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Compound probiotics mitigate alcohol-induced liver injury in mice by regulating the gut microbiota and TLR4/NF- κ B pathway

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ABSTRACT: Alcohol-related liver disease (ALD) is a worldwide disorder with limited treatment options. As a result, probiotics are emerging as a potential complementary therapy. This research investigated the efficacy of the compound probiotics (*Bifidobacterium animalis* subsp. *lactis* GOLDGUT-BB18 and *Lactobacillus acidophilus* GOLDGUT-LA100: BB18+LA100) on alleviating alcohol-induced liver damage in mice. The results indicated BB18+LA100 supplementation mitigated liver injury by normalizing transaminase levels, ameliorating lipid profiles, and reducing hepatic steatosis diminishing steatosis of liver. Simultaneously, BB18+LA100 supplementation inhibited pro-inflammatory cytokines while elevating IL-10 levels, and established redox homeostasis by elevating antioxidant defenses and diminishing lipid peroxidation. Transcriptome analyses revealed that BB18+LA100 attenuated alcohol-induced dysregulation, particularly by suppressing the TLR4/NF- κ B pathway, with efficacy comparable to silymarin. Importantly, RT-qPCR and western blot results further confirmed these transcriptomic finding, validating the inhibitory effect of BB18+LA100 on inflammatory gene and protein expression. Moreover, BB18+LA100 supplementation increased gut microbiota diversity and the abundance of beneficial taxa, modulated amino acid and carbon metabolism, and enhanced the synthesis of short-chain fatty acids (SCFAs). Collectively, BB18+LA100 exerts robust hepatoprotective effects against alcohol-induced liver injury by maintaining gut-liver axis homeostasis and coordinating microbiota-driven metabolic and inflammatory regulation, highlighting its potential as a probiotic-based strategy for the prevention and management of ALD.

Keywords: Alcohol-related liver disease, compound probiotics, TLR4/NF- κ B pathway, gut microbiota, liver injury

1. Introduction

Alcohol-related liver disease (ALD) is an important contributor to chronic liver disorders worldwide, causing a rising burden on public health and healthcare systems [1-2]. The pathophysiology of ALD encompasses a variety of conditions, such as alcoholic hepatitis and alcoholic fatty liver, as well as chronic hepatitis with fibrosis or cirrhosis, indicating a progressive disorder influenced by complex host-environment interactions [3]. The principal risk variables of ALD encompass alcohol intake, tobacco use, obesity, metabolic

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syndrome, viral hepatitis, and gender [4-5]. Inflammation, mitochondrial dysfunction, endoplasmic reticulum stress, nitrosative and oxidative stress, aberrant lipid metabolism, multiple forms of cell death, dysbiosis of the intestinal microbiota, immune dysregulation, and reduced autophagy are key processes linked to ALD that have been recently identified [6-7].

Current treatments for ALD predominantly include control alcohol consumption, nutritional therapy, and corticosteroid medication [6]. However, corticosteroids have limitations due to their restricted clinical use and moderate efficacy [8]. Recently, novel therapeutic approaches for ALD now focus on pathophysiological targets such as modulating innate immune responses, regulation of the gut microbiota, manipulation of bacteria-derived metabolites, and activation of liver regeneration [3,8]. Probiotics and their metabolites have been gaining growing attention as microbiota-targeted interventions due to their ability to restore microbial balance, improve intestinal barrier integrity, modulate immune responses, and decrease oxidative stress in animal models [9-10]. A number of researches demonstrate that probiotic intervention enhances liver function, alters gut microbiota composition, diminishes inflammatory markers, and improves lipid profiles in individuals with ALD [11]. While numerous probiotics are advantageous for ALD, their benefits are distinct strain-specific. Recent evidence suggests that multi-strain formulations may provide complementary or synergistic effects, resulting in enhanced and more consistent protection for disease. Therefore, examining combination probiotic strains is crucial for attaining more extensive hepatoprotective benefits.

Consequently, we investigated the implications of a probiotic combination, *Bifidobacterium animalis* subsp. *lactis* GOLDGUT-BB18 and *Lactobacillus acidophilus* GOLDGUT-LA100 (BB18+LA100), on alcohol-induced liver damage in mice. Employing a comprehensive methodology that incorporated biochemical, histological, transcriptomic and metagenomic analyses, we sought to discover whether BB18+LA100 supplementation could reduce oxidative stress, inflammation, lipid accumulation, and gut microbiota dysbiosis, thereby attenuating the progression of ALD. This research offers novel insights into the gut-liver axis and highlights the therapeutic potential of probiotics in the therapy of ALD.

2. Materials and methods

2.1 Chemicals and reagents

The Lieber-DeCarli liquid control and ethanol-containing diets were purchased from Xiaoshu Youtai Biotechnology Co., Ltd. (Beijing, China). Silymarin (S0292-10G) was obtained from Sigma-Aldrich, purchased via Shanghai Iright Technology Co., Ltd. (Shanghai, China). Commercial assay kits were purchased from Shanghai Xinyu Biotechnology Co., Ltd. (Shanghai, China) and utilized to determine: (1) inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, IL-10, and lipopolysaccharide (LPS); (2) liver injury biomarkers, comprising alanine aminotransferase (ALT) and aspartate aminotransferase (AST); (3) oxidative stress indicators, encompassing malondialdehyde (MDA) and glutathione (GSH); and (4) lipid profiles, including total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). The total RNA extraction kit (G3640-50T), 2 $\times$ SYBR Green qPCR Master Mix (No ROX, G3320), and SweScript All-in-One RT

SuperMix for qPCR (One-Step gDNA Remover, G3337) were obtained from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China). The Phosphate-Buffered Saline (PBS) (1×, pH 7.2-7.4) was acquired from Shanghai Share-Bio Biotechnology Co., Ltd. (Shanghai, China). The Radioimmunoprecipitation Assay Lysis Buffer (RIPA buffer), Ultra ECL Chemiluminescent Substrate Kit, One-Step PAGE Color Gel Preparation Kit (10%), 10× Tris-Buffered Saline with Tween 20 (TBST) Buffer, Protein-Free Rapid Blocking Buffer, and 5× Protein Loading Buffer (Reducing) for western blot analysis have been obtained from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China). Primary antibodies against P65 (Cat. No. 80979-1-RR) and TLR4 (Cat. No. 19811-1-AP) were used as target protein antibodies, while β -actin (Cat. No. 66009-1-Ig) served as the internal control. The HRP-conjugated goat anti-rabbit IgG secondary antibody was employed for signal detection. All antibodies were obtained from Wuhan Sanying Biotechnology Co., Ltd. (Wuhan, China). Sodium chloride, n-hexane, sulfuric acid, and anhydrous sodium sulfate were acquired from Xilong Scientific Co., Ltd. (Guangdong, China).

2.2 Bacterial strains and culture

B. animalis subsp. *lactis* GOLDGUT-BB18 and *L. acidophilus* GOLDGUT-LA100 were cultivated anaerobically in De Man Rogosa and Sharpe (MRS) broth supplemented with 0.1% cysteine. Bacterial cells were collected by centrifugation, washed thrice with sterile 0.9% saline, and subsequently resuspended in saline. The cell suspensions were adjusted to 10^9 CFU/mL. Suspensions of *B. animalis* subsp. *lactis* GOLDGUT-BB18 and *L. acidophilus* GOLDGUT-LA100 were mixed at a 1:1 ratio.

