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Diet-conditioned microbiota enhances fecal microbiota transplantation efficacy in alcoholic liver disease through caproic acid-PPARα signaling

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eLife Assessment

The study provides **valuable** findings suggesting that modifying the donor's diet improves the effectiveness of fecal transplant therapies for liver disease. Although the reported results are of value, the evidence supporting the overall conclusions is **incomplete**. In particular, causal inferences regarding the effects of microbiota composition, as well as caproic acid signaling on the phenotypes studied, need further confirmation.

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Abstract

Background and Aim Fecal microbiota transplantation (FMT) in Alcohol-related liver disease (ALD) has shown therapeutic potential, with variable efficacy and unclear mechanism. Because dietary protein influences gut microbiota composition, we hypothesized that donor dietary preconditioning could enhance FMT efficacy. We therefore examined in a murine ALD model if high-protein donor diet improves FMT outcome.

Methods ALD was induced in C57BL/6N mice using a Lieber–DeCarli ethanol diet combined with thioacetamide administration for 12 weeks. FMT was performed using stool from diet-modulated donors, and recovery was assessed on day7 post-FMT. Multi-omics analysis using 16s rRNA and mass spectroscopy was performed for Gut microbiota composition, plasma- and stool-metabolome, and hepatic proteomes. Multi-omics outcomes were validated in ALD animal and Huh7 hepatocytes.

Results Both protein-based FMTs improved ALD recovery; Veg-FMT demonstrated superior efficacy, significantly reducing hepatic injury (AST 1.2-fold, p=0.002; bilirubin 1.2-fold, p=0.03; steatosis 1.7-fold,p=0.01) and restoring gut barrier integrity (occludin 1.5-fold,p=0.04; mucin 2 2.2-fold, p=002; and plasma endotoxin 1.7-fold, p=0.02). A significant 2-fold increase was observed in *Lachnospiraceae* NK4A136, *Coriobacteriaceae* UCG-002, and short-chain fatty acids, particularly caproic acid. Functional validation confirmed that caproic acid promoted hepatic fatty acid β-oxidation through PPARα-dependent mechanisms, reducing triglyceride accumulation and lipogenesis in both cellular and animal models.

Conclusion Donor preconditioning with a plant-protein enriched diet enhances FMT efficacy in ALD by gut microbiota modulation with increased metabolites like caproic acid. These findings highlight a microbiota-metabolite-host axis through which diet-modulated FMT improves hepatic lipid metabolism and injury, and identifies a pathway via which FMT imparts its effect.

Significance

This study identifies a mechanistic basis for improving fecal microbiota transplantation (FMT) efficacy in alcohol-related liver disease (ALD) by demonstrating that dietary preconditioning of donor microbiota improves therapeutic outcomes. We show that plant protein–modulated donor microbiota supplements abstinence-associated recovery through increased production of the microbial metabolite caproic acid, which promotes hepatic fatty acid β-oxidation via PPARα signaling. These findings highlight donor dietary conditioning and microbiota-derived metabolites, rather than microbial composition alone, as important determinants of FMT efficacy. The results suggest that microbial metabolites such as caproic acid may represent potential therapeutic targets or biomarkers to enhance and standardize microbiota-based interventions in ALD. Although the current work is based on a murine model, the identified microbiota–metabolite–host metabolic axis provides a framework for future translational studies aimed at optimizing FMT strategies in liver disease.

Introduction

Among alcohol-associated liver disease (ALD) patients, a majority develop steatosis, with only 10–35% developing hepatitis, of which 8–20% eventually progress to cirrhosis (1). The pathophysiology of ALD is influenced by compositional shifts in intestinal bacteria (2,3). Alcohol intake reduces bacterial diversity and increases the abundance of *Proteobacteria* and *Enterobacteria* and decreases the abundance of *Bacteroidetes* and Firmicutes, specifically *Lactobacillus* species, leading to intestinal barrier dysfunction and endotoxemia (3–5).

Restoration of gut microbial homeostasis has therefore emerged as a promising therapeutic strategy. Fecal microbiota transplantation (FMT) has demonstrated beneficial effects in experimental models and clinical studies of alcoholic hepatitis, including improvements in liver injury and survival (5–9). However, responses to FMT remain variable, probably due to the condition of the patient or an inept donor bacterial composition (10). These findings suggest that donor microbiota characteristics may critically influence therapeutic efficacy, highlighting the need to better understand factors that shape donor microbial function.

Diet is one of the strongest determinants of gut microbiota composition and metabolic activity (11,12). Short-term dietary changes can rapidly alter microbial communities and their metabolite production. Although dietary fiber is widely recognized as a major substrate for microbial fermentation, dietary proteins that escape digestion in the small intestine also reach the colon and can serve as substrates for microbial metabolism (13–15). Microbial fermentation of amino acids produces a variety of metabolites capable of modulating host metabolic and inflammatory pathways (16,17).

Despite these associations, the influence of donor dietary conditioning on FMT efficacy in ALD remains poorly understood. In this study, we investigated whether preconditioning donor microbiota with diets enriched in vegetable- or egg-derived proteins could enhance FMT-mediated recovery in a murine model of ALD. Using integrated microbiome, metabolomic, and proteomic analyses, we demonstrate that vegetable protein–modulated microbiota significantly improve recovery from ALD. This effect is associated with enrichment of short-chain fatty acid producing bacteria and increased production of caproic acid, a microbial metabolite that activates hepatic PPARα signaling to enhance fatty acid β-oxidation and reduce hepatic lipid accumulation. These findings highlight dietary modulation of donor microbiota as a potential strategy to improve microbiota-based therapies in alcohol-related liver disease.

Results

Protein-educated FMT enhances recovery from alcohol-induced liver injury

To determine whether donor dietary conditioning improves the therapeutic efficacy of fecal microbiota transplantation (FMT), donor mice were preconditioned with either standard diet, vegetable protein diet, or egg protein diet prior to microbiota transfer (Fig. 1A). FMT was administered to ALD mice following abstinence, and liver injury parameters were assessed seven days after transplantation.

Abstinence alone resulted in partial recovery from liver injury, as indicated by increased food intake (fold change >2, $p<0.05$) and a reduction in the liver-to-body weight ratio compared with ALD mice (Additional file 1, S. Fig. 1B-C). Serum biochemical analysis confirmed successful induction of liver injury in ALD mice, with significantly elevated levels of AST, ALT, and bilirubin compared with controls. Abstinence significantly reduced these markers relative to ALD mice (Fig. 1B).

FMT further improved liver injury beyond abstinence alone. Standard FMT significantly reduced AST (2.1-fold), ALT (2.6-fold), and bilirubin (1.8-fold) compared with ALD mice (all $p<0.001$). Protein-educated FMT produced greater improvement, with Veg-FMT showing the most pronounced effect. Veg-FMT reduced AST by 2.7-fold, ALT by 3.2-fold, and bilirubin by 2.6-fold compared with ALD mice (all $p<0.001$) (Fig. 1B).

Histological examination supported these findings. Hematoxylin-eosin staining revealed marked hepatic steatosis and inflammatory infiltration in ALD mice, which were partially improved after abstinence and further ameliorated following FMT treatment (Fig. 1C). Veg-FMT produced the greatest reduction in steatosis, showing an approximately 11-fold decrease compared with ALD mice ($p<0.001$).

Consistent with the histological observations, hepatic triglyceride levels were significantly reduced following protein-modulated FMT. Veg-FMT reduced hepatic triglycerides by 1.74-fold ($p<0.001$), while Egg-FMT reduced triglycerides by 1.47-fold ($p=0.009$) relative to ALD mice (Fig. 1F).

Veg-FMT also significantly improved inflammatory and fibrogenic markers. Expression of pro-inflammatory cytokines Il6 (2.54-fold reduction, $p=0.04$), Tnfα (1.29-fold reduction, $p=0.06$), and Tlr4 (1.11-fold reduction, $p=0.05$) decreased following Veg-FMT, while the anti-inflammatory cytokine Il10 increased 1.5-fold ($p=0.02$) (Fig. 1D). Similarly, fibrogenic genes Acta2 (4.1-fold reduction, $p<0.001$) and Tgfβ (3.3-fold reduction, $p<0.001$) were significantly downregulated (Fig. 1E).

Collectively, these findings demonstrate that dietary conditioning of donor microbiota enhances FMT-mediated recovery from alcohol-induced liver injury, with vegetable protein-modulated microbiota showing the strongest protective effect.

Veg-FMT restores intestinal barrier integrity and reduces endotoxemia

Chronic alcohol exposure disrupts intestinal barrier integrity, leading to increased gut permeability and translocation of microbial products such as endotoxin, which contribute to hepatic inflammation through the gut–liver axis. To determine whether protein-educated fecal microbiota transplantation (FMT) improves intestinal barrier function, we examined intestinal histology, tight-junction protein expression, antimicrobial peptides, inflammatory markers, and circulating endotoxin levels following treatment.

Histological analysis of intestinal tissues revealed marked epithelial damage and shortening of intestinal villi in ALD mice compared with control animals (Fig. 2A). Although abstinence resulted in partial recovery of intestinal morphology, FMT treatment further improved epithelial structure. Notably, Veg-FMT restored villus architecture to near-normal morphology, whereas Egg-

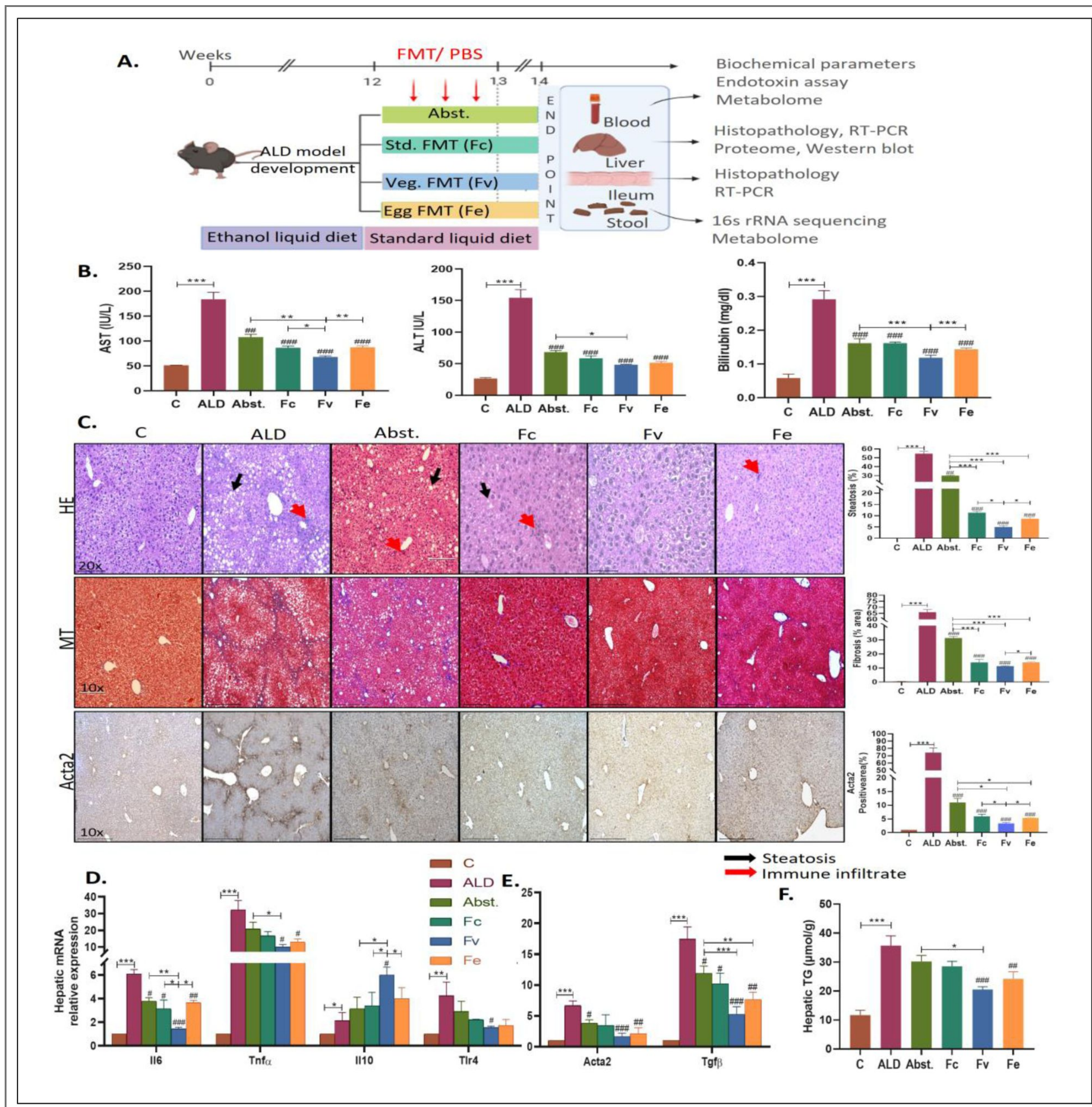

Figure 1. Protein-educated FMT improves liver function.

