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VISUAL ABSTRACT

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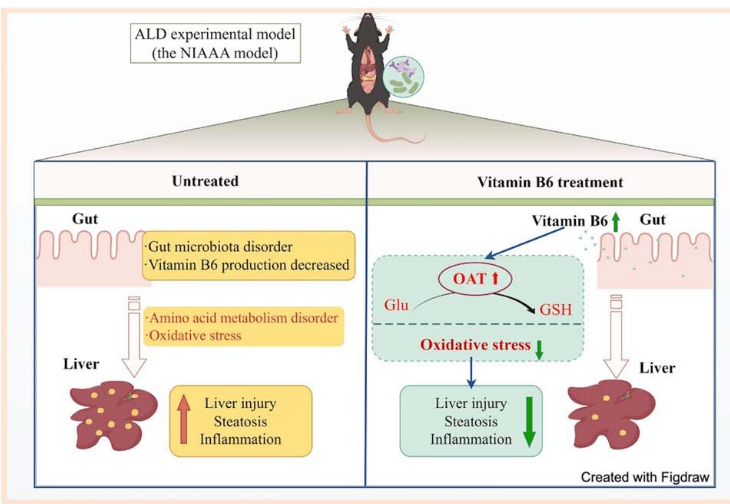

- Alcohol-induced disruption of the gut microbiome leads to decreased production of vitamin B6 in the gut.
- Reduced vitamin B6 levels exacerbate alcohol-induced disorders of amino acid metabolism in the liver.
- Vitamin B6 supplementation mitigates oxidative stress damage by restoring OAT expression levels in the liver.

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ORIGINAL ARTICLE

The gut microbiota-produced vitamin B_6 mitigates alcohol-associated liver disease by attenuating hepatic oxidative stress damage

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Abstract

Background: Alcohol-associated liver disease (ALD) is a major clinical issue characterized by progressive stages, including hepatic steatosis, liver fibrosis, cirrhosis, and HCC. Patients with long-term chronic alcoholism often present with gut microbiota dysbiosis and reduced plasma levels of vitamin B_6. This study aimed to verify that gut microbiota disruption in ALD significantly contributes to reduced in vivo production of vitamin B_6 and to investigate the role of this reduction in the pathogenesis of ALD.

Methods: The ALD was investigated utilizing the Gao-binge mouse model. Fecal microbial composition was analyzed in pair-fed mice and ALD mice to identify alcohol-induced functional changes in the microbiota. Additionally, liver protein expression profiles and liver and plasma metabolomic profiles

Abbreviations: ABX mice, antibiotic treatment mice; ALD, alcohol-associated liver disease; GSH, glutathione; KEGG, Kyoto Encyclopedia of Genes and Genomes; MDA, malondialdehyde; OAT, ornithine aminotransferase; OatΔHep, hepatocyte-specific Oat knockout; PLP, pyridoxal 5′-phosphate.

Haiyuan Shen, Liangliang Zhou, and Yuanru Yang contributed equally to this work.

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were characterized to elucidate the role of vitamin B_6 in ALD pathogenesis through integrated proteomic and metabolomic analyses. The findings were further validated using animal models and clinical patient samples.

Results: Alcohol consumption disrupted the gut microbiota in the mice, impairing the vitamin B_6 synthesis by intestinal microorganisms. Vitamin B_6 deficiency aggravated the disorder of amino acid metabolism in the liver and inhibited ornithine aminotransferase expression, thereby worsening oxidative stress damage. In patients with ALD, significant disturbances of gut microbiota were observed, along with decreased intestinal vitamin B_6 levels, which were negatively correlated with serum biochemical markers.

Conclusions: The imbalance of gut microbiota in ALD mice reduces vitamin B_6 synthesis, which affects amino acid metabolism and glutathione synthesis in the liver, thereby exacerbating ALD. These findings suggest that vitamin B_6 may play a critical protective role in ALD progression by regulating amino acid metabolism.

Keywords: alcohol-associated liver disease, amino acid metabolism, gut microbiota, multiomics analysis, vitamin B_6

HIGHLIGHTS

- Alcohol-induced disruption of the gut microbiome leads to decreased production of vitamin B_6 in the gut.
- Reduced vitamin B_6 levels exacerbate alcohol-induced disorders of amino acid metabolism in the liver.
- Vitamin B_6 supplementation mitigates oxidative stress damage by restoring ornithine aminotransferase expression levels in the liver.

INTRODUCTION

Alcohol-associated liver disease (ALD) is a major consequence of chronic alcohol consumption, representing one of the leading causes of liver-related morbidity and mortality. The incidence of ALD continues to rise annually.[1] Current treatments for ALD are limited and largely ineffective,[2] primarily due to the complex pathogenesis of the disease, which involves intricate interaction between genetics, the immune system, gut microbiota, and environmental factors. Therefore, research on the pathogenesis of ALD is an area of intense investigation in the field of chronic liver disease.

Previous studies have suggested that vitamin B_6 deficiency may contribute to the pathogenesis of various chronic liver conditions, including hepatic steatosis.[3,4] Low levels of vitamin B_6 have been associated with decreased survival rates following liver transplantation, as well as disruptions in vitamin B_6-dependent biochemical pathways, indicating its clinical relevance.[5] Chronic alcoholics frequently exhibit reduced plasma levels of pyridoxal 5′-phosphate (PLP), the coenzyme form of vitamin B_6.[6] The liver, as the primary source of PLP in plasma and the main organ responsible for ethanol oxidation, is particularly susceptible to alcohol's harmful effects. Acetaldehyde, the byproduct of ethanol oxidation, accelerates the intracellular degradation of pyridoxal phosphate.[7] In animal studies, chronic alcohol intake over 6 weeks significantly reduced PLP levels, regardless of dietary vitamin B_6 supplementation.[8] Our previous work, utilizing ultra–high-pressure liquid chromatography-quadrupole time-of-flight tandem mass spectrometry to analyze metabolomic profiles in ALD mouse models, also identified downregulation in vitamin B_6 metabolism.[9] Since vitamin B_6 is mainly obtained from diet or synthesized by gut bacteria and subsequently absorbed in the intestine,[10,11] this study investigates whether alcohol consumption disrupts gut flora, thereby impacting vitamin B_6 synthesis. Our goal is to elucidate the underlying factors contributing to reduced levels of vitamin B_6 in patients with ALD.

Growing evidence highlights the significant relationship between gut microbiota and human health. The normal gut microbiome plays essential roles in nutrient metabolism, maintaining the structural integrity of the intestinal mucosal barrier, immunomodulation, and protection against pathogens.[12] Alcohol consumption,

however, leads to a significant reduction in gut fungal and bacterial diversity, and ethanol increases intestinal permeability.[13] Patients with ALD exhibit reduced diversity of intestinal bacteria, with a lower proportion of beneficial bacteria such as *Lactobacillus, Bifidobacterium*, and *Akkermansia muciniphila*.[14,15] Furthermore, the proportions of *Bacteroidaceae* and *Prevotellaceae* are lower in patients with alcohol-associated cirrhosis,[16] while pathogenic bacteria like *Veilonella* and *Enterococcus faecalis* increase.[17] Both preclinical and clinical evidence suggest that the gut microbiota plays an important role in the development and progression of ALD.[18–20]

Furthermore, the gut microbiota is integral to the biosynthesis of B vitamins, including cobalamin, folate, pyridoxine, pantothenate, and thiamin, all of which contribute to maintaining homeostasis and promoting liver health.[21,22] Gene expression data suggest that gut microbiota affects host amino acid metabolism, leading to alterations in glutathione (GSH) metabolism, although the exact mechanisms remain unclear.[23] Vitamin B_6, as a cofactor in over 140 biochemical reactions, plays a vital role in the biosynthesis and catabolism of amino acids and neurotransmitters.[24] Its most critical functions involve amino acid biosynthesis and degradation, often through transamination reaction, as well as other processes like alpha-decarboxylation or racemization.

Based on this research background, our study employs an integrated analysis of microbiome, proteomics, metabolomics, and animal model experiments to demonstrate that alcohol consumption disrupts intestinal microbiome homeostasis, leading to reduced vitamin B_6 synthesis. This reduction, in turn, impairs amino acid metabolism and exacerbates oxidative stress damage in the liver, ultimately worsening ALD.

METHODS

Human samples

Clinical studies were conducted with patient consent and adhered to the guidelines of the Declaration of Helsinki and Istanbul. The study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. We collected serum samples from 34 individuals, comprising 21 patients with confirmed ALD and 13 healthy controls, from the First Affiliated Hospital of Anhui Medical University. Exclusion criteria for both groups included the presence of nonalcoholic liver disease, other decompensated systemic diseases, use of prebiotics and/or prebiotics, nonsteroidal anti-inflammatory drugs, antibiotics, and autoimmune liver disease (including hepatitis B, hepatitis C, and HIV). Normal liver tissue was obtained from 3 patients undergoing surgical resection for hepatic hemangioma, while liver tissue samples were collected from 5 patients undergoing OLT for alcohol-associated cirrhosis. These samples were embedded in paraffin for subsequent pathological staining. Additionally, we collected fecal samples from 11 patients with ALD and 13 healthy controls, which were stored at −80 °C for future fecal genomics data analysis.