2.3 Animal experiment design and treatment

Fig. 1A illustrates that forty male C57BL/6 mice (8 weeks old, 20 ± 2 g) were acquired from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China) and housed under controlled conditions (12-hour light/dark cycle, 25 ± 2 °C, $60 \pm 5\%$ relative humidity). All animal procedures carried out according to international ethical standards and obtained authorization the Animal Ethics Committee of Hainan University (HNUAUCC-2024-00300). Subsequent to a one-week acclimatisation period, mice had been randomly assigned to four distinct groups ($n = 10/\text{group}$): control (Ctrl), ALD model (Alc), silymarin-treated (Sily), and probiotic-treated (BB18+LA100). All mice, with the exception of the Ctrl group, were sustained on a Lieber-DeCarli liquid control diet, followed by a one-week acclimatization to a Lieber-DeCarli liquid containing ethanol diet before the intervention. The duration of the experiment was four weeks. Throughout this period, the Ctrl and Alc groups were administered daily oral gavage of normal saline, the Sily group received silymarin (100 mg/kg BW) ^[12], and each mouse for the BB18+LA100 group was administered a total dose of 10^9 CFU per day by oral gavage ^[13]. Body weight, food intake, and water intake were recorded on a weekly schedule. Subsequent to the completion of the investigation, mice were euthanised, alongside liver, colon, cecal contents, abdominal adipose tissue, and blood have been collected. Cecal contents samples were stored at -80°C for SCFAs analysis. The liver and abdominal adipose tissue were weighed to calculate the liver index and fatty index, respectively. Serum has been extracted through centrifugation at 4000

rpm for 15 min. The sections of the liver and colon were preserved in a universal tissue fixative for 24 h for histological examination. The residual tissues and serum were preserved at -80°C for future examination.

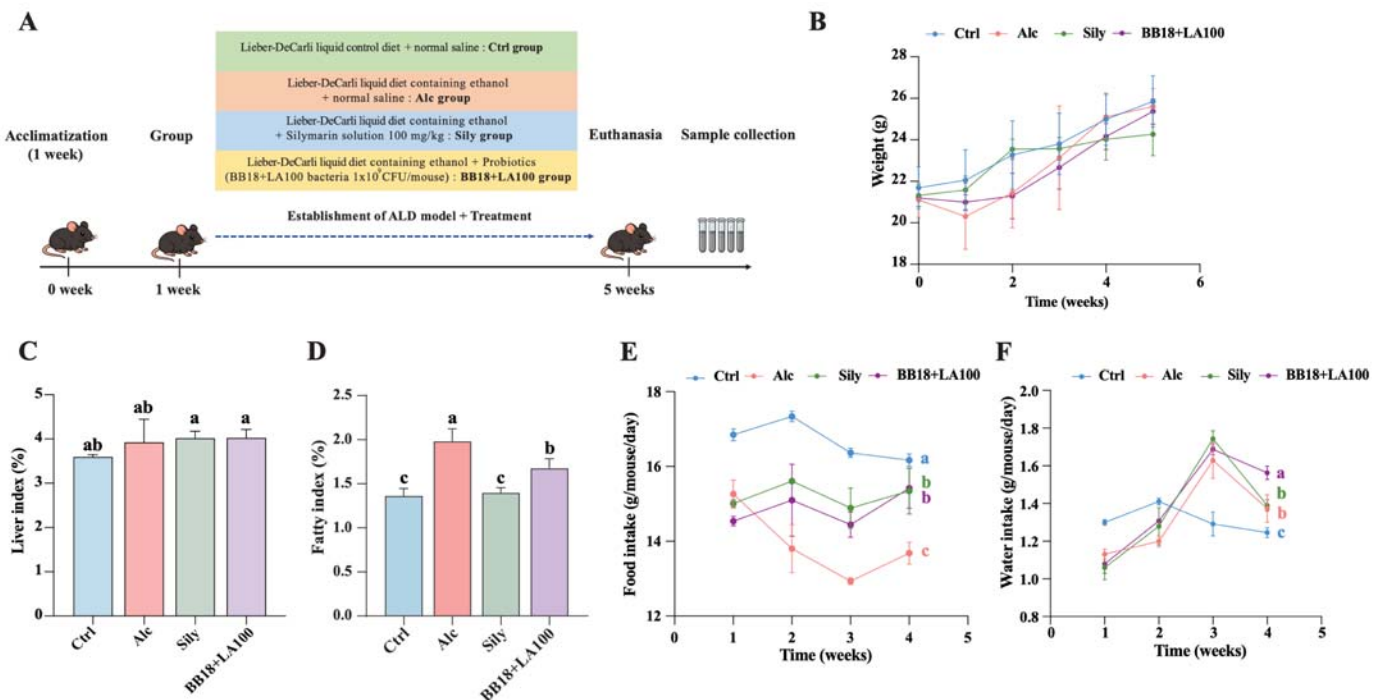


Fig. 1 Experimental animal for ALD model (A); General physiological parameters in mice: changes in body weight (B); Liver index (C); Fatty index (D), Food intake (E); Water intake (F). Data are expressed as mean $\pm$ SD. Different letters above bars indicate significant differences among groups ($P < 0.05$).

2.4 Biochemical indicators determination

Serum samples were analyzed for inflammatory cytokines, comprising $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-6 , IL-10 , and LPS. Liver tissues were used to examine oxidative stress parameters, including MDA and GSH. Liver injury markers, including ALT and AST and lipid profiles including TC, TG, LDL-C, and HDL-C were measured from both serum and liver samples. All biochemical indicators were determined with commercial assay kits following the manufacturer's guidelines.

2.5 Histopathological analysis

After being preserved in 4% paraformaldehyde at 4°C for 24 h, the liver and colon tissues were embedded in paraffin and dissected into $5\text{ }\mu\text{m}$ sections. Hematoxylin and eosin (H&E) staining was employed to stain the sections for morphological evaluation. For the purpose of identifying the buildup of lipid droplets, the liver tissue was stained with Oil Red O [12]. The IX71 microscope system (Olympus, Tokyo, Japan) was employed to acquire images, and the ImageJ software was employed to analyze the percentage area of lipid droplets [14].

2.6 Liver transcriptome sequencing and analysis

Total RNA was extracted from liver tissues of mice in the different treatment groups using Trizol reagent (Savio Biotechnology Co., Ltd., Wuhan, China). The beginning measurement of RNA quantity and purity was performed with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of

RNA was assessed through agarose gel electrophoresis. The RNA quality (RQN) was subsequently evaluated using an Agilent 5300 instrument to confirm its suitability for subsequent library preparation. RNA library construction included mRNA enrichment, fragmentation, first- and second-strand cDNA synthesis, end repair, adaptor ligation, and PCR amplification. After quality assessment, the final library was paired-end sequenced using the Illumina NovaSeq platform (Illumina, San Diego, CA, USA). Raw sequencing data was subjected to quality control, which involved the removal of adapters and the filtering of low-quality reads in order to produce clean, high-quality readings. DESeq2 R package (version 1.16.1) was employed to conduct differential expression analysis. The criteria for screening differentially expressed genes (DEGs) were $|\log_2 \text{Fold Change}| > 1.2$ and $P < 0.05$. In order to identify metabolic and signaling pathways that were substantially enriched ($P < 0.05$), KEGG pathway enrichment analysis of the identified DEGs was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>).

2.7 RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

Animal RNA extraction kits (Saviez Biotechnology Co., Ltd., Wuhan, China) were implemented to extract total RNA from mouse liver tissue. Subsequently, a commercial reverse transcription kit was implemented to convert the extracted RNA into cDNA. Key inflammatory-related genes, such as Toll-like receptor 4 (*TLR4*), C-C motif chemokine ligand 5 (*CCL5*), myeloid differentiation primary response 88 (*MYD88*), and nuclear factor kappa B (*NF-κB*), which displayed notable enrichment in the liver transcriptome analysis, were measured for expression levels using RT-qPCR. RT-qPCR was conducted with cDNA as a template in a 20 µL reaction mixture comprising 10 µL of 2× Universal Blue SYBR Green qPCR Master Mix, 7.2 µL of nuclease-free water, 0.4 µL of both forward and reverse primers, and 2 µL of cDNA. The amplification procedure comprised an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 30 s. Relative mRNA expression levels were quantified using the 2-ΔΔCT technique. The sequences of primers are displayed in Table S1.

2.8 Western blot analysis

Western blot analysis was performed to evaluate TLR4 and NF-κB proteins expression. After the homogenization in RIPA buffer and rinsed with cooled 1× PBS, liver tissues were placed on ice for 30 minutes. Following centrifuging the lysates at 12,000 rpm for 10 min at 4 °C, the supernatants were collected. 5× Protein Loading Buffer was added to each sample, followed by denaturation at 95 °C for 10 min. Equal quantities of protein were resolved by SDS-PAGE with the one-step PAGE color gel preparation kit and subsequently transferred to PVDF membranes (200 mA, 45 min). Protein-free rapid blocking agent was employed to block the membranes for 1 h at room temperature. These membranes were then incubated with primary antibodies overnight at 4°C, and secondary antibodies were incubated at room temperature. After washing three times with 10× TBST Buffer, the membranes were developed using the ultra ECL chemiluminescent substrate kit. Grayscale analysis was employed to quantify band intensities using ImageJ software ^[14].

