there were no differences in crypt height between the experimental groups (Supplementary Fig. S2A-S2B).

Using small intestine organoids, we next tested whether a decrease in tissue ω-6:ω-3 PUFA ratio would benefit intestinal stem cells (ISC) growth, proliferation and function. We observed that crypts derived from naïve fat-1 mice yielded substantially larger organoids and increased early organoid growth within 4 days in culture compared to crypts obtained from naïve WT littermates. EtOH treatment did not significantly affect the formation and growth of organoids derived either from fat-1 or WT mice (Fig. 1E-F). Although, it has been previously shown that EtOH can inhibit the growth and budding of mouse ileal organoids after 5 days of culture (29), we did not observe this effect with longer time course (after 7 days in culture, data not shown) possibly due to differences in the establishment of the cultures or growth medium. In organoids derived from the ileum of mice fed either control or ethanol containing diets, we observed a significant increase in the expression of ISC markers, Lgr5 and Bmi1, in fat-1 compared to WT mice, both in EtOH- and control diet-fed animals (Fig. 1G-H). Expression of Muc2, a goblet cells and mucus production marker, was elevated in fat-1 mice, both in EtOH-fed and PF mice, compared to WT littermates (although this increase was not significant, Fig. 1I), which was consistent with a similar goblet cell distribution in the ileal tissue of the WT and fat-1 animals (Supplementary Fig. S2C). Compared to WT, intestinal organoids derived from fat-1 experimental mice had up-regulated bactericidal permeability-increasing protein (Bpi1), an antibacterial and endotoxin-neutralizing molecule (Fig. 1J). Expression of intestinal tight junction proteins, Occl and Zo1, was similar in fat-1 and WT PF mice, while significantly higher in fat-1-EtOH compared to WT-EtOH littermates (Fig. 1K-L), which was consistent with higher ZO1 protein levels in the ileal tissue of fat-1-EtOH vs. WT-EtOH experimental animals (Fig. 1M). However, there were no appreciable EtOH-induced changes in LPS and CD14 (circulating markers of intestinal permeability) either in PF or EtOH-fed fat-1 and WT littermates (Supplemental Fig. S3A-3B).

The impact of the differential ω-6:ω-3 PUFA ratio on the intestinal mucosa transcriptome responses to EtOH challenge

To gain deeper insight into the impact of the distinct ω-6:ω-3 PUFA ratio on intestinal mucosa transcriptome responses to EtOH challenge, we performed an RNA-seq analysis of intestinal mucosal epithelial tissue (ileal segments) isolated from WT and fat-1 mice (n=3-5). Although the liver is the primary tissue responsible for EtOH metabolism, the gut is also an important, albeit quantitatively lower, first-line location for EtOH detoxification (34). There are several metabolic pathways/enzymes involved in EtOH metabolism, including alcohol dehydrogenases (Adhs), a family of dehydrogenases/reductases responsible
for oxidation of EtOH to acetaldehyde. Adh1 (expressed both in the liver and the intestine) and Adh6 (almost exclusively expressed in the small intestine (35)) were the most abundantly expressed genes in both WT and fat-1 pair-fed control animals with significantly higher levels in fat-1 mice (Fig. 2A). EtOH administration resulted in down-regulation of these genes to similar levels in both genotypes. The enzymes of the aldehyde dehydrogenase (Adh) family further catalyze the oxidation and detoxification of aldehydes, including acetaldehyde, to carboxylic acids. The most abundantly expressed Aldhs in the intestines were Aldh1b1, Aldh9a, Aldh2, and Aldh1a1. Several Aldhs were significantly higher in fat-1-PF compared to WT-PF mice, including Aldh1a1, I Aldh1a7, and Aldh3b1. Interestingly, expression of Aldh1a1 was up-regulated while Aldh9a was down-regulated by EtOH administration in both WT and fat-1 mice. The levels of Aldh1b1 and Aldh2, (the major enzyme for the acetaldehyde oxidation) were similar in all experimental groups. Although we did not evaluate the EtOH-metabolizing system in the liver, it is most likely that there were no significant differences in expression of these genes between WT and fat-1 EtOH-fed animals, as blood alcohol levels were similar in WT and fat-1 mice on the EtOH-supplemented diet (Fig. 2B).

Further analysis revealed that there were a total of 94 genes that were differentially regulated between fat-1 and WT EtOH-fed mice (80 upregulated and 14 downregulated, Fig. 2C-D). The complete list of differentially expressed genes is presented in Supplemental Table S3, and organized by biological function as determined from published literature. Twenty-five of the genes were involved in immune function (e.g., Tlr2, Tlr5, and Nos2), while 14 are important for lipid metabolism or transport (e.g., Abca12, Atn1, and Mgll). There were a number of genes differentially expressed in fat-1 vs. WT mice that are involved in cell proliferation and ISC renewal, and are potentially important for tissue repair (e.g., Adra2a, Pla2g10, and Plet1). Interestingly, the expression of tryptophan hydroxylase-1 (Tph-1, which synthesizes serotonin) was significantly increased as well as several other novel genes. For example, the prion protein gene (Prnp), the E3 ubiquitin protein ligase and ulcerative colitis (UC)-linked gene (Rnf186), and cystathionine β synthase (Cbs) were all increased in fat-1 vs. WT mice.

Inflammation and anti-microbial response to LPS-challenge in mice chronically fed EtOH

To assess the role of increased ω-3 PUFA and a concomitant decrease in the ω-6:ω-3 PUFA ratio in intestinal inflammation, we investigated the intestinal mucosa pro-inflammatory responses to LPS challenge in a setting of a chronic EtOH pre-exposure. Intestinal inflammation was significantly lower in fat-1 compared to WT mice in response to EtOH+LPS treatment, and was characterized by reduced numbers of neutrophils and mononuclear cells (Fig. 3A-B). This was also accompanied by markedly decreased levels of the pro-inflammatory cytokines INF-γ (Fig. 3C) and IL-6 in EtOH+LPS fat-1 vs WT mice (Fig. 3D). IL-1β and TNFα levels were similar in both fat-1 and WT EtOH+LPS treated mice (Fig. 3E-F). Although CXCL1 protein levels were equally induced in fat-1 and WT mice, Cxcl1 gene expression was significantly down-regulated in EtOH+LPS fat-1 mice compared WT littermates (Fig. 3G-H). Gene expression levels of Il-1β, Tnf-α, and Il-6 were consistent with their protein levels (Fig. 3I). Given that ω-3 PUFAs are precursors to specialized pro-resolving mediators that actively promote the resolution of inflammation, we tested the effect of the DHA-derived resolvins, RvD1, on intestinal inflammation caused by EtOH+LPS challenge. RvD1 treatment significantly decreased intestinal neutrophil infiltration induced by EtOH+LPS (Fig. 3I) in parallel with decreased IL-6 and CXCL1 protein levels, while TNFα levels were moderately reduced, but IL-1β levels were unchanged (Fig. 3J).

In order to maintain intestinal homeostasis and promote host defense against pathogens, different epithelial cells (mainly Paneth cells, a highly specialized population of intestinal cells located at the crypt base), produce a diverse array of antimicrobial proteins (AMPs). In our experiment, the number of Paneth cells was not observably different between the experimental groups, however, LPS administration resulted in redistribution of lysozyme staining (a marker of Paneth cells) in both WT and fat-1 EtOH+LPS treated mice (Fig. 3K). In agreement, the expression of genes encoding lysozymes, such as Lyz1 and Lyz2, was not altered by any of experimental conditions. There were also no significant differences between WT and fat-1 mice in the expression of other AMPs, including defensin and C-type lectin families in response to EtOH or EtOH+LPS treatments (Supplementary Fig. S4). In line with a previously reported
study (10), expression of intestinal alkaline phosphatase (Alpi), a glycosylphosphatidylinositol-linked protein expressed primarily in mucosal epithelia (36) and an important factor in inflammatory resolution in the mucosa (37), was significantly upregulated in fat-1-PF compared to WT-PF littermates (Fig. 3L). EtOH treatment resulted in Alpi down-regulation in both genotypes. Notably, LPS administration elevated Alpi expression in fat-1 but not WT mice resulting in significantly higher Alpi levels in fat-1 as compared to WT EtOH+LPS treated littermates. The expression of another AMP, liver enriched antimicrobial peptide 2 (Leap2), followed a similar trend as that for Alpi (Fig. 3M).

**Compositional and structural shift of gut microbiota in WT and fat-1 mice caused by chronic ethanol feeding and LPS challenge**

Because dysregulation of intestinal homeostasis and susceptibility to inflammation are often associated with alterations in the gut microbiome, we investigated how bacterial communities were impacted by chronic EtOH administration and evaluated the effects of tissue modulation of the ω-6:ω-3 PUFA ratios on these alterations. At the phylum level, *Firmicutes* and *Bacteroidetes* accounted for the major proportion of the bacterial population in all groups regardless of genotype or treatment (Fig. 4A). Six weeks of either control or EtOH-containing diet consumption resulted in significant changes in the gut microbiota in both genotypes. Importantly, the gut microbial communities of EtOH-fed mice clustered separately from naïve (baseline control) or PF mice (experimental control), indicating a unique impact of EtOH on the gut microbiome (Fig. 4B). EtOH treatment resulted in a shift in the *Firmicutes*: *Bacteroidetes* ratio (due to increased *Bacteroidetes* and decreased *Firmicutes*) in both WT and fat-1 mice compared to control PF littermates (Fig. 4C). WT and fat-1 EtOH-fed mice were differentially enriched with *Verrucomicrobiaceae*, *Clostridiaceae*, *Coriobacteriaceae*, *Peptostreptococcaceae*, *Prevotellaceae*, and several OTUs in the *Bacteroidales* order, whereas *Lachnospiraceae*, *Leuconostacaceae*, and *Pseudomonadales* were depleted in response to EtOH challenge compared to control diet feeding in both genotypes (Fig. 4D, Supplementary Fig.S5A-B). *Lactobacillaceae* and *Erysipelotrichaceae* were depleted in EtOH-fed mice compared to PF mice in WT but not fat-1 animals. Further analysis revealed several *Lachnospiraceae*, *Ruminococcaceae* (short-chain fatty acid producers), and *Bacteroidales* OTUs that were differentially enriched in EtOH-fed fat-1 mice, although microbiome diversity and richness were comparable between these two groups (data not shown). LPS-challenge in EtOH-fed mice induced a modest shift in gut microbial community structure in both WT and fat-1 mice without significant changes in microbial diversity and richness. LEfSe analysis revealed a significant expansion of *Enterobacteriaceae* and *Erysipelotrichaceae* in response to LPS challenge in WT mice, whereas several *Ruminococcaceae* and *Bacteroidales* OTUs were depleted as a result of LPS challenge in both WT and fat-1 animals (Fig. 4E). Importantly, *Porphyromonadaceae*: *Barnesiella* and *Lachnospiraceae* were differentially enriched (increased) in EtOH-fed fat-1 mice in response to LPS challenge, but not in EtOH-fed WT mice (Supplementary Fig. S5C-D).