Animals

Oat-flox mice (Strain NO. T018612) and female wild-type C57BL/6 mice (8–10 wk old) were purchased from GemPharmatech Company (Nanjing, China). Hepatocyte-specific Oat knockout ($Oat^{\Delta Hep}$) mice were generated through multiple crosses with Oat flox/flox mice and Alb-iCre mice (Strain NO. T003814, GemPharmatech). Mice were housed in the animal research center of Anhui Medical University at 22 °C on a 12-hour light-dark cycle, with free access to food and water. All animal procedures were approved by the Animal Experiment Ethics Committees of the First Affiliated Hospital of Anhui Medical University and report these in accordance with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) statement.

Animal models

To model ALD in mice, we used a modified ethanol feeding protocol based on a previous report by Bin Gao's laboratory. The short-term Gao-binge ethanol feeding model involved chronic ethanol feeding followed by a single binge. Age-matched 8- to 10-week-old female mice were fed either an isocaloric control diet or an ethanol Lieber-DeCarli diet containing 5% (vol/vol) ethanol for 10 days, following a 5-day liquid diet adaptation period. On day 11, ethanol-fed and pair-fed mice received a single dose of ethanol (5 g/kg body weight) or an isocaloric maltose dextrin solution via oral gavage, respectively. This protocol is commonly referred to as National Institute on Alcohol Abuse and Alcoholism model, a model of alcoholic liver injury by chronic ethanol feeding (10-d *ad libitum* oral feeding with the Lieber-DeCarli ethanol liquid diet) plus a single binge ethanol feeding.[25] By adjusting the vitamin b_6 content in the feed, we established National Institute on Alcohol Abuse and Alcoholism model mice with carrying levels of vitamin B_6 intake to investigate the impact of vitamin B_6 on ALD progression. After a 1-week acclimation period on a standard diet, mice were fed Lieber-DeCarli diets containing either control levels of vitamin B_6 (1.77 mg/kg pyridoxine), low vitamin B_6(VB_6^{low}, 0.17 mg/kg pyridoxine), or high vitamin B_6(VB_6^{hi}, 8.85 mg/kg pyridoxine). The customized diets were provided by Jiangsu Xietong Pharmaceutical Bio-engineering Co., Ltd.

Antibiotic treatment

After 1 week of acclimation, mice were administered a broad-spectrum antibiotic mixture. The antibiotic solution, which consisted of ampicillin (1 g/L, A9518; Sigma-Aldrich), neomycin (1 g/L, N1876; Sigma-Aldrich), metronidazole (1 g/L, M3761; Sigma-Aldrich), and vancomycin (0.5 g/L, 94747; Sigma-Aldrich), was added to the sterile drinking water and provided for 10 days.[26] The antibiotic solution was replenished every 3 days, and body weight was measured 3 times per week.

Fecal microbiota transplantation

Fresh fecal pellets (200 mg) from untreated mice (pair-fed group) and ALD model mice (EtOH group) were collected, resuspended in 2 mL sterile PBS, and centrifuged at 500*g* for 1 minute to obtain the supernatant.[27] The fecal microbial suspension was then filtered through a sterile 70 μm strainer and administered via gavage to antibiotic-treated (ABX) mice at a dose of 200 μL per mouse for 4 consecutive days.

Data availability

The 16S rRNA gene sequencing data generated in this study have been deposited in the National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/gsa) under accession number CRA012168. The proteomic and untargeted metabolomic data have been deposited in OMIX at the China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/omix) under accession numbers OMIX004706 and OMIX004710. Information on the primer sequences used for real-time PCR is provided in Supplemental Table S1, http://links.lww.com/HC9/B150. All relevant data are available in the main text, extended data, or Supplemental Materials, http://links.lww.com/HC9/B150. Additional data related to this study are available upon request from the corresponding author.

Statistical analysis

Data are presented as the mean ± SD. Statistical analysis of 2 samples was performed using a *t* test, while multiple samples were analyzed using one-way ANOVA. All statistical analyses were conducted using GraphPad Prism 9 (GraphPad, USA). A *p*-value of < 0.05 was considered statistically significant.

Additional methods are provided in the Supplemental Materials, http://links.lww.com/HC9/B150.

RESULTS

Reduced vitamin B_6 levels exacerbate ALD in mice

Preliminary studies from our group suggested significant alterations in the vitamin B_6 metabolic pathway in ALD mice. These changes involved purine, cysteine, methionine, D-glutamine, and vitamin B_6 metabolism pathways.[9] Tor validate these findings, we collected liver samples from ethanol-fed mice in accordance with the ALD model and pair-fed mice. Our results showed that vitamin B_6 levels in the liver tissue of the alcohol-fed mice were reduced (Figure 1A), indicating that alcohol consumption affects normal physiological levels of vitamin B_6. Proteomic analysis revealed that genes involved in vitamin B_6 binding (GO:0070279) were downregulated in the livers of ethanol-fed mice, suggesting that alcohol inhibits vitamin B_6 metabolism (Figure 1B).

To explore the impact of vitamin B_6 depletion on ALD progression, we modified the vitamin B_6 content in Lieber-DeCarli diets. Mice in the EtOH+VB_6^{low} group, whose dietary vitamin B_6 was reduced to one-tenth of the, exhibited significantly higher serum ALT and AST levels compared to the normal EtOH group (Figure 1C). Liver triglyceride content also significantly increased. Analysis of inflammatory cytokines in liver tissues showed that reduced vitamin B_6 exacerbated inflammation in ALD mice (Figure 1D). Pathological examination confirmed more severe hepatic steatosis and increased neutrophil infiltration in the livers from the EtOH+VB_6^{low} group (Figure 1E, F). These findings suggest that vitamin B_6 deficiency may exacerbate alcohol-induced liver damage.

Gut microbiota disruption reduces vitamin B_6 synthesis in ALD mice

To determine whether alcohol consumption reduces vitamin B_6 production by altering gut microbiota, we collected fecal samples from ethanol-fed mice and pair-fed mice for microbiota profiling using 16S RNA gene sequencing. After 16 days, alcohol-fed mice showed significant changes in gut microbial composition compared to the pair-fed mice (Supplemental Figure S1A, B, http://links.lww.com/HC9/B150). An analysis of bacterial abundance at the genus level revealed increased *Escherichia-Shigella* and *Lactobacillus* in the ethanol-fed mice, while *Parabacteroides* and *Akkermansia* decreased (Figure 2A). At the species level, 10 species increased significantly, and 3 species decreased significantly (Figure 2B, Supplemental Figure S1C, D, http://links.lww.com/HC9/B150). Functional profiling showed a notable decrease in amino acid and vitamin metabolism (Figure 2C). Our data showed intestinal