2.9 Shot-gun metagenomics sequencing and analysis

Microbial DNA from fecal samples was extracted using the Qiagen DNeasy PowerSoil Kit (QIAGEN LLC, Germantown, MD, USA) in compliance with the company's instructions. Following the extraction of genomic DNA, a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Metagenomic libraries were constructed using the Illumina NEBNext Ultra DNA Library Preparation Kit and were sequenced by Shanghai Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China).

2.10 Examination of short-chain fatty acids (SCFAs)

The quantities of SCFAs in cecal samples were quantified using gas chromatography (GC). The 100 mg portion of freeze-dried cecal contents was blended using 1 mL of pre-chilled saturated NaCl solution, homogenized, and subsequently extracted using n-hexane. The mixture was centrifuged at 12,000 rpm for 15 min at 4°C, then the supernatant was collected and passed through a 0.22 µm membrane before analysis. SCFAs contents analysis was conducted using an Agilent 7890B GC system equipped with a flame ionization detector (FID) and a DB-FFAP capillary column (30 m × 0.25 mm × 0.25 µm, Agilent). The injection volume was 1 µL (split ratio 10:1), utilizing high-purity nitrogen as the carrier gas. The temperature protocol consisted of: 90 °C for 1 min, a ramp to 150 °C at an average of 10 °C per min, followed by a ramp to 200 °C at a rate of 5 °C per min, and a hold at 200 °C for 5 min. Acetic, propanoic, butyric, valeric, isobutyric, and isovaleric acids were identified and measured by retention times and external standard curves.

2.11 Statistical analysis

The SPSS 23.0 software was employed to conduct statistical analysis across all data, and the GraphPad Prism 8.0 software was employed to visualize the results. Data are displayed as the mean ± standard deviation (SD). Tukey's multiple comparison test was preceded by a one-way analysis of variance (ANOVA). A significance level of $P < 0.05$ has been considered statistically significant.

3. Results

3.1 Effect of BB18+LA100 intervention on physiological indicators in ALD mice

To evaluate the effects of BB18+LA100 intervention on physiological changes in ALD mice, body weight, liver index, fatty index, as well as food and water intake were monitored throughout the experimental period. Body weight progressively escalated in all groups throughout the experiment's duration (Fig. 1B). Despite the Alc group demonstrating a slightly lower trend, no statistically significant variations were seen among the groups in the last week ($P > 0.05$). The liver index was marginally elevated in the Alc group compared with the Ctrl group, whereas the BB18+LA100 group showed values comparable to the Sily group. However, no significant differences in liver index were observed among the groups (Fig. 1C), indicating that neither silymarin nor BB18+LA100 supplementation significantly affected overall growth or liver mass of ALD mice. In contrast, the fatty index was markedly increased in the Alc group ($P < 0.05$) (Fig. 1D), reflecting alcohol-induced adiposity. BB18+LA100 supplementation significantly reduced the fatty index by 15.66% relative to the Alc group ($P < 0.05$).

Regarding food intake, mice fed the Lieber-DeCarli diet exhibited a progressive reduction in food consumption in the Alc group, which became significantly lower than that of the other groups during the final experimental week ($P < 0.05$). In contrast, BB18+LA100 supplementation partially restored food intake compared with the Sily group (Fig. 1E). Conversely, an inverse trend has been observed for water consumption. Prolonged Lieber-DeCarli feeding significantly elevated water consumption in the Alc, Sily, and BB18+LA100 groups, with the most pronounced effect shown in the final week, during which all three groups had markedly higher water consumption compared to the Ctrl group ($P < 0.05$). The BB18+LA100 intervention visibly reduced water consumption compared to the Alc group and exhibited a more moderate restorative effect relative to the Sily group (Fig. 1F). Collectively, these results suggest that BB18+LA100 supplementation successfully mitigated alcohol-induced adiposity, preserved regular growth metrics, and attenuated behavioural modifications in food and water consumption in ALD mice.

3.2 Effect of BB18+LA100 intervention on oxidative stress and inflammation response in ALD mice

Chronic alcohol feeding significantly reduced liver GSH levels and increased MDA concentrations in the Alc group compared with the Ctrl group ($P < 0.05$) (Fig. 2A-B). BB18+LA100 supplementation significantly increased liver GSH levels, surpassing those of the Ctrl group and reaching levels comparable to the Sily group (Fig. 2A). Meanwhile, the BB18+LA100 intervention markedly decreased liver MDA levels in comparison to the Alc group; however, MDA levels remained to be high in comparison to those in the Sily group ($P < 0.05$) (Fig. 2B). Simultaneously, alcohol exposure markedly elevated the levels of the pro-inflammatory cytokines IL-6, TNF- α , and IL-1 β (Fig. 2C-E), while simultaneously suppressing the anti-inflammatory cytokine IL-10 (Fig. 2F). BB18+LA100 supplementation effectively reversed these alterations. Specifically, IL-6 was significantly reduced to values comparable to the Sily group (Fig. 2C), whereas TNF- α showed no significant difference with the Alc group (Fig. 2D). Notably, IL-1 β were significantly lower in comparison to the Sily group ($P < 0.05$) and returned to levels similar to the Ctrl group (Fig. 2E).