(A) Study design- In separate setups, FMT was given to ALD mice (3 alternate days) from donors fed one of three diets—a standard diet, a vegetable protein diet or an egg protein diet—followed by collection of tissues and blood seven days after FMT. **(B)** Overall, FMT reduced serum AST, ALT and bilirubin levels, with an enhanced reduction in Veg-FMT (all p<0.001). **(C)** Representative micrographs of liver histology by hematoxylin and eosin (H&E) and Masson's trichome (MT) staining and immunohistochemistry for smooth muscle actin protein (α-sma) showing decreased hepatic steatosis (p<0.001) and fibrosis (MT and α-sma positive area, p=0.01in both) in protein-educated FMT with statistical analysis. **(D)** Compared with egg-FMT, Veg-FMT led to greater reductions in hepatic Il6 (p=0.04), Tnfα (p=0.06), Tlr4 (p=0.05), **(E)** Acta2 (p=0.001) and Tgfβ(p<0.001) and increased IL10 (0.02) mRNA expression. **(F)** Hepatic TGs were significantly reduced in protein-educated FMT (Veg-FMT: 1.74FC, p<0.001; Egg-FMT: 1.47FC,p=0.009). The data are presented as the means ± SEMs (standard error of the mean). *p < 0.05, **p < 0.01, ***p < 0.001 represent intergroup statistics; #p < 0.05, ##p < 0.01, ###p < 0.001 represent the ALD group (one-way ANOVA followed by Tukey's multiple comparison test).

FMT and standard FMT showed only moderate improvement (Additional file 1, S. Fig. 2A). Consistent with these histological findings, ALD mice exhibited significantly reduced expression of key tight-junction proteins, indicating impaired intestinal barrier function. Veg-FMT significantly increased mRNA expression of Zo-1 (2.04-fold, p = 0.05), Occludin (2.31-fold, p = 0.03), and Claudin-3 (1.63-fold, p = 0.01), also protein expression of Zo-1 significantly increased compared with ALD mice (Fig. 2A). These results suggest that Veg-FMT effectively restores epithelial tight-junction integrity disrupted by alcohol exposure.

In addition to structural barrier proteins, alcohol exposure also induced intestinal inflammatory responses. Veg-FMT significantly reduced expression of the pro-inflammatory cytokines Il6 (4.2-fold decrease, p = 0.002), Tnfα (5.6-fold decrease, p < 0.001), and Tlr4 (2-fold decrease, p = 0.04) compared with ALD mice, while the anti-inflammatory cytokine Il10 increased 2.8-fold (p < 0.001) (Additional file 1, S.Fig. 2B). These findings indicate that Veg-FMT attenuates alcohol-induced intestinal inflammation.

Intestinal antimicrobial defense mechanisms were also affected. Expression of the antimicrobial peptides Reg3β (1.98-fold increase, p = 0.01) and Reg3γ (2.04-fold increase, p = 0.02) was significantly elevated following Veg-FMT compared with ALD mice (Fig. 2C). Alcohol consumption degrades Muc2 in the gut mucus lining, contributing to a leaky gut and elevated endotoxemia (18). Veg-FMT also significantly increased expression of the mucin gene Muc2 expression (1.4FC, p=0.05; Fig. 2D), with a concomitant reduction in plasma endotoxin levels (1.7FC, p=0.02; Fig. 3E). A significant negative correlation was observed between Muc2 and plasma endotoxin levels (r^2= -0.86, p<0.001; Fig. 2F). These findings indicate that Veg-FMT enhances abstinence-associated restoration of intestinal barrier integrity, leading to reduced gut inflammation and endotoxemia.

Given the marked restoration of intestinal barrier integrity following Veg-FMT, we next investigated whether these effects were associated with alterations in gut microbial composition.

Veg-FMT reshapes gut microbial composition associated with improved metabolic function

Given the significant restoration of intestinal barrier integrity following Veg-FMT, we next investigated whether these improvements were associated with changes in gut microbial composition. To address this, 16S rRNA gene sequencing was performed on fecal samples collected from experimental groups after FMT treatment.

Analysis of alpha diversity using the Shannon index showed no significant differences in overall microbial diversity among the experimental groups (Additional file 1, S.Fig. 3A). However, principal coordinate analysis (PCoA) based on Bray-Curtis distances revealed clear separation between treatment groups, indicating significant differences in microbial community composition (PERMANOVA p = 0.001). The first two principal coordinates explained approximately 77% of the total variance in microbial composition (Fig. 3A).

Taxonomic profiling revealed that Veg-FMT selectively enriched several bacterial taxa associated with beneficial metabolic functions. In particular, the relative abundance of *Akkermansia*, *Lachnospiraceae* NK4A136 and *Parasutterella* increased following Veg-FMT treatment compared with ALD mice (Fig. 3B). Differential abundance analysis (FC>2, p<0.05) further identified several taxa significantly altered after Veg-FMT (Additional file 1, Fig. S. 3B, C, D, E and Additional file 2, S-table 2A). The abundances of *Coriobacteriaceae UCG-002* (4.1FC, p=0.007), *Lachnospiraceae* NK4A136 (2.25FC, p=0.02) (SCFA producers that maintain gut barrier function (19)) and *Tuzzerella* (2.45FC, p=0.006; role in lipid metabolism) exclusively increased post-Veg-FMT (Fig. 3C). The opportunistic taxa *Sporosarcina* and *Vagococcus* were exclusively decreased (1.12E-01 FC and 1.11E-02 FC, p<0.001, respectively) suggesting suppression of potentially pathogenic bacterial population. Analysis of taxa shared across treatment groups revealed enrichment of beneficial genera including *Aerococcus*, *Blautia*, and *Lactobacillus* in protein-modulated FMT recipients.

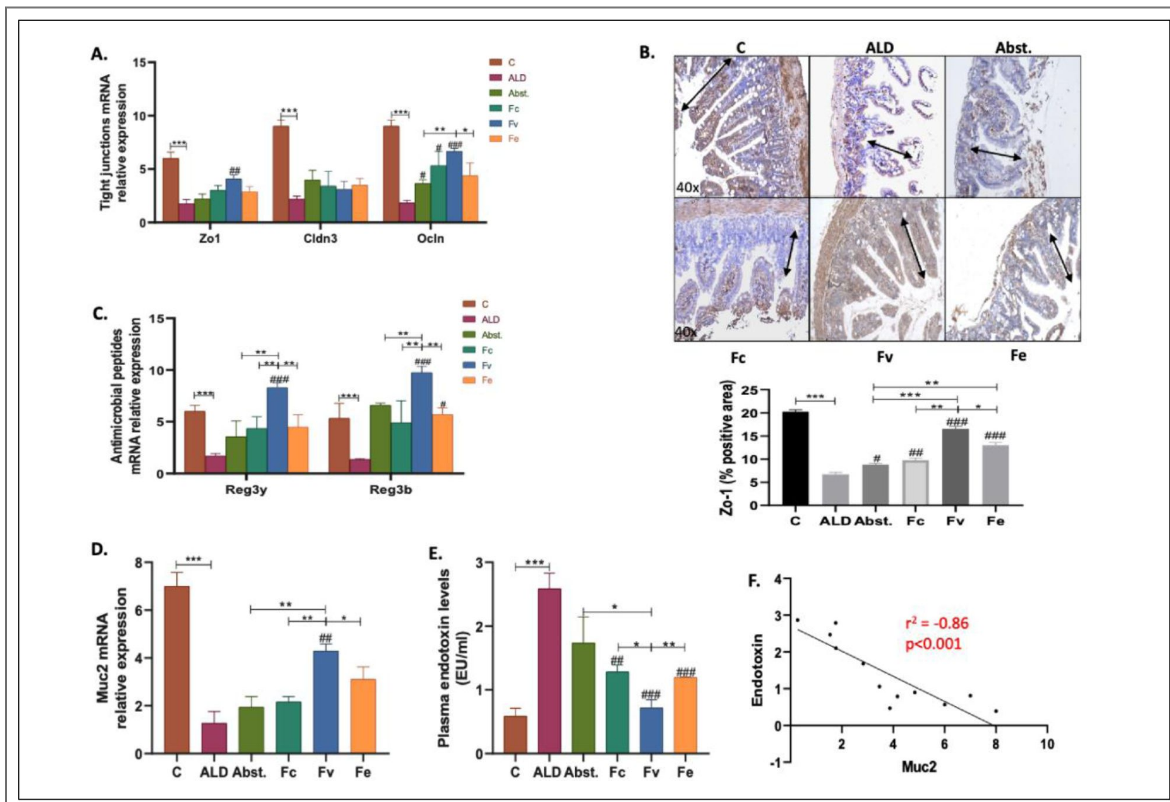

Figure 2. **Protein-educated FMT restores intestinal barrier integrity.**

(A) mRNA expression of the ileal tight junction proteins Zo-1 (p=0.04), Cldn3(p=0.01), and Ocln (p<0.001) was significantly reduced after Veg-FMT compared to ALD. **(B)** Statistical analysis revealed that the protein expression of the tight junction protein Zo-1 was markedly lower after Veg-FMT than after egg-FMT (1.27FC, p=0.004). (C) mRNA expression of the antimicrobial peptides Reg3ß (p=0.009), Reg3Γ (p=0.006) and **(D)** Muc2 (p=0.05) also decreased after veg-protein educated FMT. (**E)** Plasma endotoxin levels (p=0.02) were also significantly lower after veg-protein FMT. **(F)** Serum endotoxin and Muc2 gene expression were significantly negatively correlated (r^2=-0.86, p<0.001; Spearman's correlation). The data are presented as the means ± SEMs (standard error of the mean). *p < 0.05, **p < 0.01, ***p < 0.001 represent intergroup statistics; #p < 0.05, ##p < 0.01, ###p < 0.001 with respect to the ALD group (A-E, one-way ANOVA followed by Tukey's multiple comparison test).