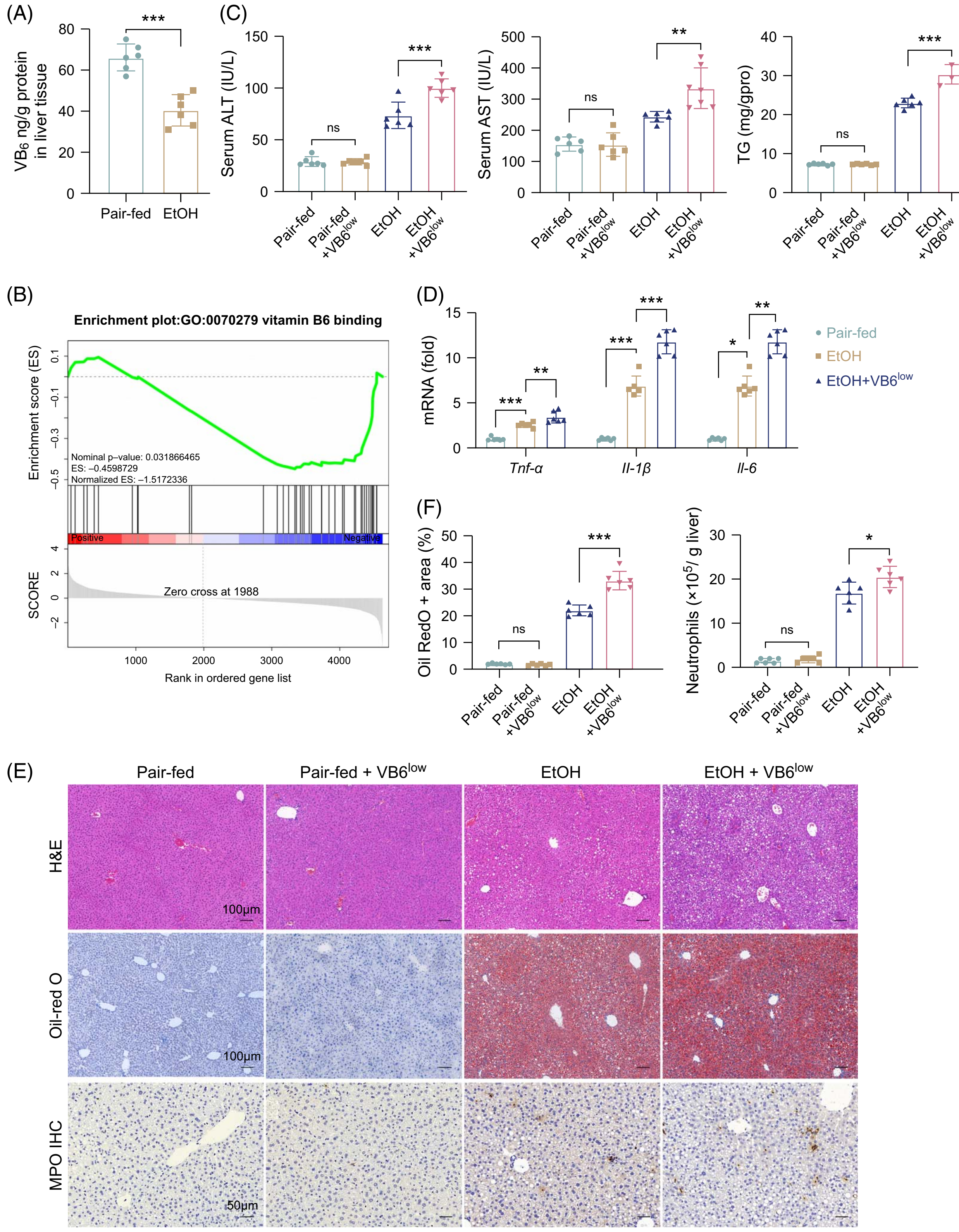


FIGURE 1 Vitamin B_6 deficiency exacerbates ALD in mice. (A) Total vitamin B_6 levels in the liver (n = 6). (B) GSEA of proteome indicated that vitamin B_6 binding signaling was downregulated in alcohol binge mice ($p < 0.05$). The experimental mice were divided into 4 groups: control group (pair-fed), control diet group with dietary vitamin b_6 reduced to one-tenth (pair-fed+VB_6^{low}), standard alcohol-fed group (EtOH), and alcohol-fed with VB_6^{low} group (EtOH+VB_6^{low}) (n = 6 per group). (C) Serum levels of ALT, AST, liver tissue levels of TG. (D) mRNA expression levels of proinflammatory cytokines. (E, F) Representative images of H&E and Oil-Red O staining (scale bar = 100 μm) and IHC staining of MPO in liver tissue (scale bar = 50 μm), Relative staining intensity is quantified and shown in the F. Abbreviations: ES, enrichment score; GO, gene ontology; H&E, hematoxylin and eosin; IHC, immunohistochemistry; MPO, myeloperoxidase; TG, triglyceride.

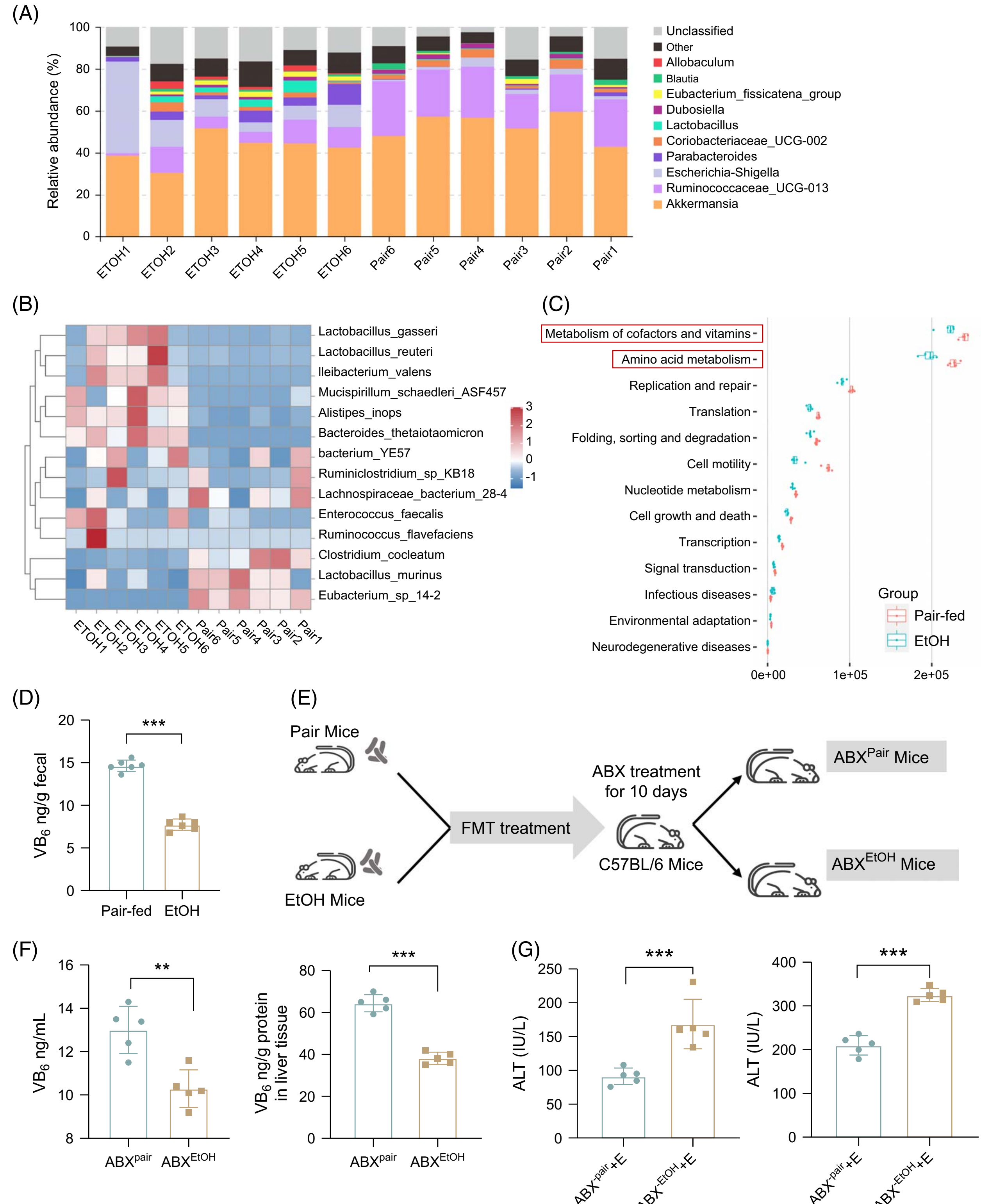


FIGURE 2 Alcohol-induced gut microbiota disruption decreases vitamin B_6 levels in mice. The intestinal contents from NIAAA model mice and pair-fed mice were collected for 16S sequencing and subsequent analysis (n = 6 per group). (A) Proportional abundance of bacteria genus in the feces of EtOH and pair mice. (B) Heatmap of the top 10 species by average abundance (each row represents a species, each column represents a sample, and the color represents species abundance. Dark blue indicates lower abundance, while red indicates higher abundance. The legend shows corresponding species abundance values). (C) Wilcoxon rank sum test of the 16S functional community profiling in ETOH mice (*x*-axis represents species abundance, and *y*-axis represents functional classification). (D) Total vitamin B_6 levels in mouse feces (n = 6). (E) Schematic of the fecal microbiota transplantation in ABX mice. Eight-week-old female mice were treated with a combination of antibiotics in drinking water for at least 10 days (ABX group). ABX mice were then orally gavaged with the fecal microbiota from 8-week-old female pair-fed mice or ETOH mice

(pooled from at least 3 cages) for 4 days (n = 5). (F) Detection of vitamin B_6 levels in intestinal and hepatic tissue. (G) Serum ALT and AST levels in 2 groups of mice after NIAAA modeling. Abbreviations: ABX, antibiotic treatment; FMT, fecal microbiota transplantation; NIAAA, National Institute on Alcohol Abuse and Alcoholism.

vitamin B_6 levels were significantly reduced in alcohol-fed mice, indicating that alcohol impairs normal vitamin B_6 synthesis (Figure 2D). To further demonstrate the role of gut microbiota in alcohol-induced vitamin B_6 production, we recolonized ABX mice with bacteria from ETOH or paired mice by fecal microbiota transplantation (Figure 2E). The levels of vitamin B_6 in both intestines and liver were significantly lower in ABX^{ETOH} mice compared to ABX^{pair} mice (Figure 2F). As expected, the ABX^{ETOH} mice exhibited markedly elevated levels of liver damage compared to the ABX^{pair} mice (Figure 2G). These results suggest that dysregulated gut microbiota contributes to vitamin B_6 deficiency in ALD mice.