We further analyzed selected bacterial taxa with roles known to be important in EtOH-induced multi-organ pathology. Specifically, *Lactobacillus*, a beneficial taxa for ALD (38) was significantly reduced in WT mice in response to EtOH alone and in response to EtOH+LPS, while levels of this bacteria were not affected by EtOH in fat-1 mice (Fig. 4F). Considering that metabolites of gut microbiota, such as short-chain fatty acids, are the primary energy source for intestinal epithelial cells, we analyzed *Roseburia*, a butyrate producing bacteria, which was enriched (although not significantly) in fat-1-PF compared to WT-PF animals, and similarly decreased in both genotypes in EtOH and EtOH+LPS treated animals. In line with a previous report (39), EtOH feeding resulted in increased abundance of *Akkermansia*, and this effect was independent on the genotype. The abundance of *Parabacteroides* was increased by EtOH+LPS in fat-1 mice. Other genera such as *Barnesiella* were significantly increased, while *Alistipes* were decreased by EtOH+LPS challenge in both genotypes. The abundance of the *Oscillibacter* genus were not affected in any experimental condition.
Impact of ω-6:ω-3 ratio modulation on the plasma metabolome following EtOH administration and LPS challenge

The intestinal microbiota communicates with the host via numerous microbial metabolites (40). The blood metabolome, a net result of metabolic activity of the gut microbiota and metabolic changes in several tissues, communicates signals between multiple organs, including the gut and the liver. Therefore, we conducted a plasma metabolomic analysis to characterize metabolic phenotypes and identify key differences between WT and fat-1 experimental mice. Principal Component Analysis demonstrated that WT and fat-1 mice differed in overall plasma metabolite composition in PF, EtOH-fed, and EtOH-fed+LPS-challenged groups (Fig. 5A). These changes are schematically presented in Fig. 5B. Overall, 18 plasma metabolites had significant differences between WT and fat-1 PF animals, including butyric acid which was significantly lower in WT, however this difference was lost in EtOH-fed mice. Chronic EtOH feeding resulted in minimal differences between WT and fat-1 mice (there were 7 differentially expressed metabolites), while LPS administration produced the majority of observed metabolomic differences (37 differentially expressed metabolites). Statistically significant changes belonged to multiple metabolic pathways, including amino-acid, carbohydrate, lipid, xenobiotic and microbial metabolism among others. Specifically, EtOH-treated WT mice compared to fat-1 littermates had higher levels of cytosine, cysteine-S-sulfate, L-5-hydroxy-tryptophan, arachidonic acid and cis-aconitate, while exhibiting lower levels of glutamine and pantothenic acid. Compared to EtOH+LPS treated fat-1 mice, WT counterparts revealed increased levels of several lipid compounds such as arachidonic acid, 9-hexadecenoic acid (a trans-isomer of palmitoleic acid), and a phospholipid O-phosphoethanolamine. Molecules involved in carnitine metabolism such as acetyl-L-carnitine and glutaryl-L-carnitine were elevated in WT-EtOH+LPS as compared to fat-1-EtOH+LPS treated mice. The most prominent alterations between EtOH+LPS treated fat-1 and WT mice were observed in protein degradation and amino-acid metabolism. Compared to fat-1 mice, WT animals treated with EtOH+LPS revealed reduced levels of tryptophan, creatine, threonine, methionine, and glutamic acids. Several acetylated amino acids and dipeptides, including O-acetylseryine, phenylacetylglucine, N-acetyl-L-glutamine, 2-Aminoadipic acid were increased in WT-EtOH+LPS vs. fat-1-EtOH+LPS mice, while the levels of N-acetylornithine were reduced. WT-EtOH+LPS mice also exhibited increased levels of urea, uridine, as well as several molecules belonging to dietary components and microbial metabolites, such as N8-acetylspermidine and 3-hydroxybenzoic acid.

Alterations in fecal bile acids and related metabolic pathways in WT and fat-1 mice

One of the essential functions provided by the gut microbiota is modulation of gastrointestinal metabolites, including their synthesis, digestion, fermentation, and secondary metabolism. Among these metabolites are bile acids (BAs). EtOH-induced alterations in BA metabolism has been found in humans and in multiple animal models (41), (42) (43), (44). We next examined the impact of decreased ω-6:ω-3 PUFA ratio on selected BAs under the EtOH+LPS experimental condition. There were no significant differences in fecal levels of primary BAs (cholic acid [CA], ursodeoxycholic acid [UDCA], or chenodeoxycholic acid [CDCA], or in the levels of secondary BAs (deoxycholic acid [DCA]) in either genotype in PF mice or in response to EtOH+LPS treatment. Notably, the secondary BA, lithocholic acid (LCA), a product of microbial metabolism of UDCA and CDCA via 7-dehydroxylation (45, 46), was the only BA markedly elevated by EtOH+LPS in WT compared to WT-PF as well as in fat-1-EtOH+LPS treated mice (Fig. 6A). Measurement of total bile acid concentrations in serum revealed a significant increase in both WT and fat-1 EtOH+LPS treated mice with no differences between genotypes (Supplemental Fig. S6).

Given the importance of BA enterohepatic circulation, we measured the relative expression of genes involved in BA metabolism in both the intestine and in the liver. In the intestine, expression of Fgf15 (FGF19 in humans), which is involved in a negative feedback loop of BA synthesis in the liver, was up-regulated by EtOH and EtOH-LPS in WT but not in fat-1 mice (Fig. 6B). Intestinal expression of genes of BA reabsorption and export (Osta, Ostb, and Asbt) were similar between genotypes and not affected in any treatment group (Fig. 6C). In the liver, genes associated with the major pathways of BA synthesis (Cyp7a1, Cyp27a1 and Cyp8b) were similarly down-regulated in response to EtOH+LPS treatment in both WT and
fat-1 mice (Fig. 6D), while markers of BA transport (Abcb11) and reuptake (Slc10a1) were equally over expressed as compared to control animals (Fig. 6E-F). Several nuclear receptors, including the farnesoid X receptor (Fxr), pregnane X receptor (Pxr), and constitutive androstane receptor (Car), are all involved in the regulation of BA synthesis, and in protection against the cytotoxic effects of BAs by increasing the expression of binding proteins, transporters, and enzymes that detoxify BAs (47). Expression of Fxr, which represses BA synthesis in the liver and activates Fgf15 in the intestine, was upregulated by EtOH in the intestine but not in the liver in either genotype. EtOH+LPS treatment further increased intestinal Fxr in WT but not in fat-1 mice (Fig. 6G-H). Interestingly Pxr, which is an LCA receptor, was differentially modulated by LPS treatment in the intestine (upregulated) and the liver (downregulated) after EtOH treatment. Compared to PF control animals, expression of Car was downregulated in EtOH and EtOH+LPS treated groups in both WT and fat-1 genotypes. Notably, expression of Car was significantly higher in the intestines of fat-1 mice compared to EtOH+LPS treated WT mice.

Hydroxylation and sulfation, key pathways for the conversion of BAs to more hydrophilic and less toxic compounds, are important mechanisms to prevent BA-induced tissue/organ damage. Hydroxylation occurs predominantly via Cyp3a11 (CYP3A4 in humans) both in the intestine and in the liver (48, 49), and sulfation via specific sulfotransferases, predominately in the liver. We measured the relative expression of major genes involved in these pathways in the intestine (Fig. 7A), as well as in the liver (Fig. 7B). Expression of Cyp3a11, Cyp2b10, and Sult1b1 was significantly higher in the intestines but not in the livers of fat-1 PF mice compared to WT PF littermates. EtOH feeding significantly decreased intestinal but not liver Cyp3a11 levels in both genotypes. Intestinal and liver Sult1b1 levels were similarly significantly reduced by EtOH in fat-1 and WT animals, while Sult1a1 expression was significantly increased in the intestines and decreased in the livers of both genotypes. Interestingly, compared to EtOH alone EtOH+LPS administration significantly induced intestinal Cyp3a11, Cyp2b10, and Sult1b1 in fat-1 mice, while expression of these genes was further decreased by EtOH+LPS in WT littermates. This pattern was not observed in the livers, where expression of Cyp3a11 and Sult1b1 were equally decreased in WT and fat-1 mice in response to EtOH+LPS administration.

Decreased ω-6:ω-3 PUFA ratio resulted in the attenuation of liver injury associated with EtOH and LPS administration

We next sought to determine if the effects of a decreased ω-6:ω-3 PUFA ratio on the gut was associated with attenuation of liver pathology caused by EtOH consumption and LPS-challenge. EtOH alone moderately increased plasma ALT levels (a marker of liver injury) in WT but not in fat-1 mice. Further, compared to WT, fat-1 mice had significantly decreased ALT levels in response to EtOH+LPS treatment (Fig. 8A), but there were no differences in hepatic triglyceride (TG) accumulation, and the degree of steatosis was similar in both genotypes (Fig. 8B-C). We next performed multiple correlation analyses to evaluate the potential link between markers of liver injury, intestinal inflammation, and the gut microbiota. ALT levels were positively correlated with intestinal CXCL1 levels (Fig. 8D), and the frequency of the bacteria genera Oscillibacter (Oscillospiraceae family in the phylum Firmicutes) and Parabacteroides (Porphyromonadaceae family in the phylum Bacteroidetes) in WT but not in fat-1 mice subjected to EtOH+LPS treatment (Fig. 8E). Negative correlation was noted between liver TG levels and the genus Alistipes (Rikenellaceae family in the phylum Bacteroidetes) in EtOH+LPS treated fat-1 mice.
DISCUSSION

In this study, we showed that the reduction in the ω-6:ω-3 PUFA ratio in fat-1 mice produced changes that attenuated many of the toxic effects of EtOH and EtOH+LPS on the intestine, and was associated with reduced liver injury. Because there were no differences in the expression of intestinal EtOH-metabolizing genes between fat-1 and WT mice, the differential effects identified are due to other mechanisms as discussed below. Our results demonstrated that endogenous ω-3 PUFA enrichment and reduction in the ω-6:ω-3 PUFA ratio preserved intestinal tight junction protein expression in both intestinal organoids and as determined in vivo by RNA-seq analysis. One potential mechanism is through down-regulation of Nos2 expression. In addition to its well-established role in innate immunity, iNOS has also been demonstrated to disrupt intestinal tight junctions (50). A reduced ω-6:ω-3 PUFA ratio also positively impacted intestinal stem cell growth and proliferation, shown in organoid cultures and in vivo. The expression of several genes were increased in fat-1 vs. WT mice that have been demonstrated to regulate proliferation of intestinal cells (e.g., adrenocorticotropin 2A, Adra2a) and renewal of the colonic mucosa in mice (e.g., aldo-keto reductase family 1, B8, Akr1b8). Finally, the expression of tryptophan hydroxylase-1 (Tph1) was also significantly up-regulated in fat-1 mice. TPH1 synthesizes 5-HT (5-hydroxytryptamine, serotonin) in the gut and although the role of 5-HT in gut health is controversial, it was recently shown that mice with enhanced uptake of 5-HT in the intestine had increased mucosal growth (51).

There were several other interesting observations related to intestinal health and disease revealed by RNA-seq analysis of the intestinal mucosa, including Plet1 with ~ 46 fold-induction in fat-1 mice vs. WT. Zepp, et al. recently demonstrated that IL17A-mediated induction of Plet1 expression was necessary for tissue repair following dextran sulfate sodium (DSS)-induced colitis (52). In a series of reports in mouse models of colitis, a number of other genes have been demonstrated to be beneficial. For example, cellular prion protein (PrPC, encoded by Prnp) and Rnf186, were elevated in fat-1 mice and each has been demonstrated to protect mice from DSS-induced colitis (53, 54). The expression of Toll-like receptor genes, (Tlr2 and Tlr5) was up-regulated in fat-1 vs. WT mice and Choteau, et al. demonstrated that Tlr2 knockout mice had greater intestinal dysfunction and inflammation when compared to WT mice (55).