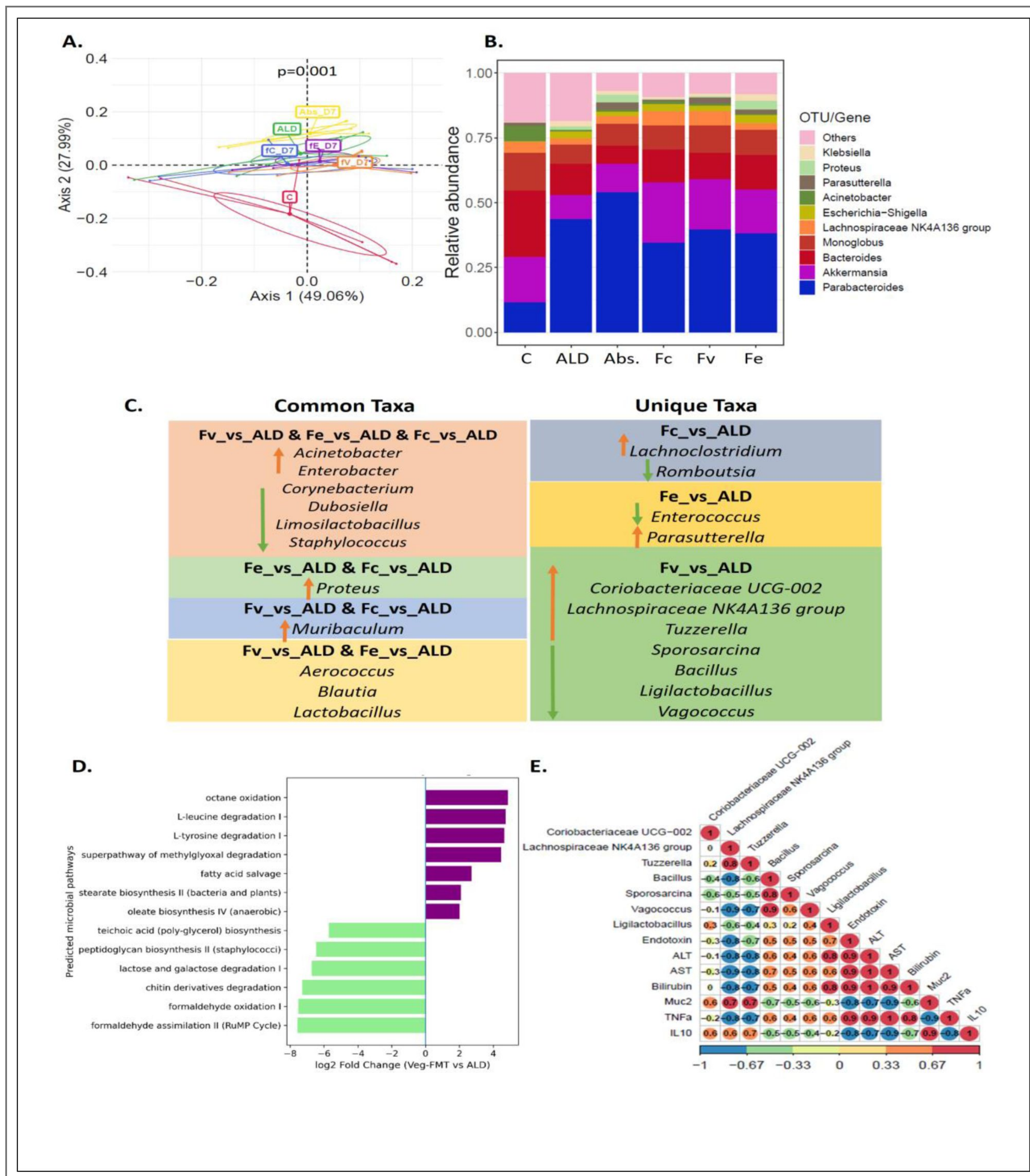

Figure 3. **Favorable gut microbial variations were observed after protein-FMT.**

(A) PCoA plot showing significant (p=0.001, PERMANOVA) differences in microbial composition post-FMT among the different treatment groups. **(B)** Comparison of the relative abundances of bacterial genera revealed increases in *Akkermansia, Lachnopiraceae* NK4A136 and *Parasutterella abundance* post-FMT. **(C)** Tables showing differentially altered taxa present commonly and uniquely in the FMT treatment groups. **(D)** Bidirectional bar plot showing selected functional pathways predicted by PICRUSt in veg-FMT **(E)** Correlations between serum injury biomarkers and differential bacterial taxa identified by Veg-FMT. Pearson's correlation values are displayed in circles. Red represents a positive correlation, whereas blue represents a negative correlation. Correlation according to the scale given at the bottom of the plot.

To further investigate the relationship between microbial changes and host physiology, correlation analysis ($r^2 > 0.7$, $p<0.05$) was performed between differentially abundant bacterial taxa and liver injury parameters (Fig. 3E). Several bacterial taxa enriched after Veg-FMT displayed strong negative correlations with liver injury markers, including serum ALT, AST, bilirubin, and TNFα levels. Notably, *Lachnospiraceae* NK4A136 and *Tuzzerella* showed significant negative correlations with these markers while positively correlating with the intestinal mucus marker Muc2. Together, these findings demonstrate that Veg-FMT promotes enrichment of beneficial microbial taxa associated with improved intestinal barrier integrity and reduced liver injury. To determine whether the observed microbial compositional changes translated into functional metabolic differences, pathway prediction analysis was performed using PICRUSt (Fig. 3D; Additional file 1, Fig. S. F and Additional file 2, S-table 2B). Compared with ALD, Veg-FMT showed significant enrichment of pathways related to amino acid degradation and fatty acid metabolism, including L-leucine degradation (log2FC = 4.74, p = 0.0017), L-tyrosine degradation (log2FC = 4.64, p = 0.0023), octane oxidation (log2FC = 4.86, p = 0.0013), and fatty acid salvage (log2FC = 2.71, p = 0.018). Additional enrichment of lipid biosynthesis pathways such as stearate biosynthesis II (log2FC = 2.09, p = 0.018) and oleate biosynthesis IV (log2FC = 2.01, p = 0.020) further indicated increased microbial fatty-acid metabolic potential. In contrast, several pathways associated with bacterial structural metabolism and carbohydrate degradation, including peptidoglycan biosynthesis II (log2FC = −6.46, p = 0.00023), teichoic acid biosynthesis (log2FC = −5.72, p = 0.0024), and lactose and galactose degradation I (log2FC = −6.74, p = 0.00064), were reduced in Veg-FMT compared with ALD. Collectively, these findings suggest that Veg-FMT enhances microbial fermentation-associated metabolic potential. Since gut microbiota–derived metabolites can regulate host metabolism through the gut–liver axis, we next examined whether Veg-FMT was associated with metabolic reprogramming in the liver.

Veg-FMT promotes hepatic metabolic reprogramming through PPARα signaling and fatty acid β-oxidation

To determine whether microbiota remodeling induced by Veg-FMT influences hepatic metabolic pathways, we performed quantitative proteomic analysis of liver tissues from control, ALD, and FMT-treated mice.

A total of 3,841 proteins were identified, and unsupervised hierarchical clustering revealed clear segregation between treatment groups, indicating substantial remodeling of hepatic protein expression following FMT (Fig. 4A). Pathway enrichment analysis of the differentially expressed proteins (DEPs; FC > 2, p < 0.05) identified several KEGG pathways associated with metabolic regulation and cellular stress responses (Additional file 1, S. Fig. 4A-E and Additional file 2, S-table 3). The apoptosis pathway, which was elevated in ALD mice, was reversed by all FMT treatments (Additional file 1, S. Fig. 4C-4E). Notably, Veg-FMT uniquely and significantly reduced unsaturated fatty acid biosynthesis (p = 0.04) and showed a marginal reduction in the reactive oxygen species (ROS) pathway (p = 0.08; Additional file 1, S. Fig. 4E).

Comparison of common and unique pathways revealed distinct metabolic effects of dietary protein-modulated FMT (Fig. 4B). Stopping alcohol increased oxidative phosphorylation, whereas all FMT treatments significantly enriched pathways related to PPAR signaling, primary bile acid biosynthesis, and endoplasmic reticulum protein processing. In addition, protein-modulated FMT specifically enhanced pathways associated with cholesterol metabolism, fatty acid degradation, and histidine metabolism (Fig. 4C, Additional file 2, S-table 4).

Among the enriched pathways, PPAR signaling emerged as a prominent metabolic pathway associated with FMT-mediated recovery. Given the well-established role of peroxisome proliferator–activated receptors (PPARs) in regulating hepatic lipid metabolism and steatosis (20,21), we further examined proteins associated with this pathway. Enrichment analysis identified 9 proteins linked to PPAR signaling in Veg-FMT ($p = 8.3 \times 10^{-6}$), 7 proteins in Egg-FMT ($p = 1.44 \times 10^{-5}$), and 5 proteins in Std-FMT (p = 0.001; Fig. 4D). Among these, PPARα expression was