Vitamin B_6 regulates amino acid metabolism in ALD

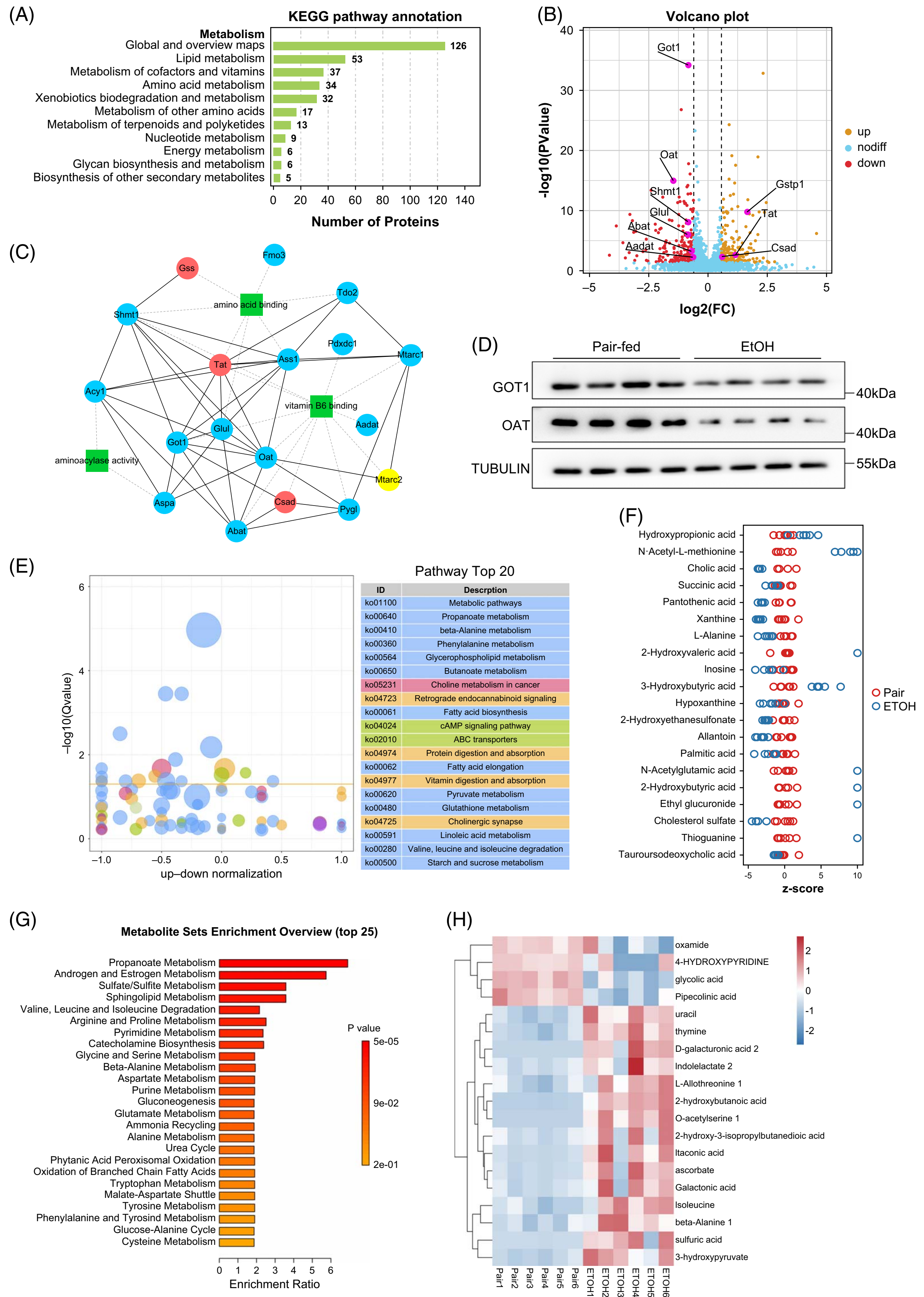
To investigate the role of vitamin B_6 metabolism in ALD, we performed proteomic analysis of liver samples from ALD and control mice. A heatmap and principal component analysis plot showed significant differences in liver protein abundance between ethanol-fed and pair-fed mice (Supplemental Figure S2A–C, http://links.lww.com/HC9/B150). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that ethanol treatment significantly affected lipid metabolism, cofactor and vitamin metabolism, and amino acid metabolism (Figure 3A). Given vitamin B_6's role as a coenzyme in amino acid metabolism, we focused on the amino acid metabolic pathway. Several proteins involved in both vitamin B_6 and amino acid binding were significantly altered in ethanol-fed mice, including glutamic-oxaloacetic transaminase 1, OAT (ornithine aminotransferase), and GSH s-transferases p1 (Figure 3B). Proteomics and metabolomics integration revealed a close relationship between amino acid metabolism and vitamin B_6 (Figure 3C). Western blot analysis confirmed the dysregulation of key proteins in alcohol-fed mice (Figure 3D). KEGG pathway analysis further indicated that amino acid metabolism, particularly GSH metabolism, is significantly impacted in ALD mice (Supplemental Figure S2D, E, http://links.lww.com/HC9/B150). Taken together, these results demonstrate that vitamin B_6 deficiency disrupts amino acid metabolism, contributing to ALD progression.

To assess the changes in liver metabolic profiles resulting from different dietary interventions, we performed liquid chromatograph-mass spectrometry on the liver samples. The Orthogonal Partial Least Squares Discrimination Analysis score map was used to represent the clustering of the samples, thereby validating the repeatability and reliability of the liver tissue metabolic spectrum in mice (Supplemental Figure S3A, http://links.lww.com/HC9/B150). Differential metabolite analysis revealed significant alterations in amino acid biosynthesis and metabolism in ethanol-fed mice (Supplemental Figure S3B, http://links.lww.com/HC9/B150). A Z-Score analysis was used to compare relative metabolite levels, showing that most of the differing metabolites were associated with amino acid and fatty acid metabolism, indicating a vital role for amino acid changes in ALD progression (Figure 3F). Pathway analysis visualized using a KEGG enrichment bubble diagram further confirmed these alterations in differential metabolites. The top 20 enriched pathways (Q-value < 0.05) predominantly involved alterations in amino acid metabolic pathways (Figure 3E). The results suggest that alcohol consumption not only disrupts lipid metabolism but also significantly impacts amino acid metabolism. Additionally, a joint analysis of metabolomics and microbiome showed a strong correlation between differential metabolites and microorganisms (Supplemental Figure S3D, http://links.lww.com/HC9/B150).

To further investigate amino acid changes in ethanol-fed mice, we performed serum metabolomics analysis. Using metabolite set enrichment analysis, we identified significant correlations between ALD and the degradation of valine, leucine, and isoleucine, as well as the metabolism of arginine, proline, glycine, and serine (Figure 3G). These amino acid changes were also visualized in a heat map (Figure 3H). Building on previous research, our study confirmed that ALD is driven not only by the harmful effects of alcohol metabolism but also by its impact on gut microbiota, which impair vitamin B_6 synthesis in patients with ALD and mice. In addition, vitamin B_6 plays a key role in metabolic pathways, including those involved in GSH production in the liver, by helping to restore alcohol-induced disorders in amino acid metabolism. This represents a significant advancement over previous studies, indicating that gut microbiota plays a crucial role in influencing host amino acid and GSH metabolism by affecting vitamin B_6 synthesis and metabolism.

Protective effect of vitamin B_6 against alcohol-induced liver injury

Based on these findings, we hypothesized that vitamin B_6 deficiency-driven amino acid metabolism disorder promotes ALD. To test this, we supplemented vitamin B_6 in a Lieber-DeCarli diet and observed its effect on National Institute on Alcohol Abuse and Alcoholism model mice. Vitamin B_6 supplementation significantly