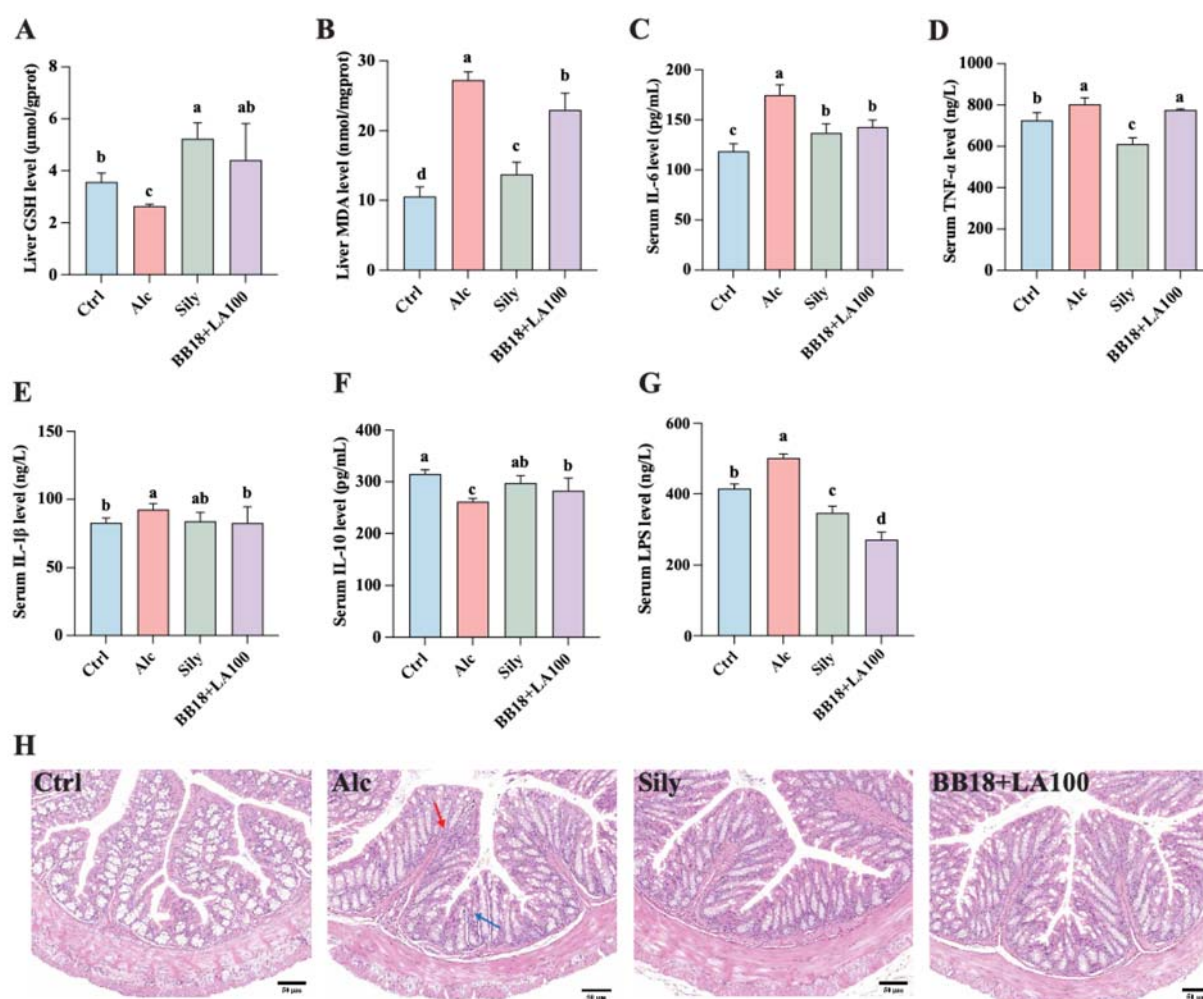


Fig. 2 Effects of BB18+LA100 on oxidative stress, inflammatory cytokines, endotoxin levels, and intestinal histology in ALD mice. Liver reduced glutathione (GSH) (A); Liver malondialdehyde (MDA) (B); Serum IL-6 (C); Serum TNF-α (D); Serum IL-1β (E); Serum IL-10 (F); Serum lipopolysaccharide (LPS) (G); Histological images of colon tissues stained with H&E (scale bar = 50 μm) (H). Red and blue arrows indicate epithelial damage and inflammatory cell infiltration, respectively. Data are expressed as mean ± SD. Different letters above bars indicate significant differences among groups ($P < 0.05$).

Moreover, BB18+LA100 treatment upregulated IL-10 production to a level comparable to the Sily group (Fig. 2F). In parallel, BB18+LA100 treatment markedly suppressed alcohol-induced endotoxemia, as evidenced by significant reduced serum LPS level, which was lower than that observed in the Sily group ($P < 0.05$, Fig. 2G). Moreover, the histological analysis of the ileum revealed severe villus damage, epithelial shedding, inflammatory infiltration, and reduced goblet cell numbers in the Alc group. In contrast, BB18+LA100 supplementation markedly preserved villus architecture and goblet cell abundance (Fig. 2H). Overall, these findings demonstrate that BB18+LA100 effectively attenuated alcohol-induced oxidative stress and inflammatory responses by restoring redox homeostasis, rebalancing pro- and anti-inflammatory cytokines, suppressing endotoxemia, and preserving intestinal barrier integrity in ALD mice.

3.3 Effect of BB18+LA100 intervention on liver injury in ALD mice

To further assess the protective efficacy of the BB18+LA100 intervention against liver injury, histological alterations in the liver were analysed by H&E staining. The Ctrl group demonstrated normal liver

architecture, marked by preserved cellular organisation and an absence of hepatocellular degeneration or necrosis. Conversely, the Alc group had significant pathological changes, including hepatocyte ballooning, cytoplasmic vacuolation, and infiltration of inflammatory cells (Fig. 3A). In comparison to the Alc group, both Sily and BB18+LA100 supplementation significantly diminished these histological abnormalities, as indicated by decreased hepatocellular degeneration, necrosis, and cellular vacuolation.

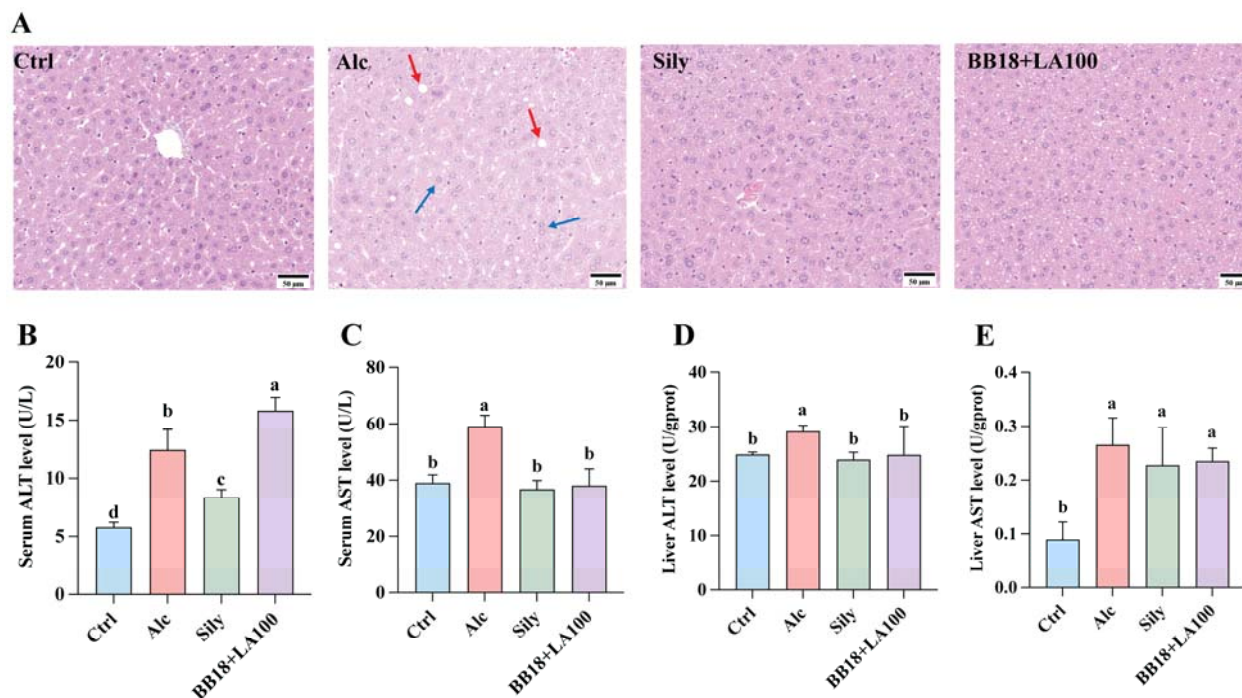


Fig. 3 Effects of BB18+LA100 on liver injury markers in in ALD mice. Histological images of H&E-stained liver section (scale bar = 50 μ m; hepatocyte ballooning (red arrows), cytoplasmic vacuolation, and inflammatory infiltration (blue arrows) (A); Serum alanine aminotransferase (ALT) (B); Serum aspartate aminotransferase (AST) (C); Liver ALT (D); Liver AST (E). Data are expressed as mean $\pm$ SD. Different letters above bars indicate significant differences among groups ($P < 0.05$).

Additionally, liver injury indicators, including ALT and AST activities in both serum and liver tissues, were assessed (Fig. 3B-E). Serum ALT and AST levels were significantly elevated in the Alc group compared with Ctrl ($P < 0.05$) and were decreased in treatment groups. Serum ALT significantly increased in the Alc group (12.46 ± 1.75 U/L) compared with the Ctrl group (5.79 ± 0.44 U/L). The Sily group showed a moderate elevation (8.36 ± 0.66 U/L), whereas BB18+LA100 supplementation further increased ALT (15.82 ± 1.14 U/L). Serum AST levels were markedly elevated in the Alc group (59.13 ± 3.90 U/L) relative to the Ctrl group (39.05 ± 2.84 U/L), while both Sily (36.77 ± 3.12 U/L) and BB18+LA100 (38.05 ± 5.92 U/L) exhibited values comparable to the Ctrl group (Fig. 3B-C). The Alc group exhibited a significant increase in liver ALT activity (29.17 ± 1.09 U/gprot, $P < 0.05$) relative to the Ctrl group (24.90 ± 0.46 U/gprot), but normalized in the BB18+LA100 (24.86 ± 5.22 U/gprot) and Sily (23.94 ± 1.37 U/gprot) groups (Fig. 3D). Similarly, liver AST activity rose in the Alc group (0.27 ± 0.05 U/gprot; $P < 0.05$) but decreased in both BB18+LA100 (0.24 ± 0.02 U/gprot) and Sily (0.22 ± 0.07 U/gprot) groups, with no significant difference between treatments (Fig. 3E). Taken together, these results demonstrate that BB18+LA100 effectively alleviated alcohol-induced liver injury, as evidenced by marked improvements in liver histopathology and normalization of serum and liver transaminase activities in ALD mice.