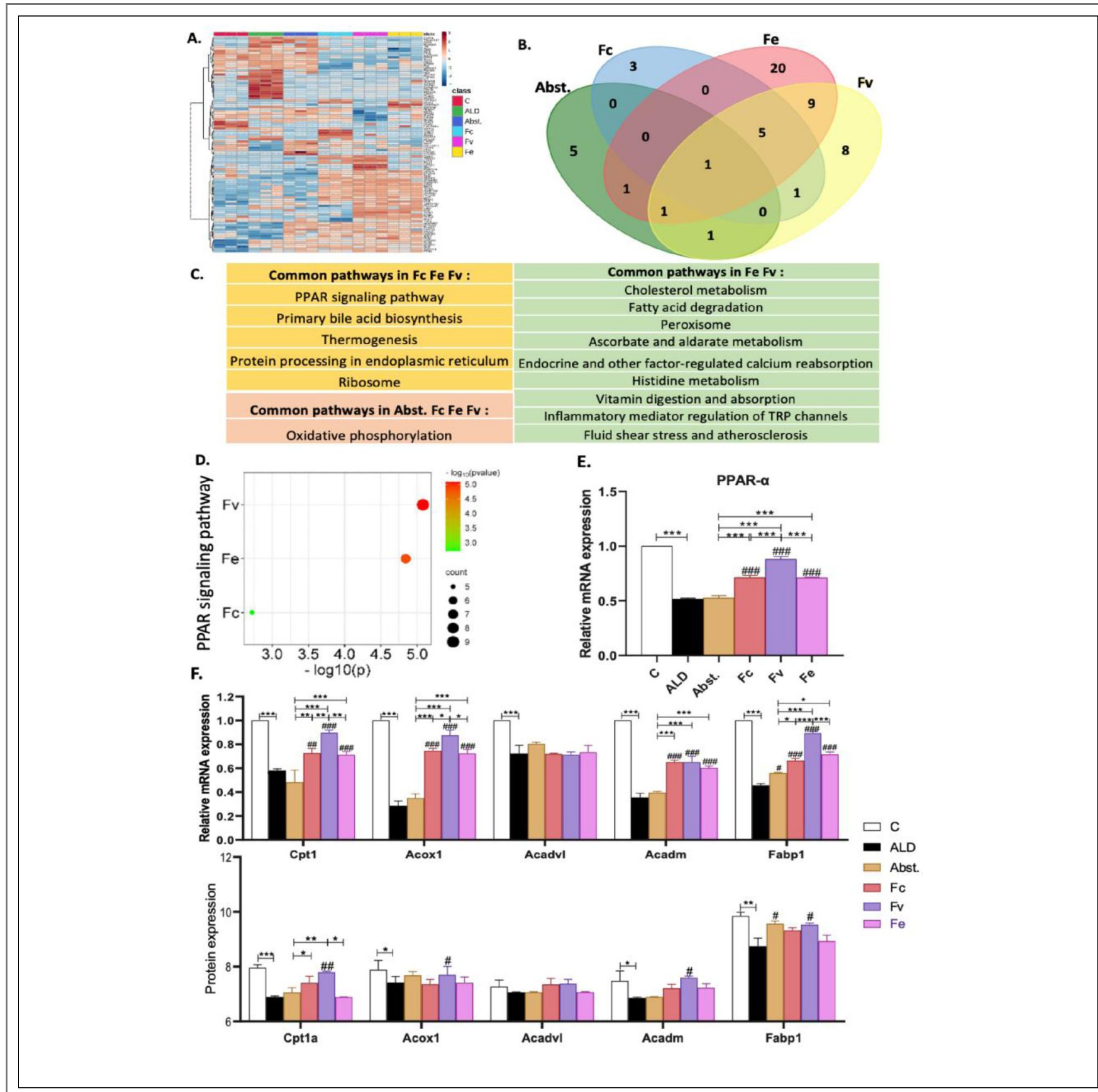

Figure 4. **Fecal microbiota transplantation alters the hepatic proteome.**

(A) Heatmap showing differentially expressed proteins whose expression was upregulated or downregulated in different groups. **(B)** Venn diagram showing the number of common and unique enriched pathways among abst, Std-FMT, Veg-FMT and egg-FMT, with the **(C)** table showing pathway names. **(D)** The PPAR signaling pathway was significantly enriched ($p=8.3\times10^{-06}$) in the Veg-FMT group. **(E)** Hepatic PPARα mRNA expression was greater in the Veg-FMT group ($p<0.001$) than in the Egg-FMT group. **(F)** mRNA expression (top panel; Cpt1 ($p=0.003$), Fabp1 ($p=0.005$) and peroxisomal Acox1 ($p=0.02$)) and protein expression (bottom panel; Cpt1a ($p=0.007$), Acox1 ($p= 0.05$), Acadm ($p= 0.04$) and Fabp1 ($p= 0.02$)) of PPARα target genes involved in fatty acid beta-oxidation increased after Veg-FMT. The data are presented as the means ± SEMs (standard error of the mean). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ represent intergroup statistics; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ represent the ALD group (one-way ANOVA followed by Tukey's multiple comparison test).

significantly increased following Veg-FMT (1.7-fold vs ALD, $p < 0.001$; 1.2-fold vs Egg-FMT, $p < 0.001$) (Fig. 4E),), whereas PPARγ expression remained unchanged across treatment groups (Additional file 1, S.Fig. 4F).

To determine whether PPARα activation translated into functional metabolic changes, we examined downstream genes involved in fatty acid β-oxidation. Both Veg-FMT and Egg-FMT increased β-oxidation–related gene expression; however, Veg-FMT produced significantly stronger effects. Compared with Egg-FMT, Veg-FMT significantly increased expression of mitochondrial Cpt1 (1.25-fold, $p = 0.003$), Fabp1 (1.25-fold, $p = 0.005$), and peroxisomal Acox1 (1.2-fold, $p = 0.02$; Fig. 4F). Consistent with these findings, protein expression of Cpt1a (1.2-fold, $p = 0.007$), Acox1 (1.1-fold, $p = 0.05$), Acadm (1.1-fold, $p = 0.04$), and Fabp1 (1.1-fold, $p = 0.02$) was significantly increased only in Veg-FMT–treated mice; Fig. 4F). Together, these findings demonstrate that vegetable protein–modulated FMT more effectively activates hepatic PPARα signaling and downstream fatty acid β-oxidation pathways, indicating enhanced hepatic lipid metabolism following Veg-FMT treatment.

Veg-FMT mediated metabolic effects are dependent on PPARα signaling

To determine whether the metabolic benefits of Veg-FMT were mediated through PPARα signaling, we pharmacologically inhibited PPARα using the antagonist GW6471 in Veg-FMT–treated ALD mice (Fig. 5A). PPARα inhibition significantly reduced hepatic PPARα mRNA expression (4.5-fold decrease, $p < 0.001$; Fig. 5B) and protein levels (2.3-fold decrease, $p = 0.01$; Fig. 5C) compared with Veg-FMT alone. Consistent with suppression of this pathway, inhibition of PPARα resulted in a significant increase in the liver-to-body weight ratio (3.3-fold, $p = 0.04$; Additional file 1, S.Fig. 5A).

Systemic markers of liver injury were also markedly elevated following PPARα inhibition, including serum AST (1.7-fold, $p = 0.003$), ALT (2-fold, $p = 0.002$), and bilirubin (2.5-fold, $p = 0.04$), accompanied by increased hepatic triglyceride accumulation (1.45-fold, $p = 0.03$; Fig. 5D). Histological analysis further revealed persistence of hepatic inflammation, steatosis, and fibrosis in the inhibitor-treated group (Additional file 1, S.Fig. 5C; Fig. 5E), indicating loss of the hepatoprotective effects observed with Veg-FMT alone.

PPARα inhibition also impaired intestinal barrier integrity, as evidenced by villus blunting, reduced ZO-1 protein expression (1.97-fold decrease, $p < 0.001$; Additional file 1, S.Fig. 5D), and significantly elevated plasma endotoxin levels (2.3-fold increase, $p < 0.001$; Fig. 5F). Consistently, expression of canonical PPARα target genes involved in fatty acid β-oxidation, including Cpt1, Acox1, and Fabp1, was markedly suppressed (5-fold, 4.3-fold, and 4.7-fold decreases, respectively; $p < 0.001$ for all; Additional file 1, S.Fig. 5E).

Interestingly, inhibition of PPARα did not significantly alter overall gut microbiota composition between groups (PERMANOVA $p = 0.644$; Additional file 1, S.Fig. 6), suggesting that the loss of protection was primarily due to disruption of host metabolic signaling rather than changes in microbial community structure.

Collectively, these findings demonstrate that activation of hepatic PPARα signaling is essential for the protective metabolic effects of Veg-FMT, linking microbiota-derived metabolic signals to host lipid oxidation and liver recovery.

Metabolomic profiling identifies caproic acid enrichment following Veg-FMT

PPARα has multiple metabolic ligands (22). Untargeted metabolomic profiling was performed on stool and plasma samples to identify microbial metabolites potentially involved in PPARα signaling. A total of 665 metabolites in stool and 522 in plasma were detected. Principal coordinate analysis demonstrated clear separation between experimental groups, with the first two components explaining ~60% of the variance in stool metabolites and ~31% in plasma

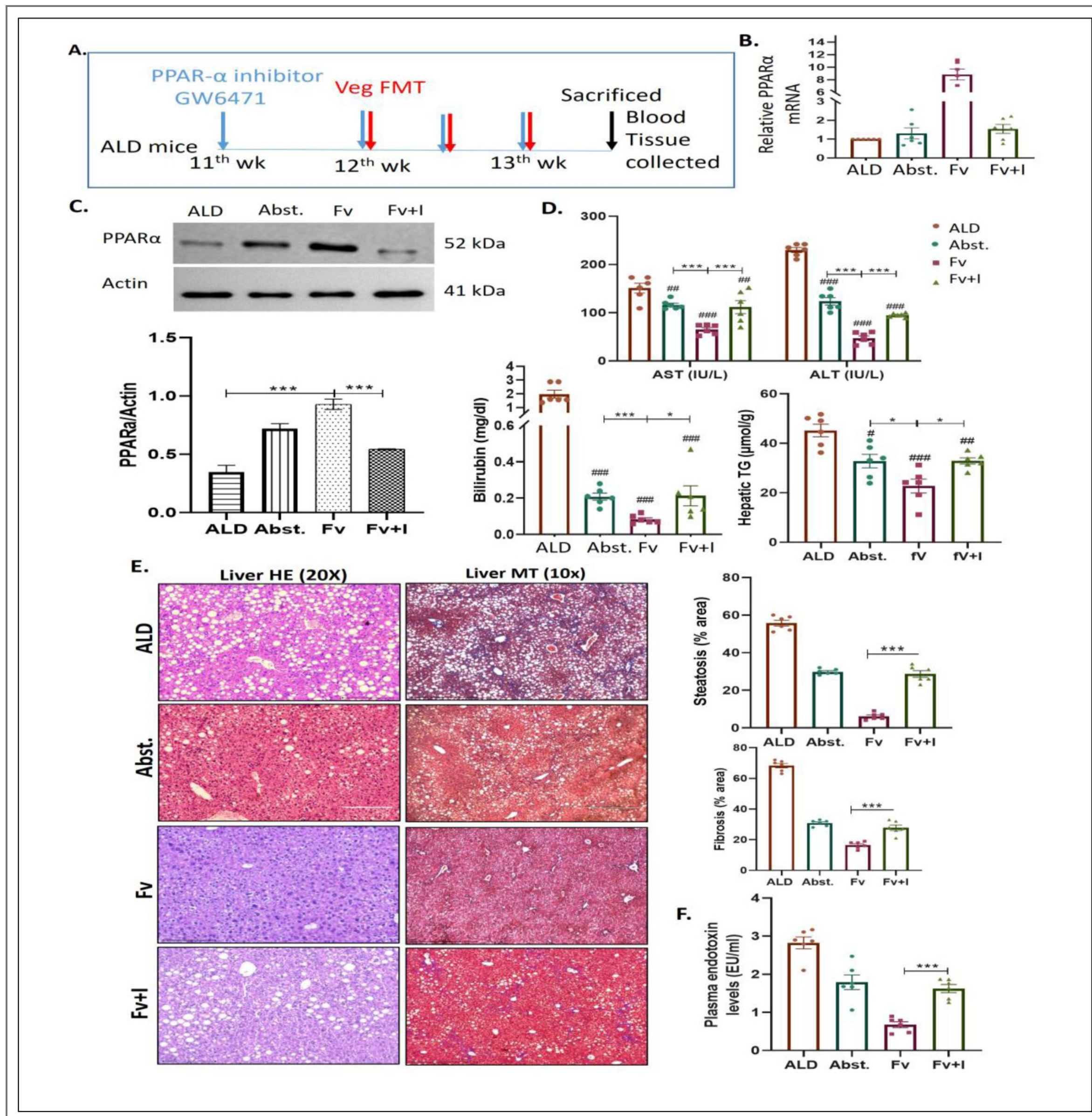

Figure 5. **Veg-FMT alleviates hepatic injury through PPARα activation.**

(A) Study design showing PPARα inhibitor administration in ALD mice with Veg-FMT. At 7 days after FMT, the tissues and blood were collected for further analyses. **(B)** mRNA (p<0.001) and **(C)** protein expression of PPARα decreased significantly (p=0.01). **(D)** Serum AST(p=0.003), ALT(p=0.002), bilirubin(p=0.04) and hepatic triglyceride levels (p=0.03) significantly increased after PPARα inhibition. **(E)** Liver histology images showing increased steatosis (p<0.001) by H&E and fibrosis(p<0.001) by MT staining after inhibition copared to veg-FMT. **(F)** Plasma endotoxin levels (p=0.001) were also increased after inhibition. The data are presented as the means ± SEMs (standard error of the mean). *p < 0.05, **p < 0.01, ***p < 0.001 represent intergroup statistics; #p < 0.05, ##p < 0.01, ###p < 0.001 with respect to the ALD group (one-way ANOVA followed by Tukey's multiple comparison test).