One of the most significant findings from this study is that endogenous ω-3 PUFA enrichment and RvD1 treatment attenuated EtOH-mediated intestinal inflammation. Altering the ω-6:ω-3 PUFA ratio has also proven beneficial in other mouse models. For example, fat-1 mice were protected from DSS-induced colitis (56) and many SPMs, including RvD1, are effective in mouse models of inflammatory bowel disease [reviewed in (57)]. In humans, the role of ω-3 PUFAs in intestinal inflammation is not well established, but there is evidence for beneficial effects in ulcerative colitis and Crohn’s disease [reviewed in (58)], however, the role of SPMs has not been investigated but there is evidence that patients with UC had low levels of DHA-derived SPMs compared to patients in remission (59). Based upon these studies and on our results, treatment with RvD1 and other SPMs may be an effective treatment strategy, alongside ω-3 PUFA supplementation, to treat inflammatory disorders of the intestine.

The intestine, among other organs exposed to environmental factors, produces a number of protective factors, including anti-microbial peptides (AMPs). Gut AMPs protect the host from pathogens and control intestinal inflammation by either an enzymatic (Alpi, alkaline phosphatase and soluble phospholipase A2) or a non-enzymatic mechanisms [e.g. defensins, cathelecidins, and C-type lectins (REG3 family)] (60). Although there were no differences in the expression of defensins between fat-1 and WT mice challenged with LPS, fat-1 mice had significantly higher intestinal expression of two antimicrobial genes, Leap2, and Alpi. LEAP2 (liver-expressed antimicrobial peptide 2), despite the name, is most highly expressed in the small intestine (61), and though there is evidence that LEAP2 has antimicrobial activity in vitro, its in vivo role is less clear. In contrast, much more is known about the function of ALPI. ALPI is an important defense mechanism in the intestine because it can inactivate LPS and regulate the microbiota [reviewed in (62)]. Interestingly, the expression of Alpi can be induced by the EPA-derived SPM, resolvin E1 (37). Another novel finding from this study was the observation of increased expression of a “non-classical” antibacterial and endotoxin-neutralizing molecule, Bpi1, in fat-1 mice-derived intestinal organoids. Bpi is transcriptionally up-regulated by lipoxins (63), and exerts a potent (nanomolar) and selective bioactivity against gram-negative bacterial species.
Our results provide evidence that tissue ω-3 PUFA enrichment favorably modulates intestinal microbiota. It is well documented that alcohol consumption resulted in alterations of the intestinal microbiota in humans and rodents (64), therefore modulation of gut microbial composition is an attractive therapeutic approach for alcohol-related pathologies, including liver disease. We previously showed that a diet enriched in saturated fatty acids prevented gut microbiota changes induced by ethanol and a diet enriched in ω-6 PUFA, linoleic acid, further attenuated intestinal and liver damage (6). In the present study, we observed significant EtOH-induced changes in both WT and fat-1 mice; however, modulation of the ω-6:ω-3 PUFA ratio did not produce marked changes in the intestinal microbiome (in α or β diversity, or phyla composition) between fat-1 and WT mice. Our data are in agreement with studies demonstrating the lack of significant changes in microbial diversity associated with ω-3 PUFA supplementation (14). However, our results are in some disagreement with the study by Kaliannan et al., that demonstrated significant differences in gut microbiota in fat-1 compared to WT mice (10), including significantly lower levels of Proteobacteria in fat-1 mice, which we did not observe in our study. This discrepancy can potentially be explained by environmental differences in the mouse facilities between our institutions (e.g., animal husbandry practices, bedding, water, etc.). This effect was convincingly demonstrated recently in a study where mice from the same vendor were fed the same Lieber-DeCarli EtOH diet but housed in two different institutions in France produced drastically different outcomes attributed to differences in the gut microbiome due to differences in animal husbandry between these two institutions (43). Nevertheless, there were several individual taxa that revealed differences associated with modulation of the ω-6:ω-3 PUFA ratio. Thus, an important observation in our study was that the EtOH-induced drop in Lactobacillus in WT was prevented in fat-1 mice. A decrease in Lactobacillus was associated with alcohol consumption in humans and in rodents (65, 66). Lactobacillus species exert multiple beneficial properties (e.g., production of antimicrobial substances (67)), and treatment with Lactobacillus rhamnosus Gorbach Goldin (LGG), for example, resulted in improvement of intestinal barrier function and subsequent protection against alcohol-induced endotoxemia and liver injury in mice (38, 68, 69). In addition, many species of lactic acid bacteria, e.g., Lactobacillus plantarum, possess enzymes involved in saturation metabolism of PUFAs generating multiple fatty acid species, such as hydroxy, o xo, conjugated, and partially saturated trans-fatty acids (70). Some of these unique lipid species exert specific physiological functions. For instance, 10-Hydroxy-cis-12-octadecenoic acid, a gut microbial metabolite of linoleic acid, attenuated intestinal epithelial barrier dysfunction, and ameliorated intestinal inflammation in DSS-induced colitis in mice by the suppression of TNFR2 up-regulation (71). In line with the observation that ω-3 PUFA supplementation was associated with an increase in abundance of butyrate-producing genera Roseburia (14), we also observed elevated levels (also not significant) of Roseburia in fat-1 mice as compared to WT littermates. Despite the relatively small changes in Roseburia, it most likely had functional consequences, as the blood butyric acid, an energy source for enterocytes and an important epigenetic modulator of gene expression, was significantly higher in fat-1 mice, but only in the PF animals. Butyrate supplementation attenuated ethanol-induced intestinal barrier and liver injury (72). In addition to butyrate production, Roseburia metabolizes linoleic acid via conjugated linoleic acids to vaccenic acid in human and animals (73), and both of these compounds are considered to be beneficial for health (74). Metabolomic analyses of plasma from fat-1 and WT mice revealed several interesting findings. ω-6 PUFA-derived arachidonic acid (AA) was elevated in the blood of WT relative to fat-1 mice (both EtOH alone, and EtOH+LPS exposures). AA and its metabolites can be pro-inflammatory and we have recently demonstrated that many AA metabolites were up-regulated in mice fed a diet high in ω-6 PUFAs and EtOH and were associated with increased liver inflammation (75). Tryptophan metabolism is a critical function of the gut microbiota that regulates many host functions including metabolism and immunity (76). Blood tryptophan was diminished in WT mice relative to fat-1 mice exposed to EtOH+LPS, while the toxic tryptophan metabolite 3-indoxyl sulfate (I3S) was increased. I3S is an aryl hydrocarbon receptor (AhR) ligand (77) that can promote systemic inflammation through immune cell activation (78). Since fat-1 mice had reduced levels of I3S, this may be one mechanism by which fat-1 mice are protected from EtOH+LPS-induced intestinal inflammation. These data also revealed lower concentrations of nicotinamide in WT
mice exposed to EtOH+LPS relative to fat-1 mice. Nicotinamide is an NAD+ precursor and the metabolism of EtOH decreases the NAD+/NADH ratio in the liver disrupting energy metabolism leading to fatty liver (79). fat-1 mice maintain levels of NAD+ precursors which could elicit protection through preservation of tissue REDOX status. We also found decreased levels of methionine but increased levels of cystathionine in the EtOH-fed and LPS-challenged WT mice. Interestingly, a recent study by Yang, et al. found increased levels of these two amino acids in human patients with alcoholic cirrhosis (80).

Important roles for ethanol-mediated alterations in BA profile and metabolism have been reported in clinical and experimental studies (81, 82). Our data demonstrated that the profile of BAs was similar between WT and fat-1 PF and EtOH+LPS treated mice. Similar expression of genes involved in BA synthesis (e.g., Cyp7a1, Cyp27a1, and Cyp8b1) in the livers of WT and fat-1 mice may explain the comparable levels of BA in these animals. LCA, the most lipophilic secondary BA, was the only BA which was significantly elevated by EtOH+LPS in WT but not fat-1 mice. Increased LCA levels were also observed in actively drinking cirrhotic patients (44). At physiological levels, LCA, may prevent TNF-α release from colonic epithelial cells in vitro, and protect against colonic inflammation in a DSS-induced colitis animal model (83). However, high LCA concentrations are toxic, particularly to the liver, as serious liver damage was noted in mice exposed to LCA (84). The expression of a number of genes important for BA detoxification was increased in fat-1 mice, suggesting that they are able to resist the generalized down-regulation of the detoxification pathway in response to LPS. Finally, we found that the expression of Nr1h4/Fxr was induced by EtOH; however, LPS-challenge did not further increase its expression in fat-1 mice, as was found in WT mice. Conversely, the expression of Nr1i3/Car was decreased by EtOH, but increased to a greater extent in fat-1 mice vs. WT in the EtOH+LPS group. Interestingly, this pattern was specific to the intestine. It was recently shown that inhibition of intestinal FXR protected mice from non-alcoholic liver disease (85). In addition, NR1I3/CAR has been demonstrated to play a role in reducing intestinal damage in mice treated with DSS (86).

In our study we used a multi-pronged approach to investigate alterations in the intestinal homeostasis caused by EtOH. While this design has significant strengths and advantages, we acknowledge that there are some limitations in our study. For example, the RNA-seq analysis was performed on total ileal mucosa RNA. Thus, with some of the genes identified, we cannot identify the specific cell type responsible for the differential expression, particularly with genes of the immune system. Therefore, future studies using single-cell sequencing would be useful in elucidating the role of the various cells of the gut. Nevertheless, these data provide a solid foundation upon which to further investigate the effect of ω-3 PUFAs on intestinal homeostasis.

There are several important implications to our findings. First, that modest changes in the ratio of ω-6:ω-3 PUFAs can lead to significant changes in the response of the intestine to EtOH with respect to attenuation of inflammation and suggests that dietary factors can have a profound effect. Secondly, this effect is mediated not only by changes in the expression of the inflammatory pathway, but also through the induction of AMP gene expression and subsequent changes in the microbiota. Indeed, AMPs are emerging as a therapeutic treatment for a number of diseases (87). In addition to these effects seen in fat-1 mice, additional pathways are involved, including that by which some hepatotoxic BAs (e.g., LCA) are detoxified through hydroxylation and conjugation in both the intestine (88) and liver (89). Furthermore, the effects of ω-3 PUFAs on the associated liver injury on the liver may be mediated by other factors, including plasma metabolites and intrinsic changes in the ratio of ω-6:ω-3 PUFAs in the livers of fat-1 mice. It is also possible that some of the changes observed in fat-1 mice are secondary to an overall attenuation in inflammation and protection of the liver from EtOH-induced damage mediated by other mechanisms. In conclusion, our results provide the basis for a model in which decreased tissue ω-6:ω-3 PUFA ratio leads to specific changes in intestinal homeostasis, the gut microbiota, BA metabolism, and plasma metabolome that coordinate to reduce intestinal inflammation and associated liver damage in the context of chronic EtOH exposure and LPS challenge (summarized in Fig. 8F).
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REFERENCES