3.4 Effect of BB18+LA100 intervention on lipid accumulation in ALD mice

To further investigate the impact of BB18+LA100 on lipid accumulation and lipid profiles, liver lipid deposition was evaluated using Oil Red O staining, while TC, TG, LDL-C, and HDL-C levels were measured in both serum and liver tissues (Fig. 4). Fig. 4A displays liver slices stained with Oil Red O. The Ctrl group exhibited a typical liver structure with no detectable lipid droplet accumulation. In contrast, the mice in Alc group demonstrated significant liver steatosis, with numerous lipid droplets present in hepatocytes. In the treatment groups, the Sily group reduced lipid droplets accumulation, nonetheless, residual vacuoles were evident. The BB18+LA100 group markedly reduced lipid droplets, restoring liver architecture to levels comparable to the Ctrl group. The quantitative analysis of Oil Red O-staining indicated an elevated level in liver lipid droplet area in the Alc group ($3.10 \pm 0.82\%$), markedly exceeding levels in all other groups ($P < 0.05$, Fig. 4B). The Sily treatment diminished the lipid droplet area to $1.19 \pm 0.15\%$, instead BB18+LA100 intervention restored lipid droplet area to $0.46 \pm 0.31\%$. More importantly, the examination of the lipid profile corroborated these findings (Fig. 4C-J).

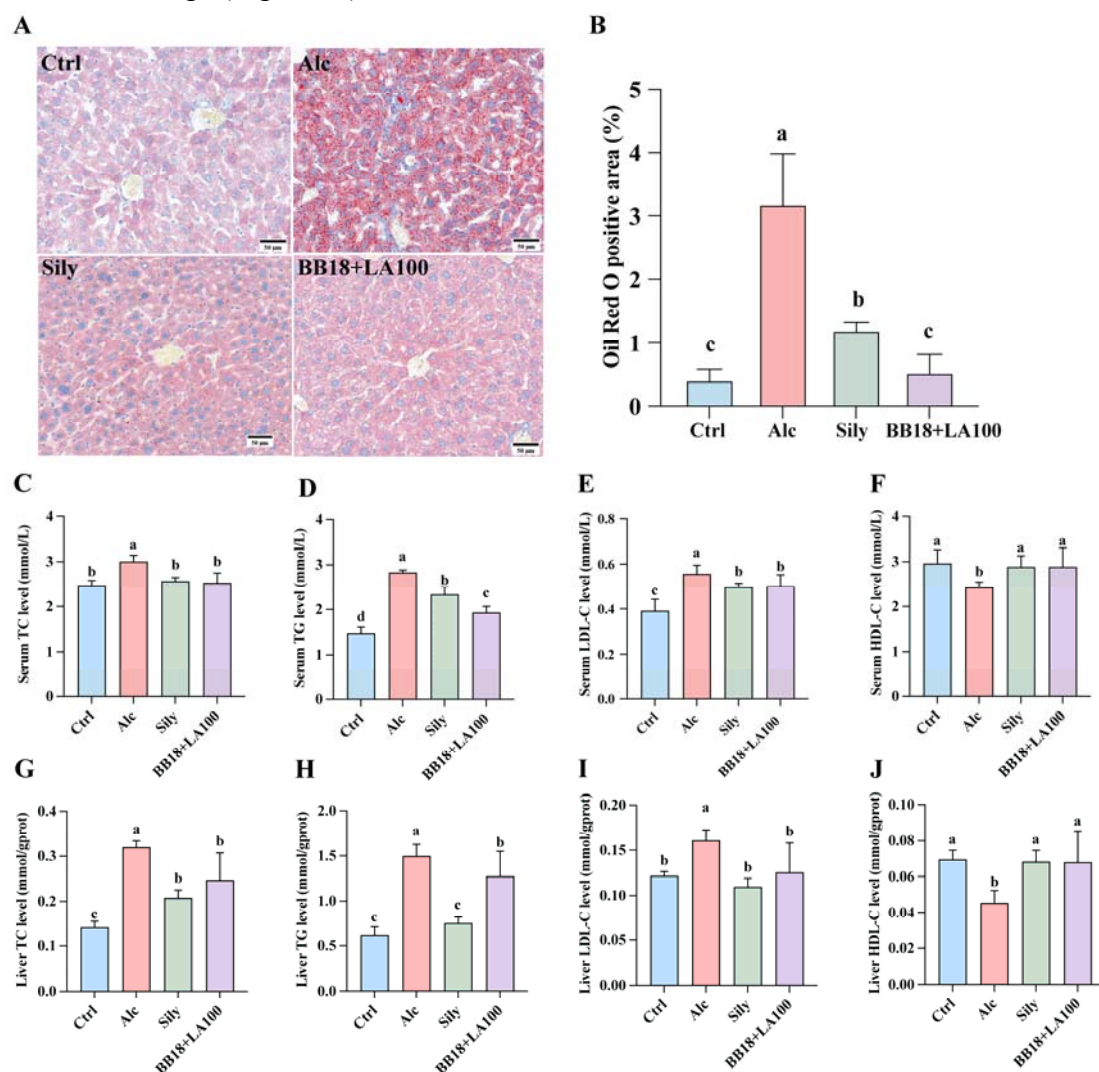
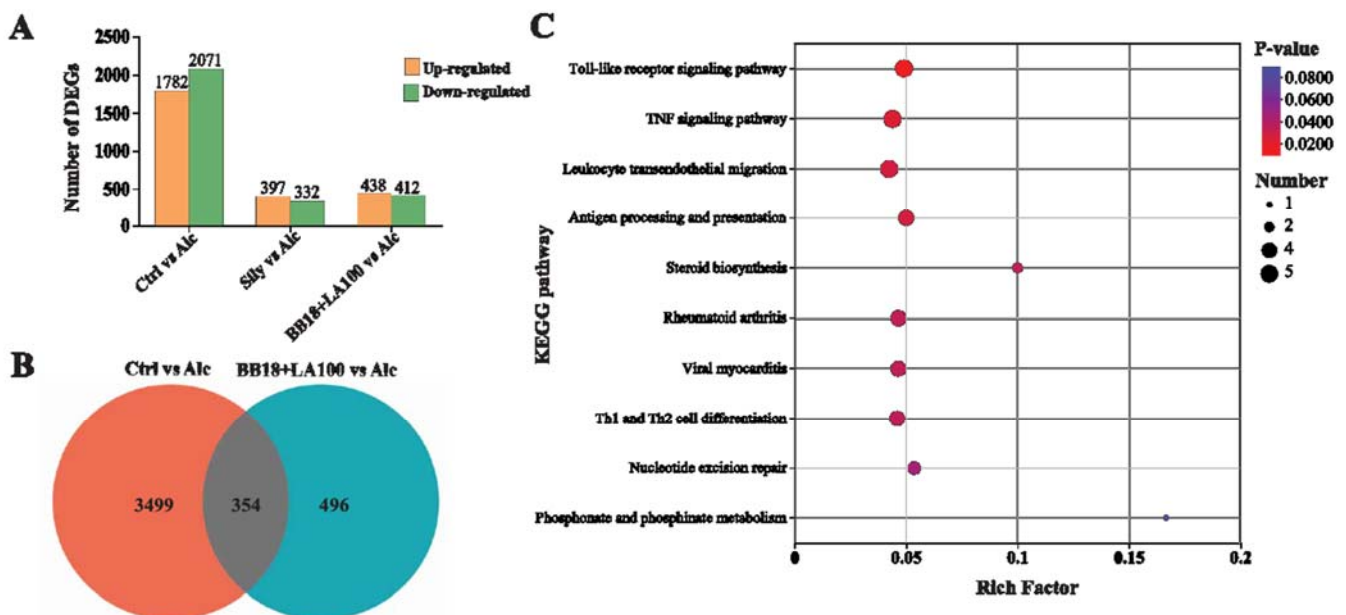


Fig. 4 Effects of BB18+LA100 on liver lipid accumulation and lipid metabolism in ALD mice. Oil Red O-stained liver sections (scale bar = 50 μ m) (A); Quantitative analysis of Oil Red O-positive area (B); Serum total cholesterol (TC) (C); Serum total triglycerides (TG) (D); Serum low-density lipoprotein cholesterol (LDL-C) (E); Serum high-density lipoprotein cholesterol (HDL-C) (F); Liver TC (G); Liver TG (H); Liver LDL-C (I); Liver HDL-C (J). Data are expressed as mean $\pm$ SD. Different letters above bars indicate significant differences among groups ($P < 0.05$).