(A)
KEGG pathway annotation
Metabolism
Global and overview maps 126
Lipid metabolism 53
Metabolism of cofactors and vitamins 37
Amino acid metabolism 34
Xenobiotics biodegradation and metabolism 32
Metabolism of other amino acids 17
Metabolism of terpenoids and polyketides 13
Nucleotide metabolism 9
Energy metabolism 6
Glycan biosynthesis and metabolism 6
Biosynthesis of other secondary metabolites 5
Number of Proteins
(B)
Volcano plot
-log10(PValue)
log2(FC)
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nodiff
down
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Abat
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Fmo3
Tdo2
amino acid binding
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Ass1
Mtarc1
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vitamin B6 binding
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Glul
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aminoacylase activity
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40kDa
55kDa
(E)
-log10(Qvalue)
up–down normalization
Pathway Top 20
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ko01100 Metabolic pathways
ko00640 Propanoate metabolism
ko00410 beta-Alanine metabolism
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ko00480 Glutathione metabolism
ko04725 Cholinergic synapse
ko00591 Linoleic acid metabolism
ko00280 Valine, leucine and isoleucine degradation
ko00500 Starch and sucrose metabolism
(F)
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N-Acetyl-L-methionine
Cholic acid
Succinic acid
Pantothenic acid
Xanthine
L-Alanine
2-Hydroxyvaleric acid
Inosine
3-Hydroxybutyric acid
Hypoxanthine
2-Hydroxyethanesulfonate
Allantoin
Palmitic acid
N-Acetylglutamic acid
2-Hydroxybutyric acid
Ethyl glucuronide
Cholesterol sulfate
Thioguanine
Tauroursodeoxycholic acid
z-score
Pair
ETOH
(G)
Metabolite Sets Enrichment Overview (top 25)
Propanoate Metabolism
Androgen and Estrogen Metabolism
Sulfate/Sulfite Metabolism
Sphingolipid Metabolism
Valine, Leucine and Isoleucine Degradation
Arginine and Proline Metabolism
Pyrimidine Metabolism
Catecholamine Biosynthesis
Glycine and Serine Metabolism
Beta-Alanine Metabolism
Aspartate Metabolism
Purine Metabolism
Gluconeogenesis
Glutamate Metabolism
Ammonia Recycling
Alanine Metabolism
Urea Cycle
Phytanic Acid Peroxisomal Oxidation
Oxidation of Branched Chain Fatty Acids
Tryptophan Metabolism
Malate-Aspartate Shuttle
Tyrosine Metabolism
Phenylalanine and Tyrosind Metabolism
Glucose-Alanine Cycle
Cysteine Metabolism
Enrichment Ratio
P value
5e-05
9e-02
2e-01
(H)
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4-HYDROXYPYRIDINE
glycolic acid
Pipecolinic acid
uracil
thymine
D-galacturonic acid 2
Indolelactate 2
L-Allothreonine 1
2-hydroxybutanoic acid
O-acetylserine 1
2-hydroxy-3-isopropylbutanedioic acid
Itaconic acid
ascorbate
Galactonic acid
Isoleucine
beta-Alanine 1
sulfuric acid
3-hydroxypyruvate
Pair1 Pair2 Pair3 Pair4 Pair5 Pair6 ETOH1 ETOH2 ETOH3 ETOH4 ETOH5 ETOH6

FIGURE 3 Proteomics and metabolomics reveal vitamin B_6 pathways in ALD intervention. Proteomic analysis was performed on liver samples from pair-fed and NIAAA model mice. (A) KEGG pathway analysis showed alterations in vitamin and amino acid metabolism pathways in ETOH mice. (B) Volcano Plot of dysregulated proteins in the livers of ALD mice. Key proteins in the vitamin B_6 binding pathway are labeled (blue: downregulated; red: upregulated; Log2 fold change > 1.5, $p < 0.05$). (C) Network diagram showing interactions between differentially expressed proteins and GO-enriched pathway related to the vitamin B_6 pathway and 2 amino acid-related pathways. (D) Western-blotting validation of the top 2 differentially expressed proteins (GOT1 and OAT) in the livers of pair-fed mice and ethanol-fed mice. (E) GO enrichment difference bubble map: the *y*-axis represents −log10 (Q-value), the *x*-axis represents the z-score, and the yellow line indicates the Q-value threshold of 0.05. The top 20 GO terms are listed to the right, with different colors representing different ontologies. (F) Z-score plot demonstrating disparities in metabolites between the 2 groups. (G) Metabolite Set Enrichment Analysis (MSEA) in serum. (H) Heatmap of differential serum metabolites. Abbreviations: GOT1, glutamic-oxaloacetic transaminase 1; KEGG, Kyoto Encyclopedia of Genes and Genomes; NIAAA, National Institute on Alcohol Abuse and Alcoholism; OAT, ornithine aminotransferase.

reduced serum ALT, AST, and the hepatic triglyceride levels in ethanol-fed mice (Figure 4A, B). Histological analysis and oil red staining confirmed that vitamin B_6 alleviated ethanol-induced liver steatosis and inflammation (Figure 4C). Flow cytometry revealed that hepatic neutrophil infiltration was significantly decreased after vitamin B_6 treatment in ethanol-fed mice (Figure 4D). Inflammation is a hallmark of ALD,[28] vitamin B_6 also reduces proinflammatory cytokines, such as IL-6 and IL-1β, in liver tissue (Figure 4E). Moreover, vitamin B_6 restored the expression of lipid metabolism-related genes, including peroxisome proliferator-activated receptor α and Sterol regulatory element-binding protein 1, in the liver (Figure 4E). Previous studies have demonstrated that oxidative stress plays an important role in the progression of ALD,[29] and our proteomic results reveal that the GSH metabolic pathway, which is enriched, is crucial to the oxidative stress response. To explore this further, we investigated whether vitamin B_6 could inhibit oxidative stress in ALD mice. The results indicated that the ethanol-fed mice exhibited decreased superoxide dismutase activity and elevated levels of malondialdehyde (MDA) and 4-Hydroxynonenal. In contrast, vitamin B_6 inhibited oxidative stress by increasing superoxide dismutase levels and reducing MDA and 4-Hydroxynonenal levels in ethanol-fed mice (Figure 4F, G). Additionally, western blot analysis confirmed that the dysregulation of glutamic-oxaloacetic transaminase 1 and OAT in ETOH mice was reversed by vitamin B_6 treatment (Figure 4G). These data indicate that vitamin B_6 protects against alcohol-induced liver damage by modulating amino acid metabolism and oxidative stress.

Vitamin B_6 alleviates liver oxidative stress by restoring amino acid metabolism

To further explore the protective mechanism of vitamin B_6 in ALD, we performed serum metabolomics analysis. The data showed that vitamin B_6 altered amino acid metabolism, increasing levels of tyrosine, proline, serine, methionine, alanine, and other amino acids in ethanol-fed mice (Figure 5A–C). Real-time quantitative fluorescent PCR confirmed that vitamin B_6 restored the dysregulated expression of key genes in ethanol-fed mice (Figure 5D). Consistent with metabolomics results, amino acid content in the liver, such as GSH and proline, was significantly reduced in ALD mice and worsened by vitamin B_6 deficiency. However, vitamin B_6 supplementation restored these levels (Figure 5E). Immunohistochemical staining of MDA in the liver sections also showed that vitamin B_6 reduced oxidative stress, while its deficiency exacerbated MDA production (Figure 5F). Taken together, these results suggest that vitamin B_6 protects the liver by attenuating oxidative stress during ethanol-induced liver injury.

Vitamin B_6 promotes GSH synthesis through OAT to reduce alcohol-induced liver injury

Proteomic analysis revealed that OAT expression was downregulated in ALD mice. OAT catalyzes the conversion of ornithine to glutamate, which plays a key role in glutamate metabolism and GSH synthesis.[30,31] Given the importance of GSH in oxidative stress, we hypothesized that vitamin B_6 mitigates oxidative stress by restoring OAT expression and promoting GSH synthesis. To test this, we used specific $Oat^{\Delta Hep}$ and control littermates ($OAT^{flox/flox}$) to establish an ALD model using a standard Lieber-DeCarli diet with increased vitamin B_6. In $Oat^{\Delta Hep}$ mice, ethanol-induced liver damage was exacerbated and could not be ameliorated by vitamin B_6 supplementation (Figure 6A). Through pathological staining of liver tissue and the measurement of hepatic triglyceride levels across groups, we consistently observed that $Oat^{\Delta Hep}$ mice exhibited increased steatosis and inflammation compared to the control group. Notably, vitamin B_6 failed to exert its therapeutic effect on $Oat^{\Delta Hep}$ mice (Figure 6B and C). Based on this observation, we analyzed the hepatic tissue samples from each group to assess oxidative stress by measuring GSH and MDA levels. Our findings revealed that genetic ablation of OAT impaired the restorative effects of vitamin B_6 on GSH levels, leading to a significant increase in oxidative