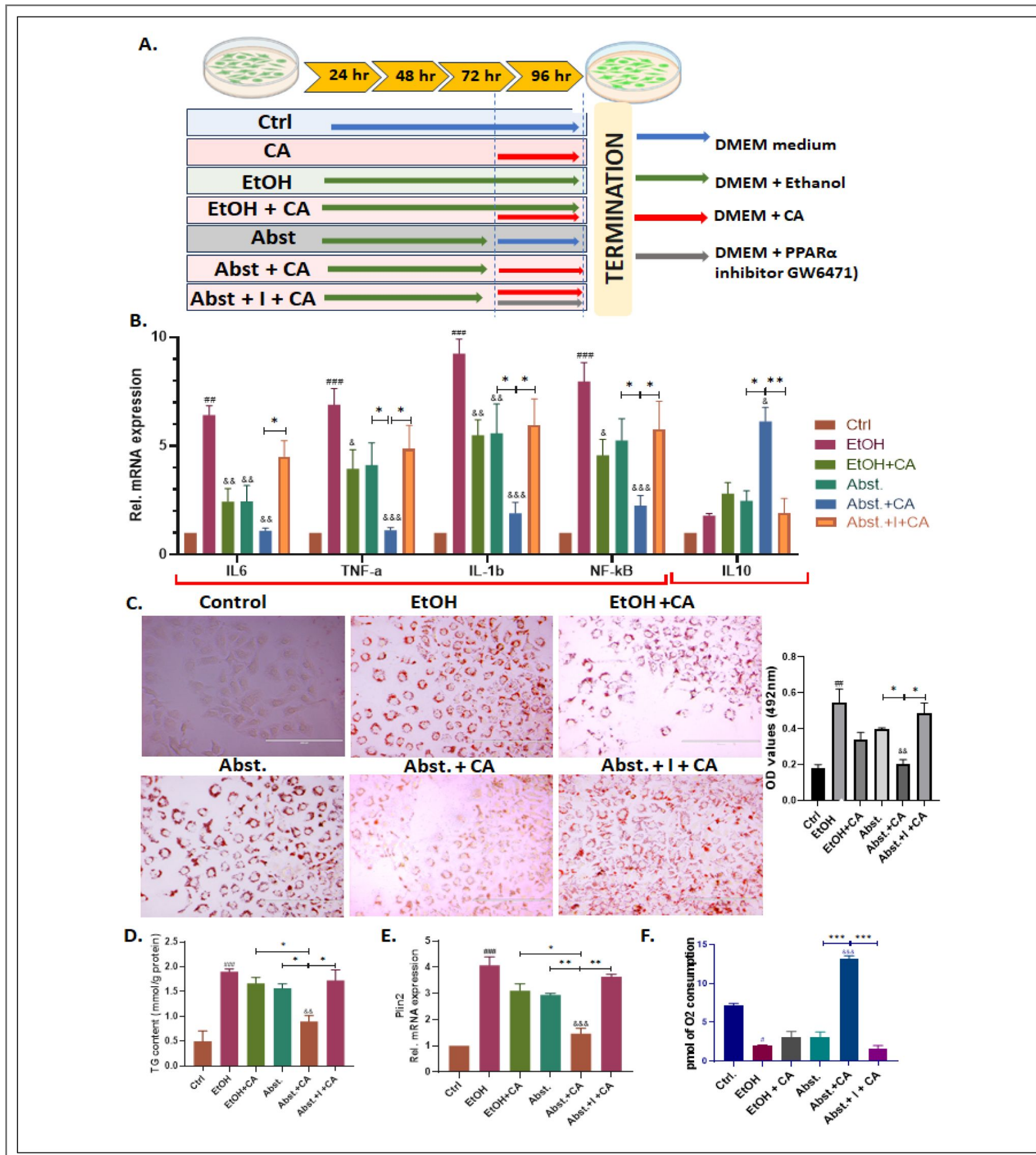

Figure 6. **Caproic acid supplementation mitigates ethanol-induced steatosis by enhancing fatty acid β-oxidation in Huh7 cells.**

(A) Study design: Huh7 cells were treated with ethanol or caproic acid alone or in combination. A PPARα inhibitor was also given with caproic acid. Injury was assessed after 24 hours. **(B)** mRNA expressions of pro-inflammatory reduced (IL6, p=0.002; TNFα, p=0.006; IL1β, p<0.001; Nfkb, p<0.001) and anti-inflammatory markers (IL10, p=0.005) increased after Abst+CA supplementation and these changes reversed in presence of PPARα inhibitor. **(C)** Cytopathology showing higher lipid accumulation in ethanol treated cells using oil-red-o staining and significantly reduced after CA supplementation (p=0.02). CA supplementation effects were reduced in the presence of PPARα inhibitor (p=0.01). **(D)** TG levels were significantly decreased after CA supplementation (p=0.05) and effects were reversed after PPARα inhibition. (E) mRNA expression of lipid droplet marker (Plin2) increased in ethanol treatment and reduced CA supplementation (p=0.002), but reversed in presence of PPARα inhibitor. **(F)** Mitofuel flexibility assay showed increased β-oxidation dependency after CA supplementation (p<0.001), but it was reduced in the presence of the inhibitor (p<0.001). The data are presented as the means ± SEMs (standard error of the mean). *p < 0.05, **p < 0.01, ***p < 0.001 represent intergroup statistics; #p < 0.05, ##p < 0.01, ###p < 0.001 with respect to the ALD and control groups. &p < 0.05, &&p < 0.01, &&&p < 0.001 with respect to ALD (one-way ANOVA followed by Tukey's multiple comparison test).

metabolites (Additional file 1, S. Fig. 7A-B). Differential metabolite analysis revealed multiple metabolites significantly altered in Abst- and FMT-treated animals compared with ALD mice (FC>1.5, $p<0.05$) (Additional file 1, S. Fig. 7C-D; Additional file 2, S-table 5 for stool and S-table 6 for plasma). Unique significantly upregulated metabolites for both stool and plasma were identified (Additional file 1, S. Fig. 7E-F; Additional file 2, S-table 7 for stool and 8 for plasma). Comparative analysis of protein-modulated FMT groups showed 72 metabolites commonly increased in stool and 7 in plasma, whereas Veg-FMT uniquely increased 61 stool and 23 plasma metabolites. Among these metabolites, two molecules were significantly enriched in both stool and plasma following Veg-FMT: N-butanoyl-homoserine lactone and caproic acid (Additional file 1, S. Fig. 7G and Additional file 2, S-table 9). N-butanoyl-homoserine lactone is a bacterial quorum-sensing molecule involved in microbial communication and biofilm regulation (23). In contrast, caproic acid (hexanoic acid) is a microbial fatty acid metabolite associated with anti-inflammatory effects and host energy metabolism (24). Caproic acid levels were significantly increased in Veg-FMT recipients in both stool (2.5-fold, $p=0.01$) and plasma (3.4-fold, $p = 0.01$), and were higher compared with Egg-FMT animals (Additional file 1, S. Fig. 7H-I).

These findings identified caproic acid as a candidate microbiota-derived metabolite potentially linking Veg-FMT–induced microbiota remodeling to activation of hepatic PPARα signaling.

Caproic acid mitigates ethanol-induced steatosis by enhancing fatty acid β-oxidation in hepatocytes

Because fatty acids can function as PPARα ligands (25), we next examined whether caproic acid could modulate PPARα signaling in hepatocytes. Ethanol-exposed Huh7 cells were treated with caproic acid (CA) in the presence or absence of the PPARα inhibitor GW6471 (Fig. 6A). Based on dose-response experiments, 100 mM ethanol and 900 μM caproic acid were selected for subsequent studies (Additional file 1, S.Fig. 8A).

CA supplementation significantly reduced ethanol-induced inflammatory responses, including IL-6 (5-fold, $p = 0.002$) and TNFα (5.5-fold, $p = 0.006$), while increasing the anti-inflammatory cytokine IL-10 (Fig. 6B). These effects were reversed in the presence of GW6471, indicating that the anti-inflammatory effects of CA are mediated through PPARα signaling.

Consistent with this observation, CA markedly suppressed lipogenic gene expression, including Srebp1c (4-fold, $p < 0.001$) and Fasn (4.5-fold, $p < 0.001$), while significantly increasing β-oxidation genes, including PPARα (8-fold, $p < 0.001$), Cpt1a (7.6-fold, $p = 0.001$), and Fabp1 (5.7-fold, $p = 0.002$) (Additional file 1, S. Fig. 8B–C). Oil Red O staining confirmed a marked reduction in lipid accumulation following CA treatment (2-fold decrease, $p = 0.02$), which was reversed by PPARα inhibition (Fig. 6C).

Consistently, intracellular triglyceride levels were significantly reduced after CA supplementation (1.7-fold decrease, $p = 0.05$; Fig. 6D), accompanied by reduced expression of the lipid droplet marker PLIN2 (2.07-fold decrease, $p = 0.002$; Fig. 6E). In contrast, genes involved in lipid export (ApoB and MTTP) showed only modest and non-significant changes, suggesting that CA-mediated steatosis reduction primarily results from enhanced fatty acid oxidation rather than increased lipid export.

To directly assess mitochondrial metabolic activity, a Mito Fuel Flex assay was performed. CA significantly increased oxygen consumption associated with fatty acid oxidation (4.2-fold, $p < 0.001$; Fig. 6F), and this effect was completely abolished by GW6471. In comparison, the known PPARα ligand butyric acid (25) induced only a modest increase in fatty acid oxidation (Additional file 1, S. Fig. 8E).

Together, these findings demonstrate that caproic acid attenuates ethanol-induced hepatocellular lipid accumulation through PPARα-dependent activation of fatty acid β-oxidation.