In present study, serum lipid profiles exhibited a consistent pattern across groups. TC, TG, and LDL-C levels observed significantly increased in the Alc group, whereas both treatments significantly reduced these lipids ($P < 0.05$). BB18+LA100 normalized TC and LDL-C to levels comparable with the Sily group and showed a significantly lower TG level than Sily (Fig. 4C-E). Serum HDL-C levels were markedly reduced in the Alc group compared with the Ctrl group ($P < 0.05$), whereas BB18+LA100 intervention restored HDL-C to levels comparable to those observed in the Sily and Ctrl groups (Fig. 4F). Consistent results were observed in liver lipid profiles. Liver TC, TG, and LDL-C level were substantially elevated in the Alc group and ameliorated by both interventions ($P < 0.05$). BB18+LA100 restored liver TC and LDL-C to levels equivalent to Sily (Fig. 4G-I). Liver HDL-C exhibited a parallel trend, being reduced in the Alc group and normalized by BB18+LA100 treatment (Fig. 4J). Collectively, these results demonstrate that BB18+LA100 effectively alleviates liver lipid accumulation and improves systemic and liver lipid profiles in ALD mice.

3.5 Effect of BB18+LA100 intervention on transcriptional profile and the TLR4/NF- κ B pathway in ALD mice

To verify the hepatoprotective mechanism of BB18+LA100, transcriptomic analysis was carried out. The results displayed significant changes in differentially expressed genes (DEGs) among the groups ($P < 0.05$). Alcohol treatment resulted in a substantial alteration in the transcriptome, with 1,782 genes upregulated and 2,071 genes downregulated, relative to the control group (Fig. 5A). Supplementation with both silymarin and BB18+LA100 significantly diminished the quantity of DEGs compared to the Alc group, indicating that probiotic and silymarin intervention has a protective effect on alcohol induced transcriptional dysregulation. A Venn diagram revealed that 354 DEGs were shared between the Ctrl vs. Alc and BB18+LA100 vs. Alc comparisons (Fig. 5B). KEGG enrichment analysis based on DEGs revealed that alcohol consumption markedly activated immunological and inflammation-related pathways, such as Toll-like receptor signaling, TNF signaling, and leukocyte transendothelial migration, and steroid biosynthesis pathways (Fig. 5C), indicating the effectiveness of probiotic treatment in mitigating alcohol-induced transcriptome alterations, particularly in inflammatory signaling pathways.



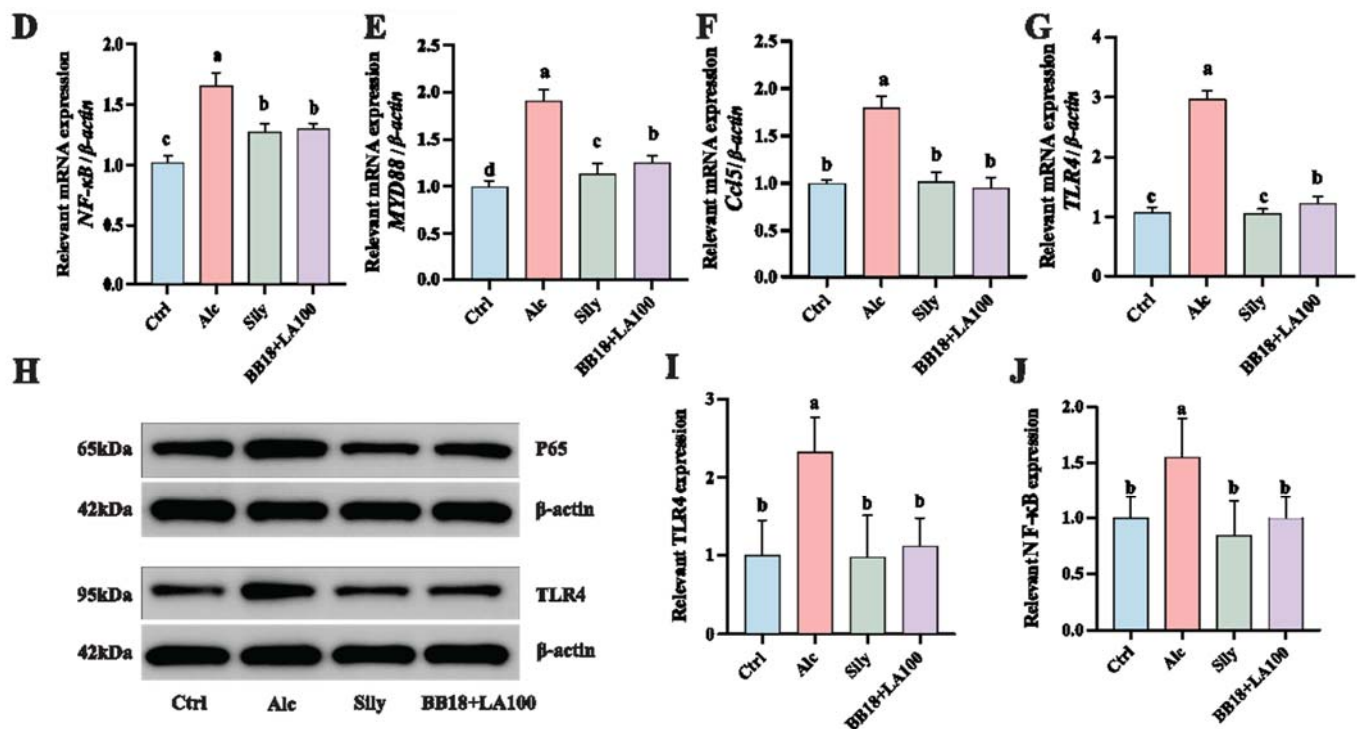


Fig. 5 Effects of BB18+LA100 on transcriptomic alterations and TLR4/NF- κ B signaling in ALD mice. Number of differentially expressed genes (DEGs) among groups (A); Venn diagram showing number of specific and sharing DEGs between Ctrl vs. Alc and BB18+LA100 vs. Alc (B); KEGG enrichment analysis of DEGs (C); Liver mRNA expression of *NF- κ B*, *MYD88*, *Ccl5*, and *TLR4* (D-G). Western blot analysis of P65 and TLR4 protein expression (H); Quantification of TLR4 and NF- κ B p65 protein expression (I-J). Data are expressed as mean $\pm$ SD. Different letters above bars indicate significant differences among groups ($P < 0.05$).

To validate the findings of transcriptomics, RT-qPCR was performed on key DEGs associated with the Toll-like receptor signaling pathway. The liver mRNA levels of *NF- κ B*, *MYD88*, *Ccl5*, and *TLR4* were markedly elevated in the Alc group ($P < 0.05$), in accordance with the trends observed in the transcriptomic analysis, indicating that the Lieber-DeCarli alcohol diet induces liver inflammation (Fig. 5D-G). Conversely, BB18+LA100 supplementation markedly decreased the levels of these pro-inflammatory genes, decreasing their levels comparable with those in the Sily and Ctrl groups, thereby validating its suppressive effect on the TLR4/NF- κ B signalling pathway. Furthermore, Western blot analysis substantiated these findings, indicating that TLR4 and NF- κ B protein expression was markedly increased in the Alc group ($P < 0.05$) whereas dramatically diminished after BB18+LA100 therapy (Fig. 5H-J). Taken together, these results indicate that BB18+LA100 alleviates alcohol-induced liver inflammation by regulating transcriptional dysregulation and inhibiting the stimulation of the TLR4/NF- κ B signalling pathway.