1. Bishehsari F, Magno E, Swanson G, Desai V, Voigt RM, Forsyth CB, et al. Alcohol and Gut-Derived Inflammation. Alcohol Res. 2017;38(2):163-71.
2. Rao R. Endotoxemia and gut barrier dysfunction in alcoholic liver disease. Hepatology. 2009;50(2):638-44.
3. Xie G, Zhong W, Li H, Li Q, Qiu Y, Zheng X, et al. Alteration of bile acid metabolism in the rat induced by chronic ethanol consumption. FASEB J. 2013;27(9):3583-93.
4. Kirpich IA, Feng W, Wang Y, Liu Y, Barker DF, Barve SS, et al. The type of dietary fat modulates intestinal tight junction integrity, gut permeability, and hepatic toll-like receptor expression in a mouse model of alcoholic liver disease. Alcohol Clin Exp Res. 2012;36(5):835-46.
5. Kirpich IA, Feng W, Wang Y, Liu Y, Beier JI, Arteel GE, et al. Ethanol and dietary unsaturated fat (corn oil/linoleic acid enriched) cause intestinal inflammation and impaired intestinal barrier defense in mice chronically fed alcohol. Alcohol. 2013;47(3):257-64.
6. Kirpich IA, Petrovino S, Ajami N, Feng W, Wang Y, Liu Y, et al. Saturated and Unsaturated Dietary Fats Differentially Modulate Ethanol-Induced Changes in Gut Microbiome and Metabolome in a Mouse Model of Alcoholic Liver Disease. Am J Pathol. 2016;186(4):765-76.
7. Huang W, Wang B, Li X, Kang JX. Endogenously elevated n-3 polyunsaturated fatty acids alleviate acute ethanol-induced liver steatosis. Biofactors. 2015;41(6):453-62.
8. Wang M, Zhang X, Ma LJ, Feng RB, Yan C, Su H, et al. Omega-3 polyunsaturated fatty acids ameliorate ethanol-induced adipose hyperlipolysis: A mechanism for hepatoprotective effect against alcoholic liver disease. Biochim Biophys Acta Mol Basis Dis. 2017;1863(12):3190-201.
9. Costantini L, Molinari R, Farinon B, Merendino N. Impact of Omega-3 Fatty Acids on the Gut Microbiota. Int J Mol Sci. 2017;18(12).
10. Watson H, Mitra S, Croden FC, Taylor M, Wood HM, Perry SL, et al. A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota. Gut. 2018;67(11):1974-83.
11. Ungaro F, Rubbino F, Danese S, D'Alessio S. Actors and Factors in the Resolution of Intestinal Inflammation: Lipid Mediators As a New Approach to Therapy in Inflammatory Bowel Diseases. Front Immunol. 2017;8:1331.
12. Musa-Veloso K, Venditti C, Lee HY, Darch M, Floyd S, West S, et al. Systematic review and meta-analysis of controlled intervention studies on the effectiveness of long-chain omega-3 fatty acids in patients with nonalcoholic fatty liver disease. Nutr Rev. 2018;76(8):581-602.
17. Jeyapal S, Kona SR, Mullapudi SV, Putcha UK, Gurumurthy P, Ibrahim A. Substitution of linoleic acid with alpha-linolenic acid or long chain n-3 polyunsaturated fatty acid prevents Western diet induced nonalcoholic steatohepatitis. Sci Rep. 2018;8(1):10953.
18. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510(7503):92-101.
19. Zehr KR, Walker MK. Omega-3 polyunsaturated fatty acids improve endothelial function in humans at risk for atherosclerosis: A review. Prostaglandins Other Lipid Mediat. 2018;134:131-40.
20. Freitas RDS, Campos MM. Protective Effects of Omega-3 Fatty Acids in Cancer-Related Complications. Nutrients. 2019;11(5).
21. Lee KR, Midgette Y, Shah R. Fish Oil Derived Omega 3 Fatty Acids Suppress Adipose NLRP3 Inflammasome Signaling in Human Obesity. J Endocr Soc. 2019;3(3):504-15.
22. Simopoulos AP. An Increase in the Omega-6/Omega-3 Fatty Acid Ratio Increases the Risk for Obesity. Nutrients. 2016;8(3):128.
23. Blasbalg TL, Hibbelen JR, Ramsden CE, Majchrzak SF, Rawlings RR. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. Am J Clin Nutr. 2011;93(5):950-62.
24. Hart CL, Morrison DS, Batty GD, Mitchell RJ, Davey Smith G. Effect of body mass index and alcohol consumption on liver disease: analysis of data from two prospective cohort studies. BMJ. 2010;340:c1240.
25. Kang JX, Wang J, Wu L, Kang ZB. Transgenic mice: fat-1 mice convert n-6 to n-3 fatty acids. Nature. 2004;427(6974):504.
26. Gustot T, Fernandez J, Szabo G, Albillos A, Louvet A, Jalan R, et al. Sepsis in alcohol-related liver disease. J Hepatol. 2017;67(5):1031-50.
27. Kang JX, Wang J. A simplified method for analysis of polyunsaturated fatty acids. BMC Biochem. 2005;6:5.
28. Collins JF, Hu Z, Ranganathan PN, Feng D, Garrick LM, Garrick MD, et al. Induction of arachidonate 12-lipoxygenase (Alox15) in intestine of iron-deficient rats correlates with the production of biologically active lipid mediators. Am J Physiol Gastrointest Liver Physiol. 2008;294(4):G948-62.
29. Lu R, Voigt RM, Zhang Y, Kato I, Xia Y, Forsyth CB, et al. Alcohol Injury Damages Intestinal Stem Cells. Alcohol Clin Exp Res. 2017;41(4):727-34.
30. Forsyth CB, Shaikh M, Bishehsari F, Swanson G, Voigt RM, Dodiya H, et al. Alcohol Feeding in Mice Promotes Colonic Hyperpermeability and Changes in Colonic Organoid Stem Cell Fate. Alcohol Clin Exp Res. 2017;41(12):2100-13.
31. Antharam VC, Li EC, Ishmael A, Sharma A, Mai V, Rand KH, et al. Intestinal dysbiosis and depletion of butyrogenic bacteria in Clostridium difficile infection and nosocomial diarrhea. J Clin Microbiol. 2013;51(9):2884-92.
32. Kozlov A, Bean L, Hill EV, Zhao L, Li E, Wang GP. Molecular Identification of Bacteria in Intra-abdominal Abscesses Using Deep Sequencing. Open Forum Infect Dis. 2018;5(2):oyf025.
33. Kirpich IA, Gobejishvili LN, Bon Homme M, Waigel S, Cave M, Arteel G, et al. Integrated hepatic transcriptome and proteome analysis of mice with high-fat diet-induced nonalcoholic fatty liver disease. J Nutr Biochem. 2011;22(1):38-45.
34. Seitz HK, Poschl G. The role of gastrointestinal factors in alcohol metabolism. Alcohol Alcohol. 1997;32(5):543-9.
35. Fu ZD, Selwyn FP, Cui JY, Klaassen CD. RNA Sequencing Quantification of Xenobiotic-Processing Genes in Various Sections of the Intestine in Comparison to the Liver of Male Mice. Drug Metab Dispos. 2016;44(6):842-56.
36. Vaishnava S, Hooper LV. Alkaline phosphatase: keeping the peace at the gut epithelial surface. Cell Host Microbe. 2007;2(6):365-7.
37. Campbell EL, MacManus CF, Kominsky DJ, Keely S, Glover LE, Bowers BE, et al. Resolvin E1-induced intestinal alkaline phosphatase promotes resolution of inflammation through LPS detoxification. Proc Natl Acad Sci U S A. 2010;107(32):14298-303.
38. Wang Y, Kirpich I, Liu Y, Ma Z, Barve S, McClain CJ, et al. Lactobacillus rhamnosus GG treatment potentiates intestinal hypoxia-inducible factor, promotes intestinal integrity and ameliorates alcohol-induced liver injury. Am J Pathol. 2011;179(6):2866-75.
39. Yan AW, Fouts DE, Brandl J, Starkel P, Torralba M, Schott E, et al. Enteric dysbiosis associated with a mouse model of alcoholic liver disease. Hepatology. 2011;53(1):96-105.
40. Rastelli M, Knauf C, Cani PD. Gut Microbes and Health: A Focus on the Mechanisms Linking Microbes, Obesity, and Related Disorders. Obesity (Silver Spring). 2018;26(5):792-800.
41. Llopis M, Cassard AM, Wrzosek L, Boschat L, Bruneau A, Ferrere G, et al. Intestinal microbiota contributes to individual susceptibility to alcoholic liver disease. Gut. 2016;65(5):830-9.
42. Wu WB, Chen YY, Zhu B, Peng XM, Zhang SW, Zhou ML. Excessive bile acid activated NF-kappa B and promoted the development of alcoholic steatohepatitis in farnesoid X receptor deficient mice. Biochimie. 2015;115:86-92.
43. Ferrere G, Wrzosek L, Cailleux F, Turpin W, Puchois V, Spatz M, et al. Fecal microbiota manipulation prevents dysbiosis and alcohol-induced liver injury in mice. J Hepatol. 2017;66(4):806-15.
44. Bajaj JS, Kakiyama G, Zhao D, Takei H, Fagan A, Hylemon P, et al. Continued Alcohol Misuse in Human Cirrhosis is Associated with an Impaired Gut-Liver Axis. Alcohol Clin Exp Res. 2017;41(11):1857-65.
45. Hirano S, Masuda N. Enhancement of the 7 alpha-dehydroxylase activity of a gram-positive intestinal anaerobe by Bacteroides and its significance in the 7-dehydroxylation of ursodeoxycholic acid. J Lipid Res. 1982;23(8):1152-8.
46. Ishii M, Toda T, Ikarashi N, Kusunoki Y, Kon R, Ochiai W, et al. Gastrectomy increases the expression of hepatic cytochrome P450 3A by increasing lithocholic acid-producing enteric bacteria in mice. Biol Pharm Bull. 2014;37(2):298-305.
47. Garcia M, Thirouard L, Sedes L, Monrose M, Holota H, Caira F, et al. Nuclear Receptor Metabolism of Bile Acids and Xenobiotics: A Coordinated Detoxification System with Impact on Health and Diseases. Int J Mol Sci. 2018;19(11).
48. Alnouti Y. Bile Acid sulfation: a pathway of bile acid elimination and detoxification. Toxicol Sci. 2009;108(2):225-46.
49. Owen BM, Milona A, van Mil S, Clements P, Holder J, Boudjelal M, et al. Intestinal detoxification limits the activation of hepatic pregnane X receptor by lithocholic acid. Drug Metab Dispos. 2010;38(1):143-9.
50. Han X, Fink MP, Yang R, Delude RL. Increased iNOS activity is essential for intestinal epithelial tight junction dysfunction in endotoxemic mice. Shock. 2004;21(3):261-70.
51. Tackett JJ, Gadotra N, Bamdad MC, Muise ED, Cowles RA. Enhanced serotonin signaling stimulates ordered intestinal mucosal growth. J Surg Res. 2017;208:198-203.
52. Zepp JA, Zhao J, Liu C, Bulek K, Wu L, Chen X, et al. IL-17A-Induced PLET1 Expression Contributes to Tissue Repair and Colon Tumorigenesis. J Immunol. 2017;199(11):3849-57.
53. Martin GR, Keenan CM, Sharkey KA, Jirik FR. Endogenous prion protein attenuates experimentally induced colitis. Am J Pathol. 2011;179(5):2290-301.
54. Fujimoto K, Kinoshita M, Tanaka H, Okuzaki D, Shimada Y, Kayama H, et al. Regulation of intestinal homeostasis by the ulcerative colitis-associated gene RNF186. Mucosal Immunol. 2017;10(2):446-59.
55. Choteau L, Vancraeyneste H, Le Roy D, Dubuquoy L, Romani L, Jouault T, et al. Role of TLR1, TLR2 and TLR6 in the modulation of intestinal inflammation and Candida albicans elimination. Gut Pathog. 2017;9:9.
56. Hudert CA, Weylandt KH, Lu Y, Wang J, Hong S, Dignass A, et al. Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. Proc Natl Acad Sci U S A. 2006;103(30):11276-81.
57. Schwanke RC, Marcon R, Bento AF, Calixto JB. EPA- and DHA-derived resolvins' actions in inflammatory bowel disease. Eur J Pharmacol. 2016;785:156-64.
58. Mozaffari H, Daneshzad E, Larijani B, Bellissimo N, Azadbakht L. Dietary intake of fish, n-3 polyunsaturated fatty acids, and risk of inflammatory bowel disease: a systematic review and meta-analysis of observational studies. Eur J Nutr. 2019.
59. Ungaro F, Tacconi C, Massimino L, Corsetto PA, Correale C, Fonteyne P, et al. MFSD2A Promotes Endothelial Generation of Inflammation-Resolving Lipid Mediators and Reduces Colitis in Mice. Gastroenterology. 2017;153(5):1363-77 e6.
60. Mukherjee S, Hooper LV. Antimicrobial defense of the intestine. Immunity. 2015;42(1):28-39.
61. Ge X, Yang H, Bednarek MA, Galon-Tilleman H, Chen P, Chen M, et al. LEAP2 Is an Endogenous Antagonist of the Ghrelin Receptor. Cell Metab. 2018;27(2):461-9 e6.
62. Fawley J, Gourlay DM. Intestinal alkaline phosphatase: a summary of its role in clinical disease. J Surg Res. 2016;202(1):225-34.
63. Canny G, Levy O, Furuta GT, Narravula-Alipati S, Sisson RB, Serhan CN, et al. Lipid mediator-induced expression of bactericidal/ permeability-increasing protein (BPI) in human mucosal epithelium. Proc Natl Acad Sci U S A. 2002;99(6):3902-7.
64. Sarin SK, Pande A, Schnabl B. Microbiome as a therapeutic target in alcohol-related liver disease. J Hepatol. 2019;70(2):260-72.
65. Bull-Otterston L, Feng W, Kirpich I, Wang Y, Qin X, Liu Y, et al. Metagenomic analyses of alcohol induced pathogenic alterations in the intestinal microbiome and the effect of Lactobacillus rhamnosus GG treatment. PLoS One. 2013;8(1):e53028.
66. Leclercq S, Matamoros S, Cani PD, Neyrinck AM, Jamar F, Starkel P, et al. Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. Proc Natl Acad Sci U S A. 2014;111(42):E4485-93.
67. De Vuyst L, Leroy F. Bacteriocins from lactic acid bacteria: production, purification, and food applications. J Mol Microbiol Biotechnol. 2007;13(4):194-9.
68. Wang Y, Liu Y, Sidhu A, Ma Z, McClain C, Feng W. Lactobacillus rhamnosus GG culture supernatant ameliorates acute alcohol-induced intestinal permeability and liver injury. Am J Physiol Gastrointest Liver Physiol. 2012;303(1):G32-41.
69. Forsyth CB, Farhadi A, Jakate SM, Tang Y, Shaikh M, Keshavarzian A. Lactobacillus GG treatment ameliorates alcohol-induced intestinal oxidative stress, gut leakiness, and liver injury in a rat model of alcoholic steatohepatitis. Alcohol. 2009;43(2):163-72.
70. Kishino S, Takeuchi M, Park SB, Hirata A, Kitamura N, Kunisawa J, et al. Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition. Proc Natl Acad Sci U S A. 2013;110(44):17808-13.