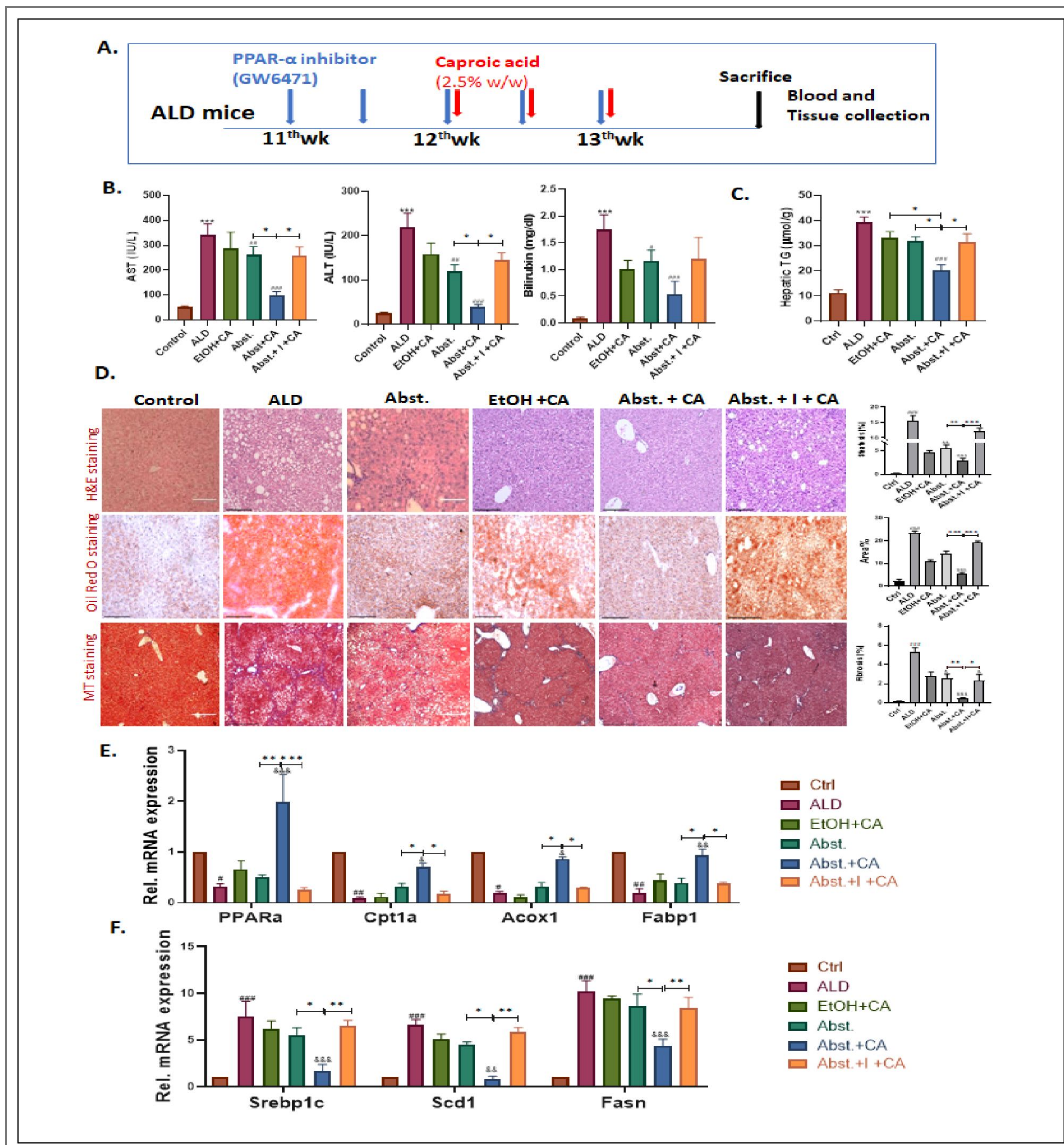

Figure 7. Caproic acid supplementation alleviated ethanol-induced liver injury through PPARα upregulation in ALD mice.

(A) Study design- ALD mice treated with caproic acid with or without a PPARα inhibitor. Injury was assessed after 1 week. **(B)** Serum AST (p=0.02), ALT (p<0.001) and bilirubin (p=0.006) levels were significantly reduced after CA supplementation. **(C)** Hepatic TGs were significantly reduced after CA supplementation (p=0.02) and these changes were reversed in the presence of PPARα inhibitor **(D)** Liver histology showing reduced steatosis (examined by H&E and Oil Red O, p<0.001 in both) and fibrosis (p=0.007) by MT staining after CA supplementation. **(E)** mRNA expression of PPARα target genes involved in fatty acid β-oxidation increased (PPARα, p<0.001; Cpt1, p=0.01; Acox1, p=0.008; Fabp1, p=0.002) and **(F)** lipogenesis-related mRNA expression decreased (Srebp1c, Scd1 and Fasn, p<0.001 in all) after CA supplementation. These beneficial effects of CA were reversed in the presence of a PPARα inhibitor. The data are presented as the means ± SEMs (standard error of the mean). *p < 0.05, **p < 0.01, ***p < 0.001 represent intergroup statistics; #p < 0.05, ##p < 0.01, ###p < 0.001 with respect to the ALD and control groups. &p < 0.05, &&p < 0.01, &&&p < 0.001 with respect to ALD (one-way ANOVA followed by Tukey's multiple comparison test).

In vivo confirmation of caproic acid-mediated hepatoprotection

To validate these findings in vivo, ALD model mice were supplemented with caproic acid with or without the PPARα inhibitor (Fig. 7A). CA supplementation significantly reduced serum markers of liver injury, including AST (3.4-fold, $p = 0.02$), ALT (5.5-fold, $p < 0.001$), and bilirubin (10-fold, $p = 0.006$), whereas these protective effects were completely abolished in the presence of the inhibitor (Fig. 7B).

Histological and biochemical analyses further demonstrated significant reductions in hepatic lipid accumulation following CA supplementation. Hepatic triglyceride levels decreased significantly (1.6-fold, $p = 0.02$; Fig. 7C), accompanied by marked reductions in H&E-positive steatotic area (5-fold, $p < 0.001$) and Oil Red O-positive lipid accumulation (4.3-fold, $p < 0.001$). CA supplementation also significantly reduced hepatic fibrosis (5-fold, $p = 0.007$; Fig. 7D). Importantly, these protective effects were not observed in mice treated with the PPARα inhibitor.

At the molecular level, CA significantly suppressed lipogenic genes, including Srebp1c (4.4-fold), Scd1 (7.3-fold), and Fasn (2.3-fold; $p < 0.001$ for all), while simultaneously enhancing expression of PPARα and downstream β-oxidation genes, including Cpt1 (7-fold, $p = 0.01$), Acox1 (4.7-fold, $p = 0.008$), and Fabp1 (4.8-fold, $p = 0.002$) (Fig. 7E-F). These transcriptional changes were abolished upon PPARα inhibition, confirming that the hepatoprotective effects of CA are PPARα dependent.

Collectively, these results demonstrate that caproic acid supplementation mitigates alcohol-induced liver injury by activating PPARα-mediated fatty acid β-oxidation pathways in vivo.

Discussion

The current study identifies veg-protein enriched donor diet followed by FMT (veg-FMT) as a therapeutic option for ameliorating alcohol-associated liver disease (ALD). We identified caproic acid, a six-carbon fatty acid produced by gut bacteria (26), as a key microbial metabolite that activates PPARα, resulting in increased fatty acid oxidation and improved hepatic lipid metabolism. A key finding is the significant role of donor preparation, particularly protein supplementation and the kind of protein in the diet, which enhances the efficacy of FMT. By modulating the donor gut microbiota through plant-based proteins, Veg-FMT accelerated and amplified abstinence related recovery, leading to reduced hepatic inflammation and fibrosis.

Veg-FMT also significantly enhanced intestinal barrier integrity with a concomitant reduction in plasma endotoxin levels, highlighting its dual action in mitigating gut epithelium damage and limiting systemic inflammation. These findings are consistent with previous studies showing that microbial interventions restore gut permeability and reduce endotoxemia in ALD models (7,27,28).

Additionally, the upregulation of antimicrobial peptides (Reg3β and Reg3γ) highlights the role of Veg-FMT in strengthening mucosal immunity. These peptides likely contribute to maintaining a healthier gut microbiota composition, which is essential for reducing alcohol-induced endotoxemia (29). These findings suggest that the benefits of Veg-FMT rely on preserving intestinal barrier integrity and mucosal defenses, which is consistent with our earlier work showing that plant protein supplementation improved hepatic stress and barrier function in ALD (30).

Donor management plays a pivotal role in the success of FMT (31). Given that diet directly influences the composition of the gut microbiota (12), numerous studies have highlighted the impact of pre-educating donor microbiota with different dietary regimens on the efficacy of FMT (32–34). Kedia et al. reported that FMT plus an anti-inflammatory diet significantly improved UC outcomes, with deep remission maintained for up to a year through ongoing dietary interventions (35). Similarly, Rinott et al. reported that diet-modulated autologous FMT, especially from green-Mediterranean diet donors, reduced weight regain and improved glycemic control, highlighting the lasting metabolic benefits of diet-driven microbiome modulation (36). In addition, Yang et al. demonstrated that FMT from methionine-restricted diet donors remodeled the gut microbiota in obese mice, increasing SCFA-producing bacteria, reducing proinflammatory taxa, and improving lipid metabolism and fat browning (33). Together, these findings highlight the importance of donor preparation, especially through tailored diets, to increase the therapeutic potential of FMT.

In addition to broader dietary regimens, specific dietary macronutrients, such as proteins, further influence the composition of the gut microbiota (11). While dietary fiber and prebiotic oligosaccharides are established substrates for gut fermentation, emerging evidence shows that small amounts of nondigestible dietary proteins and peptides also reach the colon for microbial metabolism. The differing digestibility of plant- and animal-based proteins shapes their distinct effects on the gut microbiota, with plant proteins, due to lower digestibility, being more likely to reach the colon and drive unique microbial profiles (37).

In this context, Veg-FMT, which involves plant protein-modulated microbiota, results in a compositional shift in the gut microbiota, characterized by an increased abundance of beneficial taxa such as *Akkermansia, Lachnospiraceae* NK4A136, and *Parasutterella*. *Lachnospiraceae* NK4A136 and *Tuzzerella* are known SCFA producers and mediate anti-inflammatory pathways (19). *Akkermansia* has been shown to maintain the mucus layer in the gut (38). These taxa have been associated with improved intestinal barrier function and reduced systemic inflammation. Functional pathway prediction analysis further revealed enrichment of microbial metabolic pathways associated with amino acid degradation and fatty acid metabolism following Veg-FMT, suggesting increased microbial fermentation potential that may contribute to the production of fatty-acid related metabolites (39,40).

Changes in the gut microbiota can influence liver function. Our proteomics and pathway analyses revealed that Veg-FMT significantly activated PPARα signaling, a key regulator of impaired fatty acid oxidation in ALD (41). The upregulation of PPARα and its targets (Cpt1, Acox1, and Fabp1) enhanced mitochondrial and peroxisomal β-oxidation, reducing hepatic lipid accumulation. Inhibiting PPARα reversed the protective effects of Veg-FMT, confirming its critical role. These results align with those of previous studies highlighting PPARα as a therapeutic target in ALD (42,43).

Microbial metabolites derived from dietary protein fermentation also modulate PPARα signaling. In the colon, microbial proteases hydrolyze dietary proteins into peptides and amino acids, which are further fermented into SCFAs and other metabolites (39). Among these fatty acids, caproic acid, a six-carbon chain fatty acid, emerged from our metabolomic analysis and serves as a potential ligand for PPARα activation (22). By activating PPARα, caproic acid regulates hepatic lipid metabolism, promoting fatty acid oxidation and reducing lipid accumulation. Consistently, previous studies have shown that short- and medium-chain fatty acids inhibit lipogenesis and support mitochondrial energy production (24). In both in vitro and in vivo, caproic acid supplementation alleviated ethanol-induced hepatic steatosis by promoting fatty acid β-oxidation and suppressing lipogenesis, effects largely reversed by PPARα inhibition. Thus, caproic acid, a 6-carbon fatty acid, appears central to the observed benefits. Notably, caproic acid-mediated improvement in hepatic lipid homeostasis was also associated with reduced triglyceride accumulation and downregulation of the lipid droplet-associated protein PLIN2, whereas lipid export genes (ApoB and MTP) were only modestly or non-significantly altered, indicating that steatosis attenuation was driven primarily by reduced lipid storage and enhanced fatty acid β-oxidation rather than increased VLDL export (44,45).