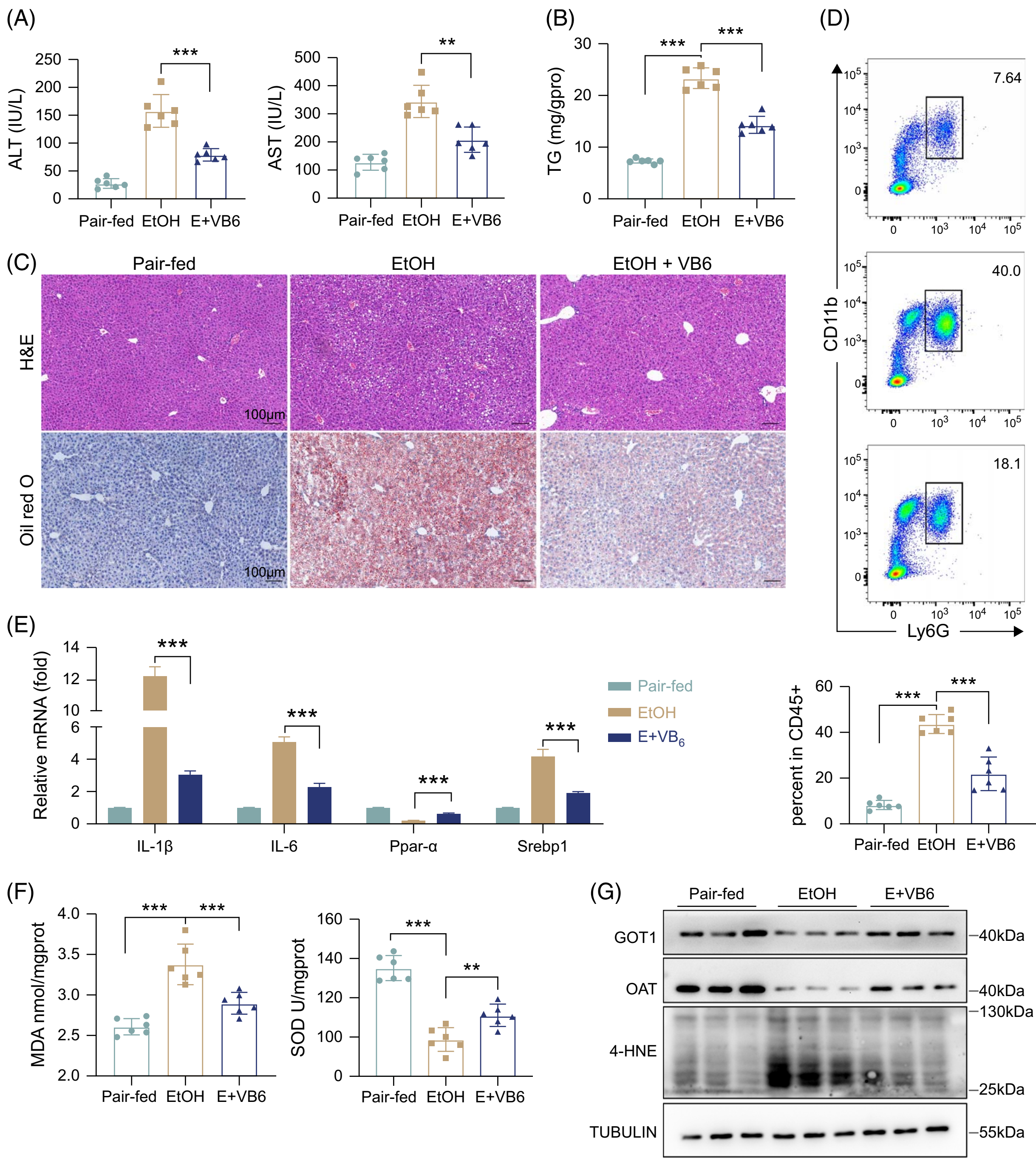

FIGURE 4 Validation of the protective effects of vitamin B_6 on ALD in mouse model. The experimental mice were divided into 3 groups: control group (pair-fed), ethanol-fed group (EtOH), and ethanol-fed with high-dose vitamin B_6 group (E+VB_6) (n = 6 per group). (A, B) Serum levels of ALT and AST (A), and liver tissue level of TG (B) from pair-fed, EtOH, and E+VB_6 group. (C) Representative images of H&E and Oil-Red O staining of liver tissues (scale bar = 100 μm). (D) Flow cytometry analysis of infiltrating neutrophils (CD45+CD11b+Ly6G+) in experimental mice. (E) mRNA expression levels of proinflammatory cytokines and lipid metabolism-related genes. (F) Levels of MDA and SOD in liver tissues. (G) Expression levels of GOT1, OAT, and 4HNE in liver tissue were detected by western blot. Abbreviations: GOT1, glutamic-oxaloacetic transaminase 1; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; OAT, ornithine aminotransferase; SOD, superoxide dismutase; TG, triglyceride.

stress damage in $Oat^{\Delta Hep}$ mice (Figure 6D). These results suggest that vitamin B_6 protects against oxidative stress-induced liver damage by enhancing GSH synthesis through the glutamate metabolism pathway via OAT regulation.

Gut microbiota dysbiosis and decreased vitamin B_6 levels in patients with ALD

To investigate differences in gut microbiota composition and vitamin B_6 levels between patients with ALD and

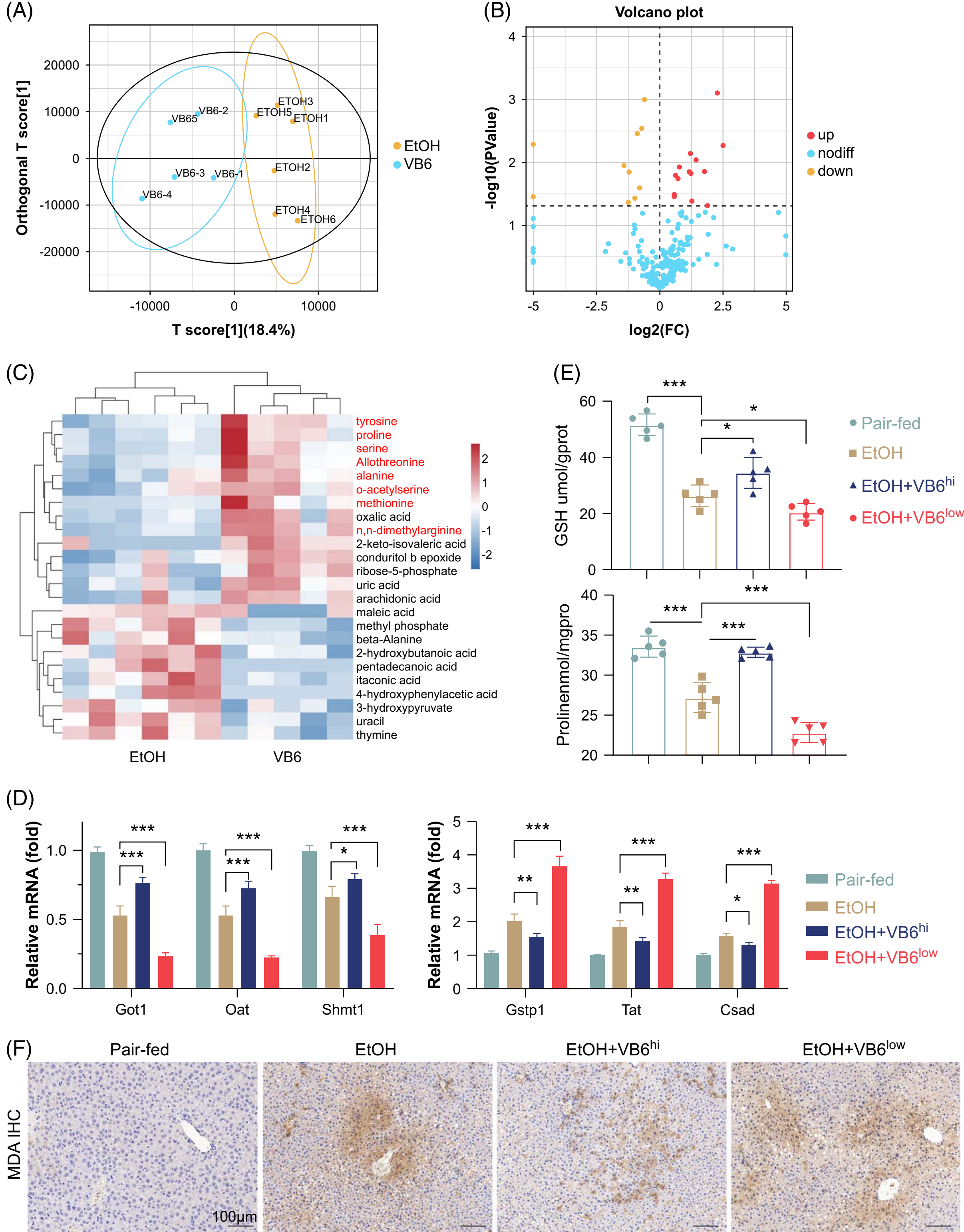

FIGURE 5 Vitamin B_6 protects against liver injury by restoring amino acid metabolism. (A) Principal component analysis of serum metabolism in ETOH and E+VB_6 mice (n = 5–6 per group). (B, C) Volcano plot (B) and heatmap (C) of differential metabolites in serum. (D) Verification of mRNA expression of predicted genes. (E) Determination of GSH and proline content in liver tissue of each group. (F) Representative images of MDA IHC staining in liver tissues (scale bar = 100 μm). Abbreviations: GOT1, glutamic-oxaloacetic transaminase 1; GSH, glutathione; GSTP1, glutathione S-transferase P1; IHC, immunohistochemistry; MDA, malondialdehyde; OAT, ornithine aminotransferase.