71. Miyamoto J, Mizukure T, Park SB, Kishino S, Kimura I, Hirano K, et al. A gut microbial metabolite of linoleic acid, 10-hydroxy-cis-12-octadecenoic acid, ameliorates intestinal epithelial barrier impairment partially via GPR40-MEK-ERK pathway. J Biol Chem. 2015;290(5):2902-18.

72. Cresci GA, Glueck B, McMullen MR, Xin W, Allende D, Nagy LE. Prophylactic tributyrin treatment mitigates chronic-binge ethanol-induced intestinal barrier and liver injury. J Gastroenterol Hepatol. 2017;32(9):1587-97.

73. Devillard E, McIntosh FM, Duncan SH, Wallace RJ. Metabolism of linoleic acid by human gut bacteria: different routes for biosynthesis of conjugated linoleic acid. J Bacteriol. 2007;189(6):2566-70.

74. Field CJ, Blewett HH, Proctor S, Vine D. Human health benefits of vaccenic acid. Appl Physiol Nutr Metab. 2009;34(5):979-91.

75. Warner DR, Liu H, Ghosh Dastidar S, Warner JB, Prodhan MAI, Yin X, et al. Ethanol and unsaturated dietary fat induce unique patterns of hepatic omega-6 and omega-3 PUFA oxylipins in a mouse model of alcoholic liver disease. PLoS One. 2018;13(9):e0204119.

76. Agus A, Planchais J, Sokol H. Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. Cell Host Microbe. 2018;23(6):716-24.

77. Schroeder JC, Dinatale BC, Murray IA, Flaveny CA, Liu Q, Laurenzana EM, et al. The uremic toxin 3-indoxyl sulfate is a potent endogenous agonist for the human aryl hydrocarbon receptor. Biochemistry. 2010;49(2):393-400.

78. Kim HY, Yoo TH, Hwang Y, Lee GH, Kim B, Jang J, et al. Indoxyl sulfate (IS)-mediated immune dysfunction provokes endothelial damage in patients with end-stage renal disease (ESRD). Sci Rep. 2017;7(1):3057.

79. Purohit V, Gao B, Song BJ. Molecular mechanisms of alcoholic fatty liver. Alcohol Clin Exp Res. 2009;33(2):191-205.

80. Yang Z, Kusumanchi P, Ross RA, Heathers L, Chandler K, Oshodi A, et al. Serum Metabolomic Profiling Identifies Key Metabolic Signatures Associated With Pathogenesis of Alcoholic Liver Disease in Humans. Hepatol Commun. 2019;3(4):542-57.

81. Brandl K, Hartmann P, Jih LJ, Pizzo DP, Argeoni J, Ventura-Cots M, et al. Dysregulation of serum bile acids and FGF19 in alcoholic hepatitis. J Hepatol. 2018;69(2):396-405.

82. Donepudi AC, Ferrer JM, Boehme S, Choi HS, Chiang JYL. Deficiency of cholesterol 7alpha-hydroxylase in bile acid synthesis exacerbates alcohol-induced liver injury in mice. Hepatol Commun. 2018;2(1):99-112.

83. Ward JBJ, Lajczak NK, Kelly OB, O'Dwyer AM, Giddam AK, Ni Gabhann J, et al. Ursodeoxycholic acid and lithocholic acid exert anti-inflammatory actions in the colon. Am J Physiol Gastrointest Liver Physiol. 2017;312(6):G550-G8.

84. Matsubara T, Tanaka N, Patterson AD, Cho JY, Krausz KW, Gonzalez FJ. Lithocholic acid disrupts phospholipid and sphingolipid homeostasis leading to cholestasis in mice. Hepatology. 2011;53(4):1282-93.

85. Jiang C, Xie C, Li F, Zhang L, Nichols RG, Krausz KW, et al. Intestinal farnesoid X receptor signaling promotes nonalcoholic fatty liver disease. J Clin Invest. 2015;125(1):386-402.

86. Hudson GM, Flannigan KL, Erickson SL, Vicentini FA, Zamponi A, Hirota CL, et al. Constitutive androstane receptor regulates the intestinal mucosal response to injury. Br J Pharmacol. 2017;174(12):1857-71.
87. Shukla PK, Meena AS, Rao V, Rao RG, Balazs L, Rao R. Human Defensin-5 Blocks Ethanol and Colitis-Induced Dysbiosis, Tight Junction Disruption and Inflammation in Mouse Intestine. Sci Rep. 2018;8(1):16241.
88. Cheng J, Fang ZZ, Kim JH, Krausz KW, Tanaka N, Chiang JY, et al. Intestinal CYP3A4 protects against lithocholic acid-induced hepatotoxicity in intestine-specific VDR-deficient mice. J Lipid Res. 2014;55(3):455-65.
89. Khan AA, Chow EC, Porte RJ, Pang KS, Groothuis GM. The role of lithocholic acid in the regulation of bile acid detoxication, synthesis, and transport proteins in rat and human intestine and liver slices. Toxicol In Vitro. 2011;25(1):80-90.
FIGURE LEGENDS

Figure 1. Effects of EtOH and endogenous conversion of tissue ω-6 to ω-3 PUFAs on intestinal morphology and homeostasis. (A-C) Ileum tissue ω-6:ω-3 PUFA ratio, and total ω-6 and ω-3 PUFAs, (n=3-5). The full panel of FAs is presented in Supplemental Table S2. (D) Representative images of stained intestinal sections with H&E or Alcian blue, 200X magnification. (E-F) Effect of EtOH on small intestine-derived organoids from naïve WT and fat-1 mice cultured for 4 days, 100X magnification (n=5). (G-H) Expression of growth and proliferation markers, (I) mucus production, (J) bacteriocidal activity, and (K-L) tight junction genes in intestinal organoids from WT or fat-1 mice fed control or EtOH diets cultured for 10 days (n=3). * p < 0.05. (L) Immunohistochemistry/confocal imaging of ZO-1 expression (200X magnification).

Figure 2. Differential gene expression in WT vs. fat-1 mice. (A) Effects of modulation of the ω-6:ω-3 PUFA ratio on EtOH metabolizing enzymes in the intestine. Data are presented as gene expression levels of the corresponding group. Lower-case letters indicate statistically significant changes between groups (a: fat-1-PF vs. WT-PF; b: WT-PF vs. WT-EtOH; c: fat-1-PF vs. fat-1-EtOH, p < 0.05). (B) Plasma EtOH levels (n=6-10). (C-D) Venn diagram and graphical representation of total number of genes differentially expressed in the PF groups.

Figure 3. Increased ω-3 PUFAs reduced LPS-mediated intestinal inflammation in mice chronically fed EtOH. (A-B) CAE-stained intestine sections and quantification of CAE-positive cells (n=6-18 mice). (C-G) Ileal pro-inflammatory cytokine expression determined by immunoassay: (C) INF-γ, (D) IL-6, (E) IL-1β, (F) TNF-α, and (G) CXCL1. (H) Ileal mRNA levels of pro-inflammatory genes (n=3-11 mice). (I) quantification of CAE-positive cells in the EtOH+LPS group treated with RvD1 (n=12-14). (J) Effects of RvD1 treatment on EtOH+LPS-induced proinflammatory cytokines (n=9-10). (K) Immunohistochemistry for lysozyme expression in the intestine. (L-M) Ileal expression of anti-microbial genes (n=3-5).

Figure 4. Effects of endogenous ω-6:ω-3 PUFA modulation on EtOH-associated gut microbiota alterations. (A) Pie charts of taxonomic composition of bacterial community at the phylum levels in stool samples from WT and fat-1 experimental mice. Statistically significant changes (p<0.05) between groups are shown: *: WT-PF vs. WT-EtOH, §: fat-1-PF vs. fat-1-EtOH, †: WT-EtOH vs. WT-EtOH-LPS, †: fat-1-EtOH vs. fat-1-EtOH-LPS. (B) Principal coordinates analysis (PCA) of gut microbiota composition based on unweighted UniFrac. P = 0.001, PERMANOVA analysis. (C) Firmicutes:Bacteroidetes ratio in experimental groups. (D-E) Cladograms showing the taxa most differentially associated with EtOH or post-EtOH LPS treatment in WT and fat-1 mice. Circle sizes in the cladogram plot are proportional to bacterial abundance. The circles represent phyla, genus, class, order, and family going from the inner to the outer circle. (F) Selected bacteria at the genus levels in WT and fat-1 experimental mice. For each analysis, n=5-10 mice per group.

Figure 5. Impact of ω-6:ω-3 PUFA ratio modulation on plasma metabolome alterations associated with EtOH administration and LPS challenge. (A) Partial least squares-discriminant (PLS-DA) analysis, (B) Plasma metabolites significantly differentially enriched in experimental groups.