However, the study has several limitations. Findings are based on an animal model and require validation in human studies for FMT application. The long-term safety and broader metabolic impacts of Veg-FMT remain unknown. Furthermore, individual microbiota variability could influence treatment reproducibility, highlighting the need for personalized approaches in future research.

Given the role of PPARα in ALD, pharmacological activation of this pathway could be explored as a therapeutic strategy. Elafibranor, a dual PPARα/δ agonist, has shown promise in improving liver function and reducing inflammation in ALD (46).

Conclusion

This study provides new insights into the mechanisms underlying the therapeutic effects of Veg-FMT. We show that preconditioning the donor microbiota with a plant-protein diet is key to enhancing FMT efficacy by promoting SCFA-producing bacteria and elevating caproic acid levels. Caproic acid, in turn, activates PPARα, increasing β-oxidation, suppressing lipogenesis, and improving hepatic injury and metabolic health. To our knowledge, this is the first demonstration of the specific pathways that mediate the benefits of FMT.

Materials and methods

Adaptation of mice to high-protein diets

Male C57BL/6 N mice (strain code-632) aged 6-8 weeks and weighing 20--25 g were procured from the Center for Comparative Medicine, ILBS, Delhi. The animals were housed under barrier housing conditions following specific pathogen-free animal husbandry practices. The ambient temperature and humidity were maintained at 21–25°C and 50–70%, respectively. The Institutional Animal Ethics Committee approved the animal protocols, and the experiments were performed following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines 2.0. The animal experiments were performed in the Experimental Animal Facility of ILBS, Delhi, during the light cycle. Autoclaved corn cob bedding material and a ventilated cage system were used for the housing. A piece of M-block wood (Kansara Scientific, India) was supplied to the mice so that they could gnaw on it to reduce stress.

The animals were acclimatized to a liquid diet for 1 week. Mice were then fed incremental alcohol in Lieber-DeCarli liquid diet (Cat#F1258SP, Bio-Serv, USA) with thioacetamide for 12 weeks to develop the ALD model as described earlier (47). Control mice were pair-fed a non-alcoholic isocaloric Lieber-DeCarli liquid diet (Cat#F1259SP, Bio-Serv, USA; calories equated with maltodextrin). For FMT the donor mice were randomly divided into three groups: control diet donor, high vegetable protein diet donor (crushed soya chunks; RSI Ltd., India), and egg protein diet donor (albumin egg flakes; Cat#GLR09.020738). All the diets were kept isocaloric (Additional file 1, S.Fig 1A). Respective diets were given for 14 days, and their fecal matter was collected at day 14. FMT recipients were fed standard liquid diet for seven days prior to FMT. The measurement of body weight was conducted every week for each animal, whereas food intake was assessed once every two days.

Fecal microbiota transplantation

For the FMT study, 6 mice were used per donor group (three donor groups: Cd, Vd and Ed). ALD was established in four groups of animals (n=16 each): Abstinence (Abst), standard FMT (Std-FMT), veg-protein FMT (Veg-FMT), and egg-protein FMT (Egg-FMT). The stool from animal donors fed high-protein or standard diets was collected and immediately processed for FMT. Fresh stool (300 mg) was resuspended in 3 ml of sterile, ice-cold 0.9% saline. Recipient ALD mice received gut washes with 200 µl of PEG-4000 (Cat#81240, Sigma Aldrich, USA). After four hours, 200 µl of the prepared fecal slurry was administered via oral gavage. Three doses of FMT were given every other day. The experiment was terminated 1 week after the last FMT dose was given.

Tissue collection

The stool was collected pre- and post-FMT. All other tissue samples were collected at the end of the experiments. Blood was obtained via retro-orbital sinuses just before euthanasia (Ketamine, 80-100mg/Kg + Xylazine, 8-10 mg/Kg). Tissue samples were weighed and divided into two groups – one set snap-frozen in liquid nitrogen, and stored at −80°C till further processing. The second group for histopathology was fixed in 10% formalin.

Biochemical tests

To investigate the effect of FMT, serum was separated from the blood by centrifugation at 3,000xg for 10 minutes. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, and triglycerides were detected by an Olympus AU5400 automatic analyzer (Olympus Optical, Tokyo, Japan). Serum endotoxin levels were measured per manufacturer's protocol using Pierce Chromogenic Endotoxin Quant Kit (#A39552, Thermofisher Scientific).

Histopathology and immunohistochemistry

Post-FMT histopathological features of liver and ileum were assessed in the formalin fixed tissue sections (4 µm thick) stained with hematoxylin-eosin (H&E) to capture immune infiltrations, steatosis (Oil red O staining) and fibrosis (Masson's trichrome staining). Immunohistochemical staining was performed to assess extent of fibrosis using antibodies against α-SMA (#MA5115467, Thermo Fischer, USA) on hepatic tissue. The sections were washed and incubated with an appropriate biotinylated secondary antibody and then with streptavidin-horseradish peroxidase complex (PolyExcel HRP/DAB Detection System Two Step Universal Kit, #PEH002, PathnSitu Biotechnologies, USA). Sections were then counterstained with hematoxylin (#S034, HiMedia). The sections were observed with the help of a light microscope (EVOSTM M5000, Thermofisher Scientific, USA) at a magnification of 10x, 20x and 40x.

Additional details of methods and materials can be found in supplementary section 1 (S1).

Data availability

The 16s fastq data have been deposited with links to BioProject accession number PRJDB35434 in the DDBJ BioProject database. The raw metabolomic files can be accessed from NGDC OMIX010622 under Bioproject PRJCA041599. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD064611. The reviewer can access the dataset by logging into the PRIDE website via the following account details: Username: reviewer_pxd064611@ebi.ac.uk, Password: M9ehP1lOAcK2 All data generated or analyzed during this study are included in the manuscript and supporting files; source data files have been provided for all figures.

Additional information

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Author Contributions

Nishu Choudhary#: Conceptualization; Data curation; Formal analysis, Methodology, Validation; Visualization; Writing – original draft, review & editing; **Ashi Mittal#:** Conceptualization; Data curation; Formal analysis, Methodology, Validation; Visualization; Writing – original draft, review & editing; **Sandeep Kumar:** Methodology, Validation; **Kavita Yadav:** Methodology, Data curation; **Anupama Kumari:** Methodology, Data curation; **Deepanshu Maheshwari:** Methodology, Data curation; **Jaswinder Singh Maras:** Resources, Data curation, Formal analysis; **Anupam Kumar:** Resources, Data curation; **Shiv Kumar Sarin:** Conceptualization, Writing - review & editing; **Shvetank Sharma:** Conceptualization, Methodology, Investigation, Resources, Supervision, Writing - review & editing. All the authors have read and approved the final manuscript.

Abbreviations

Acadm: Acyl-Coenzyme A dehydrogenase, medium chain
Acadvl: Acyl-Coenzyme A dehydrogenase, very long chain
Acox1: Acyl-Coenzyme A oxidase 1
Acta2: actin alpha 2, smooth muscle

ALT: alanine aminotransferase
ALD: Alcohol-related liver disease
APOB: Apolipoprotein B
AST: aspartate aminotransferase
Cldn3: claudin3
Cpt1: Carnitine palmitoyltransferase 1
Fabp1: Fatty acid binding protein 1
Fasn: fatty acid synthase
Fc: Std-FMT
Fe: Egg-FMT
FMT: Fecal microbiota transplant
Fv: Veg-FMT
IL: Interleukin
MTTPl; MTTP: Microsomal Triglyceride Transfer Protein
Muc2: mucin 2
Ocln: occludin
PCoA: principal coordinate analysis
PLIN2: Perlipin 2
PPARα: peroxisome proliferator-activated receptor alpha
Scd1: stearoyl-coenzyme A desaturase 1
Srebp1c: Sterol Regulatory Element Binding Protein 1c
Tgf-β: tumor growth factor beta
Tlr4: toll-like receptor 4
Tnf-α: tumor necrosis factor alpha
TGs: Triglycerides

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Additional files

Additional file 1. Supplementary information.

Additional file 2. Supplementary tables.

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Peer reviews

Reviewer #1 (Public review):

Summary:

The authors aimed to determine whether dietary conditioning of fecal microbiota donors can influence the therapeutic efficacy of fecal microbiota transplantation (FMT) in alcohol-associated liver disease (ALD). Specifically, they tested whether donor diets enriched in vegetable or egg-derived proteins alter microbiota composition and function in ways that enhance recovery from alcohol-induced liver injury. Using a murine ALD model, the study integrates microbiome profiling, metabolomics, proteomics, and functional assays to identify mechanisms underlying improved outcomes. The authors propose that vegetable protein-conditioned microbiota promote beneficial microbial remodeling and increased production

of caproic acid, which in turn activates hepatic PPARα signaling and enhances fatty acid β-oxidation, thereby reducing steatosis and inflammation.

Strengths:

The study is ambitious and methodologically comprehensive. The central idea, that donor diet can modulate FMT efficacy in ALD, is compelling and potentially impactful. It combines in vivo disease models, microbiome analysis (16S rRNA sequencing), metabolomics and proteomics, pharmacological inhibition experiments, and in vitro validation in hepatocytes. This multi-layered approach is a clear strength and allows the authors to explore the gut-liver axis. The comparison between different protein sources (vegetable vs egg) is very interesting, and the PPARα inhibition experiments provide relatively strong functional support for the involvement of host metabolic signaling pathways in mediating the observed effects.

Weaknesses:

Despite the comprehensive scope of the manuscript, several aspects of the study limit the strength of its mechanistic conclusions. The causal attribution to caproic acid remains incomplete. While caproic acid is identified and functionally tested, there is no direct demonstration that it is necessary for the Veg-FMT phenotype in vivo. The metabolomics data suggest multiple candidate metabolites, but these are not systematically explored. The study identifies specific bacterial taxa and, separately, key metabolites, but does not establish a direct connection between microbial composition and metabolite production. The use of GW6471 supports involvement of PPARα but does not fully establish specificity, as off-target effects cannot be excluded. Finally, it is not fully clear whether effects are exclusively microbiota-driven or could partially reflect the transfer of diet-derived metabolites.

The authors successfully demonstrate that donor dietary conditioning influences the therapeutic efficacy of FMT in a murine model of ALD. The data convincingly show that vegetable protein-conditioned microbiota is associated with improved liver injury, reduced inflammation, and enhanced intestinal barrier integrity compared with controls or an egg protein-enriched diet. While the proteomic and gene expression data suggest activation of pathways related to fatty acid β-oxidation, these measurements do not directly demonstrate increased metabolic flux. The use of the PPARα antagonist GW6471 provides important functional support for the involvement of this pathway, as inhibition attenuates the protective effects of Veg-FMT. However, this approach primarily establishes pathway dependency rather than directly confirming enhanced β-oxidation activity. The authors may therefore wish to moderate their interpretation or clarify this distinction, particularly given the relatively modest fold changes observed in several targets. The role of caproic acid as a central mediator is plausible but not definitively established. Finally, the link between microbiota composition, metabolic function, and host signaling remains partly correlative. Overall, the study achieves its primary aim at a phenotypic level, but some of the mechanistic claims would benefit from more cautious interpretation or additional validation.