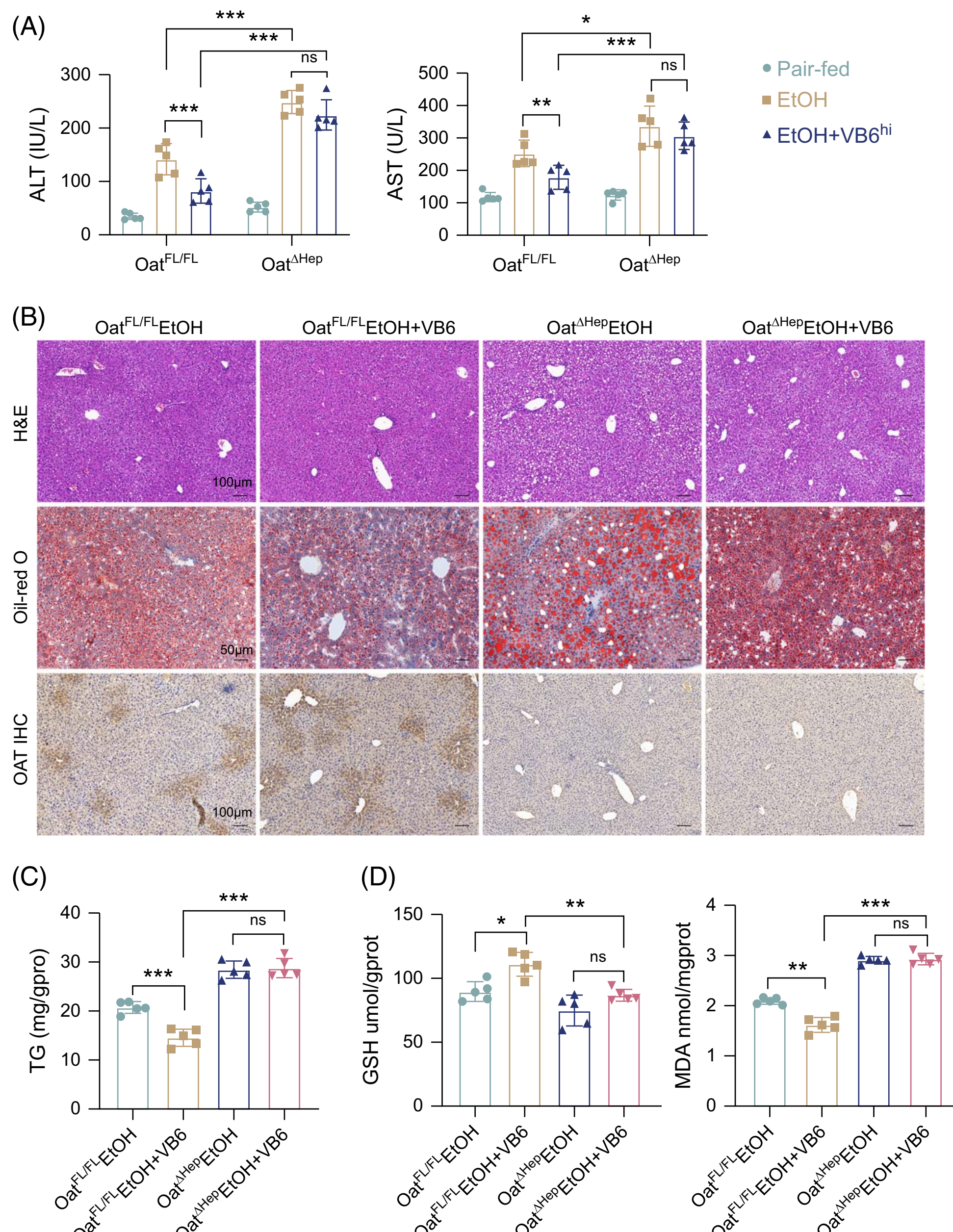

FIGURE 6 Oat gene ablation exacerbates alcohol-induced liver oxidative stress. (A) Levels of ALT and AST in the serum across groups. (B) Representative images of H&E, Oil-Red O, and OAT IHC staining in liver tissues. (C, D) Determination of TG (C), GSH, and MDA (D) content in liver tissue of each group (n = 5). Abbreviations: GSH, glutathione; H&E, hematoxylin and eosin; IHC, immunohistochemistry; MDA, malondialdehyde; OAT, ornithine aminotransferase; TG, triglyceride.

healthy groups, we analyzed fecal samples from 11 patients with ALD and 13 healthy controls. KEGG pathway analysis showed significant differences in amino acid and vitamin metabolism between the 2 groups (Figure 7A). Analysis of bacterial diversity revealed distinct microbial compositions in patients with ALD compared to healthy controls (Figure 7B and Supplemental Figure S4A–D, http://links.lww.com/HC9/B150). At the genus level, relative abundance analysis showed that *Bacteroides, Bifidobacterium,* and *Klebsiella* were significantly more abundant in ALD group, while *Blautia*, *Faecalibacterium*, and *Agathobacter* were more abundant in healthy controls (Figure 7B). At the species level, the relative abundances of *Eubacterium rectale* and *Chlorobaculum parvum* declined, whereas *Bacteroides vulgatus* and *Bifidobacterium pseudocatenulatum* increased in the ALD group (Figure 7C, Supplemental Figure S4D, http://links.lww.com/HC9/B150). These results were consistent with findings from ALD mouse models, showing reduced abundance of *Blautia* and *Ruminococcaceae*, and increased levels of *Enterobacteriaceae* and *Lachnospiraceae*, which are

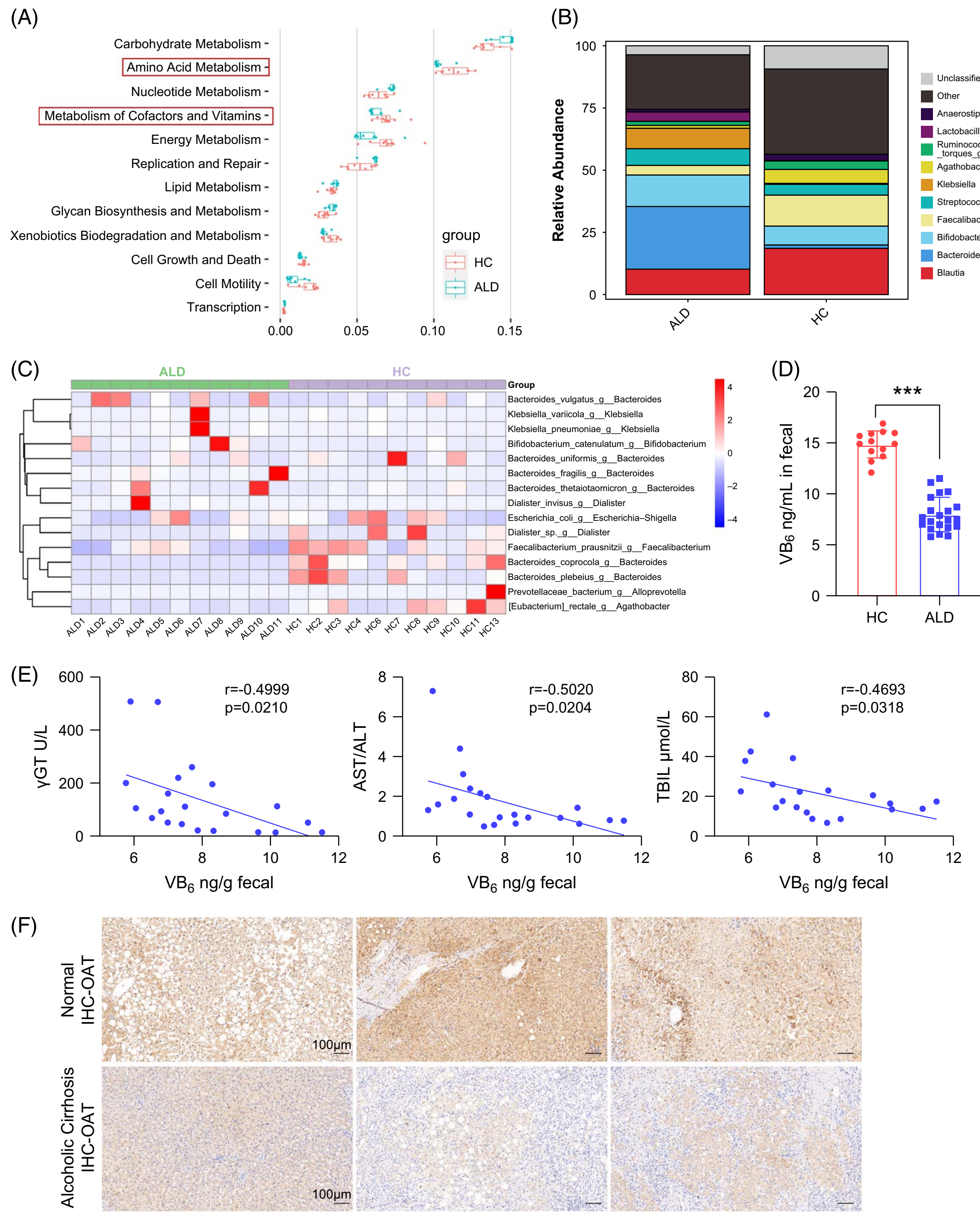

FIGURE 7 Revalidation of intestinal microflora disorders and reduced vitamin B_6 levels in patients with ALD. Fecal samples were collected from healthy controls and 11 patients who were diagnosed with ALD for 16S RNA sequencing and follow-up analysis. (A) Wilcoxon rank-sum test of 16S functional community profiling in patients with ALD to predict the biological functions of microbial communities. (B, C) Average relative abundances (B) and heatmap (C) of the microbial community at the genus level. (D) Vitamin B_6 levels in feces samples from healthy individuals and patients with ALD. (E) Correlation between vitamin B_6 concentrations and γGT, AST/ALT, and TBIL levels in all subjects (Spearman correlation). (F) Representative images of OAT IHC staining (scale bar = 100 μm). Abbreviations: ALD, alcohol-associated liver disease; γGT, γ-glutamyl transpeptidase; HC, healthy control; IHC, immunohistochemistry; OAT, ornithine aminotransferase.