Figure 6. Effects of modulation ω-6:ω-3 PUFA ratio on EtOH and LPS-mediated alterations in fecal BA profiles and related metabolic pathways. (A) Fecal primary and secondary BA concentrations (n=3-7) (B) Expression of genes related to BA detoxification in ileum (RNA-seq) and in the liver as determined by qRT-PCR in WT and fat-1 mice. Expression of genes involved in BA synthesis (D), transport (E) reuptake (F) in the liver. (G) and (H), expression of Nrlh4/Fxr, Nrl12/Fxr, and Nrl13/Car in the ileum and liver, respectively (n=3-5). BA: bile acid; CA: cholic acid, UDCA: CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; Et: ethanol; LCA: lithocholic acid.
Figure 7. BA detoxification gene expression. Expression of genes involved in the metabolic inactivation of BA in the ileum (A, RNA-seq, n=3-5) and liver (B, qPCR, n=5-10). *, p<0.05, one-way ANOVA.

Figure 8. Decreased ω-6:ω-3 PUFA ratio resulted in the improvement of liver injury associated with EtOH and LPS administration (A) Serum ALT levels (B) representative H&E-stained liver sections (400X), (C) Liver triglycerides, (D) Correlation between intestinal CXCL1 protein expression and ALT, (E) Heatmap to illustrate Spearman correlations of the relative abundance of microbiome at the genus levels and parameters of liver injury WT and fat-1 mice in the EtOH+LPS group (n=5, statistical significance indicated as * p <0.05). The r values are represented by gradient colors, where red and blue cells indicate positive and negative correlations, respectively; *P < 0.05. (F) Model showing potential mechanism by which decreased tissue ω6 PUFA and increased ω3 PUFA results in a specific changes in intestinal homeostasis, the gut microbiota, and BA metabolism that coordinate to ameliorate liver injury in mice chronically fed EtOH and challenged with LPS. Panels A-E, n=3-12 mice per group.
**Supplemental Figure S1.** (A) Breeding scheme of littermate fat-1 and WT mice. fat-1 mice were born at the expected Mendelian ratios and developed normally. (B) Representative ethidium bromide-stained agarose gel of PCR genotyping results of fat-1 mice. (C) Experimental design.

**Supplemental Figure S2.** Morphometric analysis of the ileal mucosa in response to EtOH treatment. (A) Villi length, (B) Crypt height. 10-20 complete villus-crypt junctions of ileal segments from individual mice were measured, n=4-8 animals/per group. (C) Evaluation of Goblet cells in ileal sections stained with Alcian-Blue.

**Supplemental Figure S3.** (A) Blood LPS. (B) Blood CD14 levels, n=6-17.

**Supplemental Figure S4.** Differential gene expression of antimicrobial genes. Columns are specific treatment groups as defined (A-G). Fold-change values are color-coded from blue to red (low to high, respectively). *, P<0.05, one-way ANOVA. Et, EtOH.

**Supplemental Figure S5.** Effect of endogenous ω-6:ω-3 PUFA modulation on EtOH and EtOH+LPS-associated gut microbiota alterations. Linear discriminant analysis (LDA) effect size method was performed to compare taxa between PF and EtOH-fed groups (A-B), and between EtOH or post-EtOH LPS treatment in WT and fat-1 mice (n = 5). The bar plot lists the significantly differential taxa based on effect size (positive or negative LDA score (green and red, respectively). Taxa with LDA score (log 10) > 2 and significance of a < 0.05 determined by Wilcoxon signed-rank test are shown.

**Supplemental Figure S6.** Total plasma bile acid concentrations, n=6-15.
SUPPLEMENTARY MATERIALS AND METHODS

Blood collection, tissue and fecal sample collection

At the time of sacrifice, all mice were deeply anesthetized with ketamine/xylazine, and blood was collected from the inferior vena cava using heparinized syringes. Whole blood was centrifuged at 1000xg for 15 min at 4 °C, and plasma aliquoted and stored at -80 °C for further analysis. The intestines were removed and flushed with PBS to remove luminal contents. The ileum and portions of liver tissue from the same hepatic lobe for each mouse were snap-frozen in liquid nitrogen and stored at -80 °C or were fixed in 10% neutral buffered formalin and embedded in paraffin for further analysis. Fecal samples were collected from individual mice directly into microfuge tubes and stored at -80 °C for further analysis. To determine the impact of LPS on EtOH-mediated changes in the gut microbiota, the fecal material was collected from the same mouse prior to and 24 hours after LPS challenge.

RNAseq analysis

Total RNA was purified from ileum tissue using Trizol reagent (Thermo Fisher) from 3-5 mice/group. Contaminating genomic DNA was removed by digestion with DNase I (TURBO DNA-free kit, Thermo Fisher) and RNA further purified and concentrated using the GeneJET RNA cleanup and concentration micro kit (Thermo Fisher). RNA integrity was determined by analysis on the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). All samples had an RNA integrity number ranging from 7 to 9. Library preparation and sequencing was performed by the University of Louisville Center for Genetics in Molecular Medicine core facility using the Illumina TruSeq Stranded mRNA library prep kit (part no. 20020594). Briefly, Poly A purification and RNA fragmentation were performed on 600 ng total RNA and Superscript II was used to generate cDNA, and then RNA template was removed. Second strand synthesis was performed with incorporation of dUTP to ensure stranded libraries, and double-stranded cDNA was purified with AMPure XP beads. 3’ Ends were adenylated, indexing adapters were ligated onto the ends, and libraries once again purified with AMPure beads. Fifteen cycles of PCR were used to enrich DNA fragments, followed by two AMPure bead clean-up steps. Libraries were loaded onto an Agilent DNA 1000 chip and validated on an Agilent 2100 Bioanalyzer. Quantitation was performed with the Illumina Library Quantification Kit, ABI Prism qPCR Mix from Kapa Biosystems (Wilmington, MA). Three dilutions were tested in triplicate. Libraries were diluted to 10nM, pooled, further diluted and denatured and analyzed on a NextSeq 500 v2 150 cycles High Output kit (cat#FC-404-2002) in a 2×75bp PE.

RNA-seq bioinformatic analysis

Analysis was conducted by the NIH-funded Kentucky Biomedical Research Infrastructure Network Bioinformatics Core. Each lane of sequencing data was concatenated for each sample, resulting in a total of forty-eight fastq files, two for each sample (paired reads R1 and R2). Quality control (QC) of the raw sequence data was performed using FastQC (version 0.10.1) (90). The FastQC results indicate sequence trimming was not necessary for the samples. The sequences were directly aligned to the Mus musculus reference genome assembly (mm10.fa) using tophat2 (version 2.0.13) (91) guided by Ensembl build 82 mouse transcripts (Table 1). The input reads for each sample ranged from approximately 10 to 17 million, with a concordant alignment rate of approximately 85%. To confirm expression of the fat-1 transgene, we aligned each of the original reads to the C. elegans genome (ce10). A small fraction of the reads (less than 1%) mapped to C. elegans, as expected. We then extracted the reads from the region of the fat-1 gene and constructed a UCSC genome browser track for these regions. The track can be accessed at the URL: http://genome.ucsc.edu/cgi-bin/hgHubConnect?hgHub_do_redirect=on&hgHubConnect.remakeTrackHub=on&hgHub_do_firstDb=on&position=chrIV:13,315,62213,318,582&hubUrl=http://bioinformatics.louisville.edu/~rouchka/FAT1_hub_v2/hub.txt
Differentially expressed genes between experimental groups were identified, using the tuxedo suite of programs including cuffdiff2 (version 2.2.1) (92, 93). A p-value cutoff ≤ 0.05, q-value cutoff ≤ 0.05 with FC ≥ 2 was used to determine differential expression. DEGs at p-value cutoff ≤ 0.05, q-value cutoff ≤ 0.05 with log2 FC of 1 for the pairwise comparisons were used for further analysis of enriched Gene Ontology Biological Processes (GO:BP) (94, 95) and KEGG Pathways (96) using categoryCompare (97). The GEO accession number for the RNAseq data reported in this paper is GSE133253.

Fecal bile acid analysis

Bile acid analysis from fecal samples was performed by the Wayne State University Lipidomic Core Facility (supported by NIH grant S10RR027926). Samples (adjusted to a maximum volume of 1-2 ml) were spiked with a mixture of internal standards consisting cholic acid-d5, deoxycholic acid-d5, lithocholic acid-d5, and glycocholic acid-d5 (10 ng each) for recovery and quantitation. The samples were then extracted for bile acids using a similar procedure described earlier (98, 99). Briefly, the internal standard spiked samples were applied to conditioned C18 cartridges, washed with water followed by hexane and dried under vacuum. The cartridges were eluted with 0.5 ml methanol containing 0.1% formic acid. The eluate was dried under a gentle stream of nitrogen. The residue is re-dissolved in 50 µl methanol-0.1% aqueous ammonium hydroxide (1:1) and subjected to LC-MS analysis. HPLC was performed on a Prominence XR system (Shimadzu) using a Luna C18 (3µ, 2.1×150 mm) column. The mobile phase consisted of a gradient between A: methanol-water- (5:95 v/v) and B: methanol, both containing 0.1% ammonium hydroxide. The gradient program with respect to the composition of B was as follows: 0-1 min, 50-80%; 1-8 min, 80-84%; and 8-10 min, 84-100%. The flow rate was 0.2 mL/min. The HPLC eluate was directly introduced to the ESI source of QTRAP5500 mass analyzer (ABSCIEX) in the negative ion mode with following conditions: curtain gas: 35 psi, GS1: 35 psi, GS2: 65 psi, temperature: 600 °C, ion spray voltage: -4500 V, collision gas: low, declustering Potential: -180 V, and entrance potential: -12 V. The eluate was monitored by Multiple Reaction Monitoring (MRM) to detect unique molecular ion – daughter ion combinations for each of the cholic acids (cholic, deoxycholic, chenodeoxycholic, urso-, litho-, tauro-, glyco-, taurocheno-, and glycocheno-cholic acids) with 100 msec dwell time for each transition. Optimized collisional energies (40 – 80 eV) and collision cell exit potentials (–10 V) were used for each MRM transition. The data were collected using Analyst 1.6.2 software and the MRM transition chromatograms were quantitated by MultiQuant software (both from ABSCIEX). The internal standard signal in each chromatogram was used for normalization for calculation of recovery as well as relative quantitation of each analyte.

Blood Metabolomic Analysis

2DLC-MS/MS analysis. To extract polar metabolites, 100 µL plasma was mixed with 400 µL methanol for protein precipitation. After 3 min of vigorous vortex mixing, the mixture was centrifuged at 14,000 rpm at 4°C for 20 min. Two hundred microliter of supernatant was dried by speed vacuum at 4°C to remove methanol followed by freeze dry to remove water. The dried sample was then dissolved in 100 µL 20% acetonitrile for 2DLC-MS analysis.

2DLC-MS/MS analysis was carried out on a Thermo Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled with a Thermo DIONEX UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). A reversed phase chromatography (RPC) column and a hydrophilic interaction chromatography (HILIC) column was configured in parallel mode (100) to separate polar metabolites. The RPC column was an ACQUITY UPLC HSS T3 column (150 × 2.1 mm, 1.8 µm, Waters Corporation, Milford, MA, USA) and the HILIC column was a SeQuant ZIC-chILIC column (150 × 2.1 mm, 3 µm, Merck KGaA, Darmstadt, Germany) equipped with a SeQuant ZIC-chILIC guard column.