Likely impact of the work on the field, and the utility of the methods and data to the community:

The work addresses an important and underexplored question: how donor characteristics influence FMT efficacy. By introducing donor diet as a modifiable variable, the study has potential implications for optimizing microbiota-based therapies. The datasets (microbiome, metabolomics, and proteomics) may also be valuable to the community, as they provide a resource for exploring gut-liver metabolic interactions. The translational impact will, however, depend on validation in human systems and a clearer identification of causal mechanisms.

https://doi.org/10.7554/eLife.111301.1.sa2

Reviewer #2 (Public review):

The manuscript explores a valuable strategy for optimizing Fecal Microbiota Transplantation (FMT) efficacy in alcoholic liver disease through donor dietary intervention. I have identified several critical logical gaps, missing links in the evidence chain, and methodological ambiguities that require detailed explanation and supplementation.

(1) While the Methods section states that each recipient mouse group consisted of 16 animals, microbiome sequencing was performed on only 4 samples per group. This sample size is insufficient, and the high inter-individual variability observed reduces the statistical power and representativeness of the data. I recommend increasing the sequencing sample size or, at a minimum, explicitly acknowledging the risk of false positives due to the small sample size in the Discussion.

(2) The layout of Figure 4 should be adjusted. Panel A should be enlarged for better visibility, while Panel B should be reduced in size to balance the figure composition.

(3) A rationale should be provided for the selection of egg white protein as the animal protein control. Does this adequately represent animal proteins in general? Could the results differ if casein or whey protein were used? The current choice limits the generalizability of the conclusions, and this limitation should be addressed.

(4) The ALD model was established over 12 weeks, yet the FMT intervention consisted of only 3 administrations with a 1-week observation period. In the context of such a severe liver injury model, a 1-week recovery period appears insufficient to observe genuine fibrosis reversal, which typically requires a longer timeframe. The authors should discuss whether short-term FMT can truly induce structural remodeling or if the observed effects are transient.

(5) The results rely heavily on PICRUSt2 for functional prediction. As prediction does not equate to factual validation, the authors should exercise caution in their wording within the Discussion. Alternatively, I recommend supplementing the study with shotgun metagenomic sequencing to verify the existence of these pathways rather than relying solely on predictive algorithms.

(6) Although Egg-FMT was less effective than Veg-FMT, it performed better than the standard FMT or abstinence groups. Why is the effect of egg white protein intermediate? Is this due to rapid digestion resulting in insufficient substrate, or differences in metabolite production? A deeper comparative analysis of the Egg-FMT group is required, rather than treating it merely as a negative control.

(7) Relying solely on the "inhibitor blocking effect" proves only that Caproic acid's function is dependent on the PPARα pathway, not that it directly acts on PPARα. To claim direct activation, the authors must demonstrate direct binding between Caproic acid and the PPARα protein (e.g., via SPR or MST assays). Alternatively, a luciferase reporter assay driven specifically by PPARα response elements (PPRE) should be conducted. If Caproic acid induces luminescence, it would confirm transcriptional activation of PPARα rather than mere downstream activation.

https://doi.org/10.7554/eLife.111301.1.sa1

Author response:

We thank the Reviewing Editor, Senior Editor, and both reviewers for their constructive evaluation of our manuscript. We are encouraged that the reviewers found the central question, whether donor dietary conditioning modulates FMT efficacy in ALD, compelling

and the multi-omics framework a strength. Their critiques converge on a shared theme: the manuscript's mechanistic claims around caproic acid and PPARα signaling currently rest on associative and pathway-level evidence, and would benefit from more direct causal testing and more guarded language. We agree, and we outline below the revisions we plan to undertake.

> ***Public Reviews:***
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> ***Reviewer #1 (Public review):***
>
> *While the proteomic and gene expression data suggest activation of pathways related to fatty acid β-oxidation, these measurements do not directly demonstrate increased metabolic flux. The use of the PPARα antagonist GW6471 provides important functional support for the involvement of this pathway; however, this approach primarily establishes pathway dependency rather than directly confirming enhanced β-oxidation activity. The role of caproic acid as a central mediator is plausible but not definitively established. Finally, the link between microbiota composition, metabolic function, and host signaling remains partly correlative.*

We thank the reviewer for this thoughtful assessment. We agree that the GW6471 inhibition experiments primarily support pathway dependency rather than direct activation of PPARα by caproic acid, and we will revise the manuscript accordingly to avoid overstating mechanistic conclusions. However, we would like to clarify that the objective of the current study was not to directly quantify metabolic flux. We agree that metabolic flux should not be used here. We will be modifying this in the text to make it clear that we measured mitochondrial beta oxidation as a response to caproic acid.

To functionally assess alterations in fatty acid β-oxidation capacity, we performed Seahorse Mito Fuel Flex assays, which demonstrated altered dependency and utilization of fatty acid oxidation pathways in response to caproic acid treatment. We will further clarify this distinction in the revised.

In addition, we agree that the role of caproic acid as a central mediator and the relationship between microbiota composition, metabolite production, and host signaling remain partly correlative. Therefore, we will moderate the interpretation throughout the manuscript and incorporate additional correlation analyses between microbial taxa, caproic acid levels, and disease-associated metabolic parameters to strengthen the microbiota-metabolite-host association while acknowledging the associative nature of these findings.

> ***Reviewer #2 (Public review):***
>
> *(1) While the Methods section states that each recipient mouse group consisted of 16 animals, microbiome sequencing was performed on only 4 samples per group. This sample size is insufficient, and the high inter-individual variability observed reduces the statistical power and representativeness of the data. I recommend increasing the sequencing sample size or, at a minimum, explicitly acknowledging the risk of false positives due to the small sample size in the Discussion.*

We thank the reviewer for this important comment. We would like to clarify that microbiome sequencing was performed on 6 samples per group and not on 4 samples per group, and we will revise the Methods section to improve clarity regarding the number of biological replicates analyzed. The 4 samples were used only for whole proteome analysis.

In addition, several previously published murine microbiome studies investigating gut microbial alterations in liver disease and FMT interventions have used comparable sample sizes (typically 5-8 animals per group) for 16S rRNA sequencing analyses [1–3]. Nevertheless, we agree that inter individual variability may influence microbiome analyses, and therefore

we will explicitly acknowledge this limitation and the possibility of reduced statistical power in the revised Discussion section. We will also ensure that interpretations derived from microbiome compositional analyses are presented more cautiously.

> *(2) The layout of Figure 4 should be adjusted. Panel A should be enlarged for better visibility, while Panel B should be reduced in size to balance the figure composition.*

We thank the reviewer for this suggestion. We will revise the layout of Figure 4 accordingly by enlarging Panel A for improved visibility and reducing the size of Panel B to achieve a more balanced figure composition.

> *(3) A rationale should be provided for the selection of egg white protein as the animal protein control. Does this adequately represent animal proteins in general? Could the results differ if casein or whey protein were used? The current choice limits the generalizability of the conclusions, and this limitation should be addressed.*

We thank the reviewer for this important suggestion. In the revised manuscript, we will provide additional rationale for selecting egg albumin as the animal-derived protein source. Egg albumin was chosen because it is a well-characterized protein with high biological value, rapid digestibility, standardized composition, and has also been used in our previous ALD-related dietary intervention studies for experimental consistency [4].

We agree that egg albumin does not represent all animal protein sources. Due to its rapid digestion and absorption, relatively less substrate may reach the distal gut for microbial fermentation compared with more complex proteins. In contrast, proteins such as casein or whey may generate distinct microbial and metabolite profiles and potentially different host responses.

Accordingly, we will explicitly acknowledge this limitation in the revised manuscript and clarify that our findings should not be generalized to all animal-derived proteins.

> *(4) The ALD model was established over 12 weeks, yet the FMT intervention consisted of only 3 administrations with a 1-week observation period. In the context of such a severe liver injury model, a 1-week recovery period appears insufficient to observe genuine fibrosis reversal, which typically requires a longer timeframe. The authors should discuss whether short-term FMT can truly induce structural remodeling or if the observed effects are transient.*

We thank the reviewer for this important and thoughtful observation. We agree that a one-week post-FMT observation period appears insufficient to conclude complete structural remodeling or durable fibrosis reversal in a chronic 12-week ALD model. Though it should be noted that the results achieved with the one week intervention suggest otherwise in this animal model of ALD. As can be observed from the immunohistochemistry of abstinence and treatment groups, which was further quantified for steatosis and fibrosis, there is a __% and __% reduction respectively in the treatment group. Thus we can safely conclude that in the given animal model, an alternate day FMT for 3 doses can reverse steatosis and fibrosis.

In the revised manuscript, we will explicitly clarify this distinction.

> *(5) The results rely heavily on PICRUSt2 for functional prediction. As prediction does not equate to factual validation, the authors should exercise caution in their wording within the Discussion. Alternatively, I recommend supplementing the study with shotgun metagenomic sequencing to verify the existence of these pathways rather than relying solely on predictive algorithms.*

We thank the reviewer for this important suggestion and agree that PICRUSt2-based analyses represent predictive functional inference rather than direct validation of microbial metabolic

activity. We will explicitly acknowledge in the Results and Discussion that PICRUSt2 outputs are inferences rather than measurements, and we will integrate our metabolomics data to show where predicted microbial pathways (fatty acid salvage, β-oxidation related pathways) coincide with measurable metabolite shifts, providing observational support for the predictions.

We would like to avoid doing metagenomic analysis to substantiate PICRUST2 findings primarily because metagenomic analysis would provide information on the set of genes each species carries, and not the functional state of the resulting pathways. To read out the pathways we would be left with the same two options of PICRUS2 or metabolome analysis. Yes, if we perform transcriptome analysis we can reach to a conclusion on which pathways are active. Which is likely to be similar to the readout we get from the end result of these pathways – the metabolome.

> *(6) Although Egg-FMT was less effective than Veg-FMT, it performed better than the standard FMT or abstinence groups. Why is the effect of egg white protein intermediate? Is this due to rapid digestion resulting in insufficient substrate, or differences in metabolite production? A deeper comparative analysis of the Egg-FMT group is required, rather than treating it merely as a negative control.*

We thank the reviewer for this insightful observation. We agree that the Egg-FMT group demonstrated an intermediate phenotype and should not be interpreted merely as a negative control. We will modify the text in the manuscript to mention the outcomes with egg protein, wherever it missing. In the revised manuscript, we will modify the language accordingly and expand the Discussion.

> *(7) "Relying solely on the 'inhibitor blocking effect' proves only that Caproic acid's function is dependent on the PPARα pathway, not that it directly acts on PPARα. To claim direct activation, the authors must demonstrate direct binding between Caproic acid and the PPARα protein (e.g., via SPR or MST assays). Alternatively, a luciferase reporter assay driven specifically by PPARα response elements (PPRE) should be conducted. If Caproic acid induces luminescence, it would confirm transcriptional activation of PPARα rather than mere downstream activation."*

We thank the reviewer for this important and insightful suggestion. We agree that the current inhibitor-based experiments primarily support the involvement of the PPARα pathway and do not definitively establish direct interaction or transcriptional activation of PPARα by caproic acid. Accordingly, in the revised manuscript, we will moderate our interpretation and avoid statements implying direct activation based solely on the current data.

We also agree that direct validation experiments such as SPR/MST-based binding assays or PPREdriven luciferase reporter assays would substantially strengthen the mechanistic conclusions. We are currently planning additional experiments to further evaluate the direct action of caproic acid on PPARα and will incorporate these analyses in future revisions and follow-up studies.

With the pending experiments we request the Editors to kindly provide us a time of about 2 months to send back the revised manuscript.

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