3.6 Effect of BB18+LA100 intervention on the composition and metabolic function of gut microbiota in ALD mice

Dysbiosis of gut microbiota is increasingly recognised as a substantial factor in the progression of ALD. The Fig. 6A-C illustrate that alcohol consumption significantly decreased α -diversity of gut microbiota, while treatment with silymarin or BB18+LA100 substantially alleviated these effects. The Chao1 and Sob indices were significantly higher in both treatment groups compared with the Alc group ($P < 0.05$), comparable to the levels observed in the Ctrl group (Fig. 6A-B). The Simpson index was markedly elevated in the BB18+LA100

group compared to the Alc and Sily groups ($P < 0.05$), signifying improved species evenness of gut microbiota (Fig. 6C). Principal coordinate analysis (PCoA, Bray-Curtis algorithm) was employed to conduct β -diversity analysis, which revealed distinct separation among groups (Fig. 6D). This indicates that there have been significant modifications in the composition of gut microbiota. The Alc group was distinctly separated from the Ctrl group, demonstrating that alcohol consumption caused significant gut microbiota dysbiosis. In comparison to the Alc group, the gut microbiota profiles of both Sily and BB18+LA100 groups were distinct. The BB18+LA100 group displayed closer clustering to the Ctrl group, suggesting that the probiotic supplement was more efficacious in reinstating gut microbial structure.

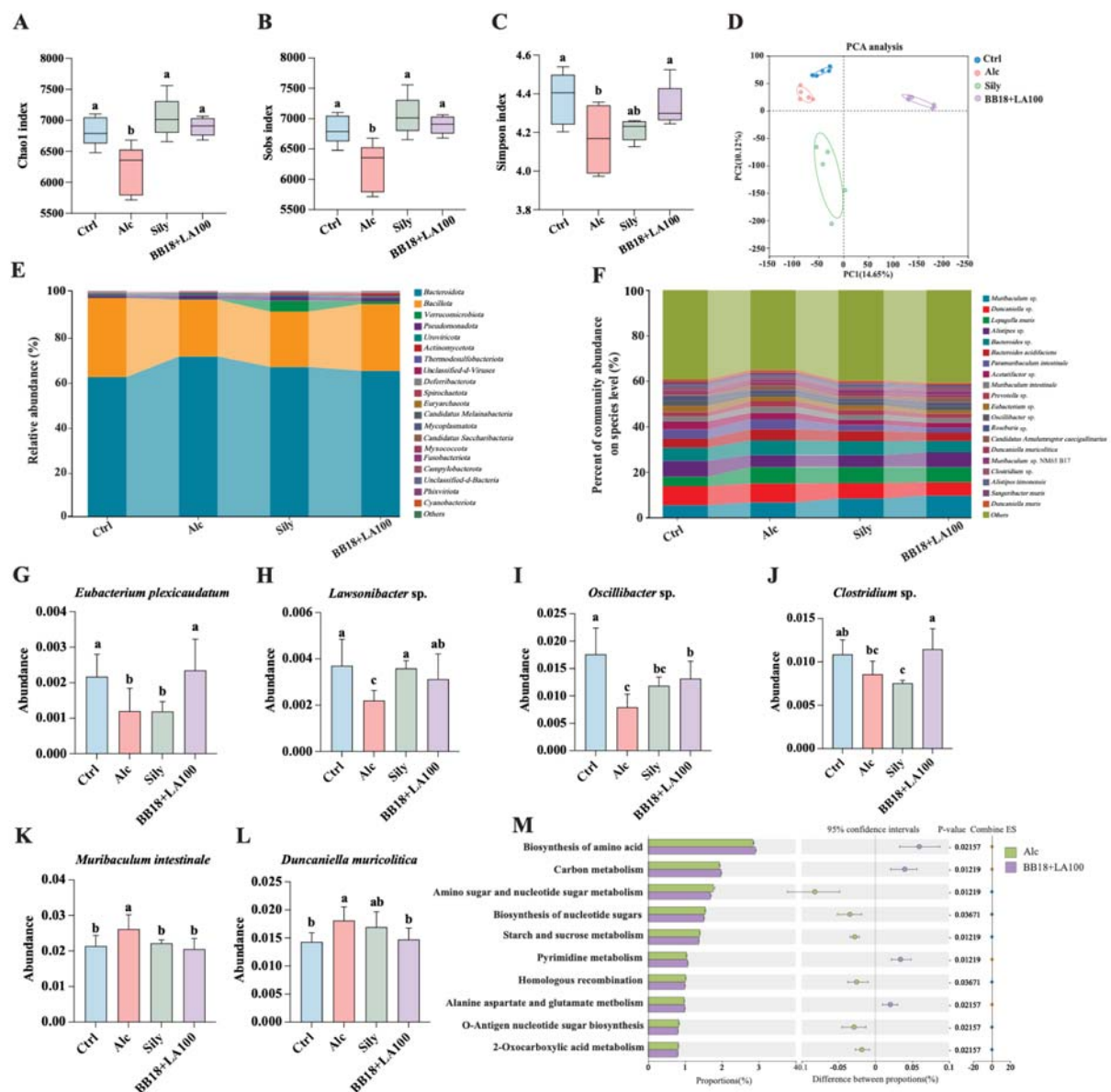


Fig. 6 Effects of BB18+LA100 on gut microbial community in ALD mice. Chao1 index (A); Sobs index (B); Simpson index (C); β -diversity of the intestinal microbiota (D); Phylum-level community histogram (E); Species-level community histogram (F); Relative abundance of differentially expressed bacteria at the species level (G-L); KEGG pathways of differentially expressed bacteria (M). Data are expressed as mean $\pm$ SD. Different letters above bars indicate significant differences among groups ($P < 0.05$).

Bacteroidota and Bacillota were the dominant phyla (Fig. 6E). Alcohol consumption decreased the abundance of Bacillota while increasing Bacteroidota abundance, however both Sily and BB18+LA100

groups reestablished the Bacillota/Bacteroidota homeostasis. The abundance of Verrucomicrobiota also exhibited an increase subsequent to BB18+LA100 treatment. At the species level, the top abundance 10 taxa were *Muribaculum* sp., *Duncaniella* sp., *Lepagella muris*, *Alistipes* sp., *Bacteroides* sp., *B. acidifaciens*, *Paramuribaculum intestinale*, *Acetatifactor* sp., *Muribaculum intestinale*, and *Prevotella* sp. (Fig. 6F). *Eubacterium plexicaudatum*, *Lawsonibacter* sp., and *Oscillibacter* sp., identified as key differential species, revealed a notable reduction in the Alc group, whereas their abundance in the BB18+LA100 group were comparable to that in the Ctrl group, suggesting that BB18+LA100 supplementation restored beneficial microbial populations associated with intestinal homeostasis (Fig. 6G-I). Interestingly, the abundance of *Clostridium* sp. was reduced in the Sily group but increase in the BB18+LA100 group compared with the Alc group (Fig. 6J). Conversely, *Muribaculum intestinale* and *Duncaniella muricolitica* exhibited an increase in the Alc group, then returning to their initial levels upon BB18+LA100 administration (Fig. 6K-L).

The functional examination of the gut microbiota indicated that, in comparison to the Alc group, there was a significant upregulation of pathways associated with amino acid metabolism, carbon metabolism, and pyrimidine metabolism in the BB18+LA100 group, whereas pathways associated with lipopolysaccharide biosynthesis and inflammation were downregulated (Fig. 6M). Collectively, these results demonstrate that BB18+LA100 reshapes the gut microbiota by enhancing beneficial taxa, suppressing potentially harmful taxa, and improving microbial metabolic functions, thereby alleviating alcohol-induced gut microbial dysbiosis in ALD mice.

3.7 Effect of BB18+LA100 intervention on the SCFAs contents in ALD mice

To assess the impact of BB18+LA100 supplementation upon gut microbe-produced metabolites in ALD mice, SCFAs concentrations in cecal contents were measured. The quantity of acetic, propanoic, butyric, valeric, isobutyric, and isovaleric acids have been measured (Fig. 7). Alcohol consumption significantly diminished overall SCFAs levels, as evidenced by substantially reduced concentrations in the Alc group comparing to the Ctrl group ($P < 0.05$). BB18+LA100 supplementation efficiently restored acetic and propanoic acid levels to values comparable to those of the Ctrl group (Fig. 7A-B). Similarly, butyric acid concentrations were markedly elevated, reaching levels closer to those obtained in the Sily group (Fig. 7C). Valeric and isobutyric acid concentrations exhibited slight elevations in the BB18+LA100 group; however, no statistically significant differences were observed in comparison to the Alc group ($P > 0.05$) (Fig. 7D-E). Isovaleric acid concentrations were markedly increased in the BB18+LA100 group relative to the Alc group ($P < 0.05$) (Fig. 7F). The findings suggest that BB18+LA100 supplementation partially reconstituted alcohol-disrupted SCFAs profiles, particularly acetate, propionate, butyrate, and isovalerate, which indicates an enhancement in microbial metabolic activity that may improve gut barrier function and mitigate inflammatory responses in ALD mice.