For 2DLC separation, the mobile phase A was water with 0.1% formic acid for RPC and 10 mM ammonium acetate (pH adjusted to 3.25 with acetate) for HILIC. The mobile phase B was acetonitrile with 0.1% formic acid for both RPC and HILIC. The mobile phase composition was varied from 5% to 100% B for RPC and 95% to 5% B for HILIC. The flow rate was 0.4 mL/min for RPC and 0.3 mL/min for HILIC.
The column temperature was set at 40 °C for both columns. The injection volume was 2 μL to each column. All samples were analyzed in full MS scan mode acquire full MS data, and pool samples which combined samples from the same group were analyzed in dd-MS² scan mode under collision energy of 20, 40 and 60 eV to acquire MS/MS data. The full MS and MS/MS data were collected in both negative and positive ionization mode, respectively.

Data processing was performed using MetSign software for spectrum deconvolution, metabolite assignment, cross-sample alignment, normalization, and statistical test (101-103). To identify metabolites, the MS/MS data of pooled samples were first matched to our in-house MS/MS database that contains the parent ion m/z, MS/MS spectra, and retention time of metabolite standards. MS/MS data without a match in the in-house database were then analyzed using Compound Discoverer software (version 2.0, Thermo Fisher Scientific, Inc., Germany). The remaining peaks that did not have a match were then matched to the metabolites in our in-house MS database. Pairwise two-tail t test was performed to determine the whether a metabolite has a significant abundance difference between sample groups, where the p-value threshold was set to p ≤ 0.05 with up to 1000 sample permutation.

**Gut Microbiota Analysis**

**Bacterial DNA extraction.** DNeasy Powersoil Kit (Qiagen, Venlo, Netherlands) was used to isolate bacterial DNA from mouse fecal samples. Fecal samples were added to the PowerBead tube plus Solution C1 according to manufacturer’s recommendations with the following modifications. The tubes were secured to a Vortex Adapter, mixed for 10 minutes on high then moved to Thermomixer (Eppendorf, Hamburg, Germany) and heated to 70°C for 10 min with shaking. The suspensions were centrifuged, supernatants transferred to new tubes, Solution C2 added, incubated on ice, centrifuged and repeated with Solution C3. Supernatants were then transferred to clean tubes, Solution C4 added, vortexed and bound to the MB Spin Column and washed twice. Purified DNA was eluted from the column with Elution Buffer (C6) and stored at -20 °C for further analysis.

**Microbial DNA sequencing.** 16S rRNA gene (V1-V3 region) was amplified using primers 27F (‘5-AGAGTTTGATCCTGGCTCAG-3’) and 534R (5’-ATTACCGCGGCTGCTGG-3’) with barcodes to allow multiplex Illumina deep sequencing as described previously (32). The PCR reaction contained 1.0 U Accuprime Taq High Fidelity Polymerase (Invitrogen, Carlsbad, CA), 1x PCR buffer II, 0.2 μM forward primer, 0.2 μM reverse primer and 2 μL DNA. Amplification was performed as follows: denaturation at 95°C for 2 minutes, 25 cycles of denaturation at 95°C for 20 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 5 minutes. PCR products for each sample were analyzed on a SYBR Safe 1% agarose gel (Invitrogen, Carlsbad, CA) to ensure an expected size of 600 base pairs. The amplicons were excised, purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Bethlehem, PA), quantified using the Qubit HS DNA quantification kits (Invitrogen, Carlsbad, CA), then pooled and purified. qPCR was performed using the Library Quant Kit (Kapa Biosystems, Wilmington, MA) and followed by Illumina paired-end sequencing using MiSeq Reagent kit V3 and PhiX control V3 kit (Illumina, San Diego, CA). Illumina sequencing generated 802,086 raw reads. After trimming and quality control, 501,036 joined paired end sequence reads were used for analysis (averaging 6,770 reads per sample). Good’s estimator of coverage was higher than 0.99 for all samples (0.9909 ± 0.0005).

**Bacterial sequence and diversity analysis.** Raw Illumina MiSeq 301 bp x 2 reads were filtered and trimmed with exact matches to barcode and primer and with a minimum average quality score of 30 (both paired ends) using custom scripts written in R. Paired end reads were then joined using FLASH (https://ccb.jhu.edu/software/FLASH/) with a minimum overlap length of 10 bp. USEARCH (https://www.drive5.com/usearch/) was employed to generate OTUs and assign taxonomy with a 97% similarity criteria against the SILVA v108 rRNA database (https://www.arb-silva.de/) as a reference. All subsequent tables were generated in R. Processed OTUs in each sample were used to calculate alpha diversity measures of Chao and Shannon-Weaver indexes. Beta diversity was calculated using UniFrac distance metric. To evaluate the bacterial community similarity among samples, principal coordinate
analysis (PCoA) utilizing UniFrac distances was generated and visualized using Qiime (http://qiime.org/). Functional prediction and analysis was performed using PICRUSt (104). Differentially significant features between combinations of paired group for both taxonomic and functional assignments were identified using LEfSe (105).
SUPPLEMENTARY REFERENCES

from: http://bioinformatics.babraham.ac.uk/projects/fastqc/.

2. Kim, D., G. Pertea, C. Trapnell, H. Pimentel, R. Kelley and S. L. Salzberg. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14: R36.

3. Trapnell, C., D. G. Hendrickson, M. Sauvageau, L. Goff, J. L. Rinn and L. Pachter. 2013. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol. 31: 46-53.

4. Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L. Rinn and L. Pachter. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc. 7: 562-578.

5. Consortium, G. O. 2004. The Gene Ontology (GO) database and informatics resource. Nucleic acids research. 32: D258-D261.

6. Ashburner, M., C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, A. P. Davis, K. Dolinski, S. S. Dwight, J. T. Eppig, M. A. Harris, D. P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J. C. Matese, J. E. Richardson, M. Ringwald, G. M. Rubin and G. Sherlock. 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 25: 25-29.

7. Kanehisa, M. and S. Goto. 2000. KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research. 28: 27-30.

8. Flight, R. M., B. J. Harrison, F. Mohammad, M. B. Bunge, L. D. Moon, J. C. Petruska and E. C. Rouchka. 2014. categoryCompare, an analytical tool based on feature annotations. Front Genet. 5: 98.

9. Maddipati, K. R. and S. L. Zhou. 2011. Stability and analysis of eicosanoids and docosanoids in tissue culture media. Prostaglandins Other Lipid Mediat. 94: 59-72.

10. Maddipati, K. R., R. Romero, T. Chaiworapongsa, S. L. Zhou, Z. Xu, A. L. Tarca, J. P. Kusanovic, H. Munoz and K. V. Honn. 2014. Eicosanomic profiling reveals dominance of the epoxygenase pathway in human amniotic fluid at term in spontaneous labor. FASEB J. 28: 4835-4846.

11. Klavins, K., H. Drexler, S. Hann and G. Koellensperger. 2014. Quantitative metabolite profiling utilizing parallel column analysis for simultaneous reversed-phase and hydrophilic interaction liquid chromatography separations combined with tandem mass spectrometry. Anal Chem. 86: 4145-4150.

12. Wei, X., W. Sun, X. Shi, I. Koo, B. Wang, J. Zhang, X. Yin, Y. Tang, B. Bogdanov, S. Kim, Z. Zhou, C. McClain and X. Zhang. 2011. MetSign: a computational platform for high-resolution mass spectrometry-based metabolomics. Anal Chem. 83: 7668-7675.

13. Wei, X., X. Shi, S. Kim, K. S, J. S. Patrick, J. Binkley, M. Kong, C. McClain and X. Zhang. 2014. Data dependent chromatographic peak model-based spectrum deconvolution for analysis of LC-MS data. Anal. Chem. 86: 2156-2165.

14. Wei, X., X. Shi, S. Kim, L. Zhang, J. S. Patrick, J. Binkley, C. McClain and X. Zhang. 2012. Data preprocessing method for liquid chromatography-mass spectrometry based metabolomics. Anal Chem. 84: 7963-7971.

15. Kozlov, A., L. Bean, E. V. Hill, L. Zhao, E. Li and G. P. Wang. 2018. Molecular Identification of Bacteria in Intra-abdominal Abscesses Using Deep Sequencing. Open Forum Infect Dis. 5: ofy025.
16. Langille, M. G., J. Zaneveld, J. G. Caporaso, D. McDonald, D. Knights, J. A. Reyes, J. C. Clemente, D. E. Burkepile, R. L. Vega Thurber, R. Knight, R. G. Beiko and C. Huttenhower. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol.* **31**: 814-821.
17. Segata, N., J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W. S. Garrett and C. Huttenhower. 2011. Metagenomic biomarker discovery and explanation. *Genome Biol.* **12**: R60.
SUPPLEMENTAL TABLE S1. **Primer Sequences for qPCR assays**

| Gene | Forward (5’-3’) | Reverse 5’-3’ | Accession no |
|------|----------------|--------------|--------------|
| 18S  | CTCAACACGGGAAACCTCAC | 5’-CGCTCCACCAACTAAGAAGCGG-3’ | NR_003278 |
| Bmi1 | CGCTTGGCTCGCATTTT | TAAAAGATCCCCGGAAGAGCGG | NM_007552.4 |
| Bpi1 | ATCTCCACCGACCTGTTCGG | TCCTGTGGAACAGTGGATCAG | NM_177850.3 |
| Lgr5 | TTCAATCCCTGCCTAGATG | ATGGCTTGCAGGCTGATAA | NM_010195.2 |
| Muc2 | AAGTTTGCCCCCTGGCTATG | GCAAACAGTCCTTGAGTG | NM_023566.3 |
| Ocln | ACCCGGAAAGAGATGGATCG | CATAGTCAGATGGGTTGGA | U49185.1 |
| ZO1  | TGGGAAAGCACAAGTGAGAC | GCTGGCCCTCCTTTAAAC | D14340.1 |
| Cyp3a11 | AGGGAAGCATCCAGGAGGATCAG | GTCCCATATCGGTAGAGGAGC | NM_007818.3 |
| Sult1a1 | TGATCTATCAGGGTGCAAGC | CAGAGTTCAAGACCTGAAA | NM_133670.2 |
| Sult1b1 | TGAGGTGCTCCATCTCAGT | GCAGCAGGCTTCAATGAGA | NM_001356943.1 |
| fat-1 | CGGTTTCTGGATGGATCCCAC | CACAGGAACGGGAAAGGAA | NM_001028389.4 |
| Gdf-5 | AAGCCCTCAGTCAGTTGTCG | AAAACATGAAAGGAGTGGG | NM_008109.3 |
## SUPPLEMENTAL TABLE S2  Intestinal Fatty Acid Composition
| Common name | Symbol | μg/mg | WT PF |  | Fat1 PF |  | Two-way ANOVA P value |  |
|--------------|--------|------|-------|---|--------|---|----------------------|---|
| Lauric       | C12:0  | 0.20±0.05 | 0.62±0.14 * | 0.32±0.10 | 0.25±0.09 | 0.10 | 0.24 | 0.03 |
| Myristic     | C14:0  | 0.01±0.00 | 0.02±0.00 | 0.01±0.00 | 0.01±0.00 | 0.60 | 0.41 | 0.09 |
| Palmitic     | C16:0  | 12.9±2.27 * | 7.65±1.76 | 7.14±1.70 | 0.05 | 0.39 | 0.03 |
| Palmitoleic  | C16:1  | 2.38±0.64 | 1.32±0.51 | 1.09±0.43 | 0.22 | 0.38 | 0.10 |
| Stearic      | C18:0  | 2.56±0.14 | 3.54±0.20 | 3.05±0.33 | 3.00±0.26 | 0.11 | 0.92 | 0.08 |
| Oleic        | C18:1  | 35.18±6.18 * | 16.81±4.45 | 19.39±5.48 | 0.01 | 0.38 | 0.04 |
| Linoleic     | C18:2(n6) | 12.96±1.87 * | 7.31±1.31 | 8.02±1.74 | 0.01 | 0.45 | 0.03 |
| γ-Linolenic  | C18:3(n6) | 0.05±0.005 | 0.10±0.01 * | 0.05±0.007 | 0.06±0.008 | 0.0004 | 0.12 | 0.01 |
| α-Linolenic  | C18:3(n3) | 0.20±0.04 | 0.22±0.07 | 0.24±0.03 | 0.13 | 0.05 | 0.18 |
| Arachidonic  | C20:0  | 0.12±0.01 * | 0.11±0.02 | 0.10±0.01 | 0.06 | 0.35 | 0.04 |
| Pauillonic   | C20:1  | 0.46±0.06 * | 0.24±0.04 | 0.28±0.05 | 0.004 | 0.44 | 0.01 |
| Eicosadienoic| C20:2(n6) | 0.08±0.006 * | 0.04±0.005 | 0.06±0.006 | <0.0001 | 0.10 | <0.02 |
| Dihomo-γ-linolenic | C20:3(n6) | 0.31±0.02 * | 0.15±0.01 | 0.24±0.01 * | <0.0001 | 0.008 | 0.12 |
| Arachidonic  | C20:4(n6) | 1.73±0.04 * | 1.04±0.09 | 0.91±0.05 | 0.06 | <0.0001 | 0.70 |
| Eicosapentaenoic (EPA) | C20:5 (n3) | nd | 0.30±0.12 | 0.24±0.01 | nd | nd | nd |
| Docosahexaenoic (DHA) | C22:6(n3) | 0.49±0.03 | 0.55±0.06 | 0.61±0.02 | 0.12 | 0.099 | 0.63 |
| Docosatetraenoic | C22:4(n6) | 0.22±0.019 * | 0.09±0.009 | 0.08±0.01 | 0.04 | <0.0001 | 0.007 |
| Docosapentaeenoic (DPA) | C22:5 (n3) | 0.16±0.01 * | 0.10±0.01 | 0.16±0.01 * | 0.0005 | <0.0001 | 0.23 |
| Lignoceric   | C24:0  | 0.05±0.008 * | 0.05±0.01 | 0.02±0.004 | 0.77 | 0.48 | 0.006 |
| Nervonic     | C24:1  | 0.05±0.006 | 0.05±0.006 | 0.06±0.003 | 0.001 | 0.01 | 0.63 |