closely associated with vitamin synthesis and metabolism.[32,33]

Clinical serum tests further confirmed reduced vitamin B_6 levels in patients with ALD, which were negatively correlated with biochemical indicators of liver damage, such as γ-glutamyl transpeptidase, AST, and AST/ALT levels (Supplemental Figure S5A–C, http://links.lww.com/HC9/B150). We then further measured vitamin B_6 levels in the fecal samples and found that patients with ALD had significantly lower levels of vitamin B_6 compared to healthy controls (Figure 7D). Additionally, intestinal vitamin B_6 levels in patients with ALD were negatively correlated with serum γ-glutamyl transpeptidase, AST/ALT, and total bilirubin levels (Figure 7E, Supplemental Figure S5C, http://links.lww.com/HC9/B150). Immunohistochemical analysis of liver tissue from patients with ALD revealed decreased OAT expression compared to normal liver tissues from patients with benign diseases (Figure 7F, Supplemental Figure S5D, http://links.lww.com/HC9/B150). These findings suggest that patients with ALD experience significant dysbiosis in their gut microbiota and reduced vitamin B_6 levels. Furthermore, the levels of vitamin B_6 and OAT expression in the liver may serve as potential indicators of the severity of alcohol-associated liver damage.

DISCUSSION

In this study, we integrated the microbiome, proteomics, and metabolomics analyses to construct a comprehensive multiomics landscape of ALD. This allowed us to better understand ALD pathogenies. We demonstrated that alcohol-induced gut microbiota dysregulation inhibits its vitamin B_6 synthesis and that the resulting vitamin B_6 deficiency, particularly in the liver, exacerbates amino acid metabolism disorders associated with ALD, thereby worsening ALD injury. We propose that vitamin B_6 plays a role in ALD by mitigating alcohol-induced oxidative stress through the maintenance of OAT expression in hepatocytes. Our findings are supported by both animal models and clinical samples.

Vitamin B_6 is a water-soluble vitamin essential for human health, existing in the body in the forms of pyridoxine, pyridoxal, and pyridoxamine. It plays multiple physiological roles, including acting as a cofactor for enzymes involved in amino acid metabolism, such as transaminases, decarboxylases, and dehydrases.[7] Meanwhile, it is essential for maintaining the normal functioning of the nervous and immune systems.[34,35] The liver is the major organ responsible for converting dietary vitamin B_6 to PLP, the active form of vitamin B_6. Within the liver, vitamin B_6 is involved in numerous enzymatic reactions, including those related to PPAR metabolism, amino acid metabolism, and several detoxification processes that are essential for liver function.[5,36] Vitamin B_6 is primarily absorbed in the jejunum, where it is converted from dietary sources into free vitamin B_6 by enzymes such as pyridoxal phosphatase before entering the bloodstream. Bacteria in the intestinal tract, particularly in the large intestine, can also produce vitamin B_6, and recent studies have identified specific bacteria species involved in its synthesis and metabolism, including *Mimosasporium fragile, Clostridium perfringens, Lactobacillus Clostridium fragilis, and Lactobacillus*, among others.

Clinical evidence suggests that chronic alcohol consumption is often associated with reduced plasma levels of vitamin B_6, possibly due to acetaldehyde-induced degradation of intracellular pyridoxal phosphate.[7] Since food intake and microbial synthesis are key sources of vitamin B_6 in mammals, it is worthwhile to explore whether these sources are diminished in witpatientsh ALD and to investigate the underlying mechanisms.[37] Alcohol is known to alter the composition of gut microbiota in both rodents and humans. However, it is not yet clear whether alcohol-induced gut microbiota imbalances affect vitamin B_6 availability. Recent evidence indicates that gut dysbiosis is linked to the severity of ALD.[16,38,39] Our research confirmed the negative impact of alcohol binge drinking on the gut microbiota of mice through amplicon sequencing analysis, showing a decrease in beneficial bacteria such as *Akkermansia muciniphila* and probiotics like *Lactobacillus*.[40] Conversely, harmful strains like *Escherichia coli*, which may exacerbate ALD, increased in abundance. These findings were further validated through microbiome analyses conducted on patients with ALD.

Through comprehensive analysis of 16sRNA sequencing results from patients and mice with ALD, we identified several bacteria associated with vitamin and amino acid production and metabolism. For instance, *Blautia* abundance was decreased in patients and mice with ALD, and this genus has been linked to vitamin D deficiency.[41] Additionally, there was an increase in the abundance of *Enterobacteriaceae*,[42] associated with vitamin D, and *Lachnospiraceae*,[43] associated with vitamin B_{12}, but a decrease in *Ruminococcaceae*, which play a key role in vitamin production and tryptophan metabolism.[34] Through in vivo experiments such as fecal microbiota transplantation, we demonstrated that reduced vitamin B_6 levels in the livers of ALD model mice were mediated by gut microbiota changes. Furthermore, vitamin B_6 levels in the feces of patients with ALD were negatively correlated with biochemical markers of liver injury. For the first time, we have shown that chronic alcohol consumption not only directly causes liver damage but also aggravates alcohol-associated liver injury due to insufficient vitamin B_6 production resulting from gut microbiota disturbances. While our study used a model of early alcohol-induced liver injury, it may not fully replicate the phenotype of advanced ALD in patients. Future research should consider chronic ethanol feeding models and focus on the dynamic changes in microflora and vitamin B_6 levels

in patients with advanced ALD, such as those with alcohol-associated cirrhosis or HCC.

Another significant finding of the current study is the critical role of amino acid metabolism in the progression of ALD. Multiple studies have confirmed that amino acid metabolism disorders are linked to various liver diseases, including HE and cirrhosis.[44] In these conditions, abnormal amino acid metabolism results in altered amino acid profiles in both blood and liver, contributing to disease progression. For example, cystine is a key precursor for GSH synthesis, which is essential for maintaining cellular redox balance, detoxification, and protection against oxidative stress.[45] Abnormal expression of cystine transporter SLC7A11/xCT has been linked to the progression of liver diseases such as hepatic carcinoma by regulating cystine and glutamate metabolism.[46,47] In liver diseases, dysregulated autophagy can further disrupt amino acid metabolism, impairing liver physiological function.[48,49] Through proteomics and metabolomics analyses, we identified amino acid metabolism-related pathways and differentially expressed proteins that may be influenced by vitamin B_6. Notably, the expression levels of these proteins, particularly OAT, were significantly altered in the liver of ALD mice but were restored after vitamin B_6 supplementation. These findings suggest potential therapeutic targets. Based on previous research and our experimental results, we demonstrate that OAT plays a crucial role in mediating the effects of vitamin B_6 on ALD progression. By participating in the glutamate metabolic network and supplying precursors for GSH synthesis, OAT helps alleviate alcohol-induced oxidative stress. However, further studies are needed to explore the specific molecular mechanisms underlying the effects of vitamin B_6 on these proteins and to investigate whether other metabolic enzymes involved in amino acid metabolism play additional roles in this context.

In conclusion, our study reveals that disruptions in gut microbiota during ALD progression lead to decreased vitamin B_6 production, which in turn exacerbates alcohol-induced amino acid metabolism disorders in the liver and worsens oxidative stress damage. Supplementation with vitamin B_6 may therefore represent a promising therapeutic strategy for ALD.

AUTHOR CONTRIBUTIONS

Haiyuan Shen performed most of the experiments and drafted the manuscript. Liangliang Zhou and Yuanru Yang performed most of the experiments and analyzed the 16S rDNA, DIA, and metabolomics data. Hang Shu, Dongqing Wu, Simin Yang, Linxi Xie, Lei Yang, Shanfei Tian, Xinru Zhang, Rui Ma, Ling Jiang, Man Jiang, Hao Zhang, Yan Wang, and Hejiao Zhang performed the experiments and analyzed the data. Shan Gao, Long Xu, and Hua Wang supervised the study and revised the manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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