* P < 0.05 PF vs. EtOH; ** P < 0.05 WT+EtOH vs. fat-1+EtOH; *** P < 0.05 WT-PF vs. fat-1-PF
| GENE SYMBOL | log2FC fad-1 vs. WT | q value | GENE SYMBOL | log2FC fad-1 vs. WT | q value | GENE SYMBOL | log2FC fad-1 vs. WT | q value | GENE SYMBOL | log2FC fad-1 vs. WT | q value |
|-------------|---------------------|--------|-------------|---------------------|--------|-------------|---------------------|--------|-------------|---------------------|--------|
| igsd4c      | 2.121               | 0.012  | Aba1d       | 1.714               | 0.028  | Baco26      | 1.549               | 0.012  | Knt1        | 1.760               | 0.012  |
| Igmd        | 1.059               | 0.028  | Acars1b     | 2.474               | 0.012  | Lmo3e       | 2.660               | 0.041  | Gels1       | 2.762               | 0.012  |
| Igtd1       | 1.781               | 0.046  | Acaro       | 1.803               | 0.024  | Thrp4       | 1.869               | 0.012  | Ahr2        | 1.119               | 0.028  |
| Igur1-18     | 2.123               | 0.046  | Acu2c       | 2.282               | 0.012  | Armc28      | 1.900               | 0.012  | Armc29      | 1.990               | 0.041  |
| Igur1-38     | 1.971               | 0.021  | Angle4a     | 1.182               | 0.012  | Armc30      | 1.945               | 0.012  | Chgb        | 1.268               | 0.012  |
| Igur2-8      | -4.241              | 0.012  | Cer1d       | 1.326               | 0.012  | Armc31      | 2.144               | 0.012  | Cnmdh       | 2.389               | 0.012  |
| Igur1-17     | -2.190              | 0.012  | Cer2b       | 1.481               | 0.046  | Armc32      | 2.397               | 0.012  | Cnrl        | 2.517               | 0.012  |
| Igur2-339    | -3.457              | 0.012  | Fchh        | 1.442               | 0.012  | Crnbr1      | 1.162               | 0.012  | Cstl5       | 2.590               | 0.012  |
| Igur4-55     | -2.543              | 0.034  | Fsnr1       | 1.432               | 0.012  | Fsta2       | 1.714               | 0.041  | Cstl6       | 1.805               | 0.012  |
| Igur5-43     | -1.956              | 0.012  | Gln2d       | 1.102               | 0.012  | Gsc2        | 1.267               | 0.021  | Gspc4       | 3.319               | 0.046  |
| Igur6-18     | -2.905              | 0.012  | Gpd1        | 1.543               | 0.012  | Gsc4        | 1.907               | 0.012  | Gspc5       | -2.604              | 0.012  |
| Igur6-15     | 1.523               | 0.034  | Gprc1       | 1.080               | 0.028  | Hspa1       | 1.401               | 0.012  | Gspc6       | 1.503               | 0.012  |
| Igur6-31     | -2.200              | 0.013  | Hspa2       | 1.017               | 0.013  | Hspa17      | 1.394               | 0.034  | Hspc1       | 1.343               | 0.012  |
| Igsa        | 1.590               | 0.046  | Hspa3       | 1.507               | 0.012  | Hspa3      | 1.311               | 0.012  | Hspc2       | 2.269               | 0.012  |
| Gcsc3a      | 1.483               | 0.034  | Hspa5       | 1.507               | 0.012  | Hspa3      | 1.311               | 0.012  | Hspc3       | 2.534               | 0.012  |
| Gs22        | 2.114               | 0.021  | Hspa5       | 1.507               | 0.012  | Hspa3      | 1.311               | 0.012  | Hspc3       | 2.534               | 0.012  |
| Cc6f        | 2.688               | 0.021  | Hspa5       | 1.507               | 0.012  | Hspa3      | 1.311               | 0.012  | Hspc3       | 2.534               | 0.012  |
| Cyfl7       | 1.876               | 0.012  | Hspa5       | 1.507               | 0.012  | Hspa3      | 1.311               | 0.012  | Hspc3       | 2.534               | 0.012  |
| Cc5d6b       | 3.288               | 0.012  | Igsf5       | 2.299               | 0.012  | Igsf10      | 3.263               | 0.029  | Igsf10      | 2.322               | 0.029  |
| Cg4h4       | 1.047               | 0.034  | Igsf4       | 2.707               | 0.012  | Igsf10      | 3.263               | 0.029  | Igsf10      | 2.322               | 0.029  |
| Noc2        | -1.501              | 0.013  | Igsf5       | 1.389               | 0.012  | Igsf10      | 3.263               | 0.029  | Igsf10      | 2.322               | 0.029  |
| Rias1        | 2.070               | 0.012  | Igsf5       | 1.534               | 0.012  | Igsf10      | 3.263               | 0.029  | Igsf10      | 2.322               | 0.029  |
| Secs3        | -1.417              | 0.012  | Igsf5       | -1.112              | 0.028  | Igsf10      | 3.263               | 0.029  | Igsf10      | 2.322               | 0.029  |
| Tsl2         | 1.615               | 0.046  | Igsf5       | 1.534               | 0.012  | Igsf10      | 3.263               | 0.029  | Igsf10      | 2.322               | 0.029  |
| Tlr5         | 2.049               | 0.012  | Igsf5       | 1.534               | 0.012  | Igsf10      | 3.263               | 0.029  | Igsf10      | 2.322               | 0.029  |
Ileum tissue

A  B  C

D

E  F

G  H  I

J  K  L

M

ZO-1

Downloaded from www.jlr.org by guest on March 6, 2020
**Figure 3**

Panel B: Graph showing fold changes in gene expression for WT and fat-1 mice under different conditions.

Panel C: Graphs illustrating the fold change in gene expression for Cxcl1 and Il-6 in WT and fat-1 mice.

Panel D: Fold changes in gene expression for Il-1β and Tnf-α in WT and fat-1 mice.

Panel E: Graphs depicting the fold change in gene expression for Il-1β and Tnf-α in WT and fat-1 mice.

Panel G: Graphs showing fold changes in gene expression for IRNA-seq in WT and fat-1 mice.

Panel H: Comparison of RNA-seq fold changes in WT and fat-1 mice.

Panel K: Images of lysosyme gene expression in WT and fat-1 mice.

Panel L: Graphs showing fold changes in gene expression for Alpi in WT and fat-1 mice.

Panel M: Graphs illustrating the fold change in gene expression for intestinal leptin in WT and fat-1 mice.
FIGURE 5

Enriched Diminished in WT EtOH vs. fat-1 EtOH

Enriched Diminished in WT EtOH+LPS vs. fat-1 EtOH+LPS
FIGURE 7

A

Ileum, RNAseq

RNA levels in WT and fat-1 mice

B

Liver, RT-PCR

Expression levels of Cyp3a17 and Sult1a1 in WT and fat-1 mice
Correlation coefficient, $r$

Genus-level Statistical significance

* $P < 0.05$

ALT, U/L Intestinal CXCL1, pg/mg tissue

$E = r = 0.6562, p = 0.0549$

WT-Et+LPS

EtOH+LPSPF

Fat-1

Fat-1

ALT UL

Intestinal CXCL1, pg/mg tissue

$F = 0.0025, p = 0.0549$

Liver TG, mg/g liver

ALT UL

Intestinal CXCL1, pg/mg tissue

$E = r = 0.6562, p = 0.0549$

WT-Et+LPS

EtOH+LPSPF

Fat-1

Fat-1

ALT UL

Intestinal CXCL1, pg/mg tissue

$F = 0.0025, p = 0.0549$
Supplemental FIGURES
SUPPLEMENTAL FIGURE 1

A

fat-1<sup>+/−</sup>  

WT C57BL/6J  

fat-1<sup>+/−</sup>  

C57BL/6J

B

1. fat<sup>1+/−</sup>
2. WT
3. fat<sup>1+/−</sup>
4. fat<sup>1+/−</sup>

fat-1  

Gdf5

C

EtOH+LPS  

LPS  

24h  

WT  

fat-1  

Pair-fed  

Isocaloric Diet

EtOH  

1-2%  

4%  

5%  

6%  

w1  

w2  

w3  

w4-6
Supplemental Figure 3

A

Plasma Endotoxin (EU/mL)

PF EIOH

WT fat-1

* 0.0 0.5 1.0 1.5

B

CD14, pg/mL

PF EIOH

WT fat-1

0 500 1000 1500
### Supplementary Figure 4

| Case | LC22 | A | B | C | D | E | F | G |
|------|------|---|---|---|---|---|---|---|
| Def45 | - | - | - | - | - | - | - | - |
| Def46 | - | - | - | - | - | - | - | - |
| Def47 | - | - | - | - | - | - | - | - |
| Def48 | - | - | - | - | - | - | - | - |
| Def49 | - | - | - | - | - | - | - | - |
| Def50 | - | - | - | - | - | - | - | - |
| Def51 | - | - | - | - | - | - | - | - |
| Def52 | - | - | - | - | - | - | - | - |

**Statistical significance** *P < 0.05*

- Fold change

**Alpha Defensin Family**

- Def45
- Def46
- Def47
- Def48
- Def49
- Def50
- Def51
- Def52