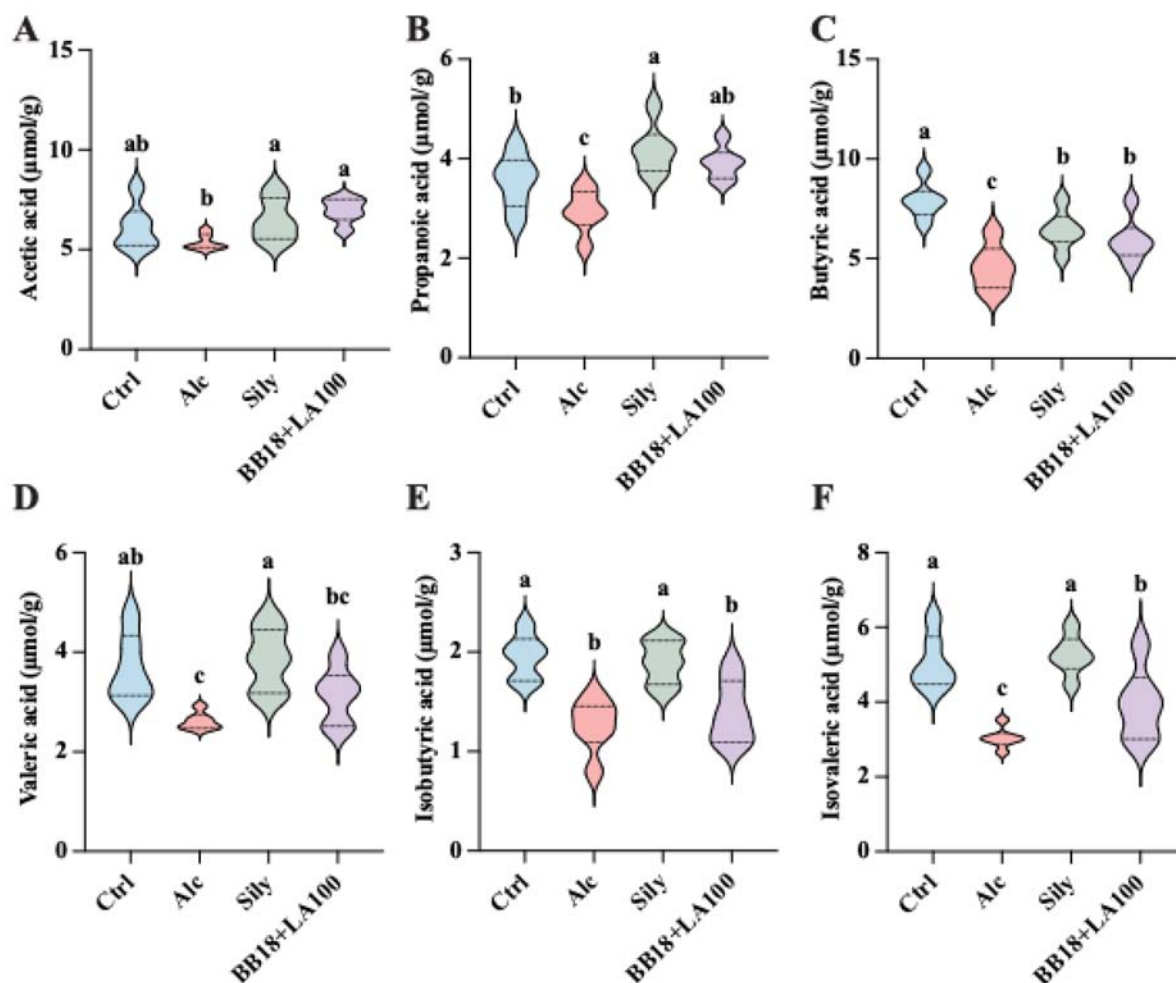


Fig. 7 Effects of BB18+LA100 on short chain fatty acids (SCFAs) variation in ALD mice. Acetic acid (A); Propanoic acid (B); Butyric acid (C); Valeric acid (D); Isobutyric (E); Isovaleric acid (F). Data are expressed as mean $\pm$ SD. Different letters above bars indicate significant differences among groups ($P < 0.05$).

4. Discussion

ALD represents a major global health burden resulting from chronic alcohol consumption and is driven by the convergence of metabolic dysregulation, oxidative stress, and persistent inflammation [12]. Increasing evidence highlights gut dysbiosis as a critical contributor to ALD pathogenesis, suggesting that manipulation of the gut-liver axis may serve as a promising therapeutic strategy [9]. Recently, multi-strain probiotic supplementation has emerged as a potential intervention to mitigate alcohol-induced liver injury [15-17]. In the present study, a Lieber-DeCarli ethanol diet-induced ALD mouse model was employed to evaluate the hepatoprotective efficacy of the compound probiotics BB18+LA100 and to elucidate their underlying mechanisms along the gut-liver axis.

The close interplay between the gut and the liver underlies the critical role of the intestinal microbiota in maintaining liver health. Regulation of the gut-liver axis is governed by intestinal barrier integrity, gut microbiota composition, and normal liver function [18]. Disruption of this axis by chronic alcohol exposure leads to gut microbiota dysbiosis, compromised intestinal barrier integrity, and enhanced translocation of bacterial products into portal circulation, thereby initiating liver inflammation and injury [19]. Consistent with

this pathogenic framework, alcohol-fed mice in our study exhibited clear signs of gut barrier disruption and endotoxemia, supporting the central role of intestinal dysfunction in ALD progression.

Intestinal epithelial injury represents an early and pivotal event in alcohol-induced gut-liver axis dysregulation. Ethanol consumption damages the intestinal mucosa, increases epithelial permeability, and facilitates the leakage of bacterial components, particularly LPS, into the portal vein [20]. In this study, BB18+LA100 supplementation significantly preserved intestinal architecture, as evidenced by improved villus morphology and superior maintenance of goblet cell populations compared with alcohol-fed mice. Goblet cells play an essential role in mucus secretion, antimicrobial peptide production, and epithelial defense against microbial invasion [21]. Serum LPS concentrations were significantly elevated in alcohol-fed mice compared to the Ctrl group, whereas BB18+LA100 supplementation dramatically restored LPS levels toward normal. The preservation of goblet cells and epithelial integrity by BB18+LA100 was accompanied by a marked reduction in circulating LPS levels, indicating attenuation of alcohol-induced endotoxemia and restoration of intestinal barrier function.

Histopathological and biochemical analyses collectively demonstrated that BB18+LA100 markedly alleviated alcohol-induced liver injury. Hepatocyte ballooning, degeneration, and acidophil bodies are hallmark histological features of alcohol-associated steatohepatitis, likely driven by ethanol-induced oxidative stress and hepatocellular damage [22]. Serum ALT and AST levels serve as indirect indicators of liver injury severity, as lipid peroxidation of hepatocellular membranes facilitates the leakage of these enzymes into the circulation [23–24]. Consistently, BB18+LA100 supplementation significantly ameliorated these pathological alterations, accompanied by reduced serum ALT and AST levels. Similar protective effects have been reported for other probiotic formulations containing *Lactobacillus* and *Bifidobacterium* species [16,18], supporting the robustness of probiotic-based interventions in ALD.

Moreover, our findings comprehensively indicate the involvement of gut microbiota modulation, oxidative stress attenuation, anti-inflammatory activity, and lipid metabolism regulation in the hepatoprotective effects of BB18+LA100. Shotgun metagenomic sequencing revealed that BB18+LA100 supplementation significantly restored microbial diversity and shifted the overall microbiota structure toward a control-like configuration. Notably, beneficial taxa such as *Eubacterium plexicaudatum* and *Oscillibacter* sp. were enriched in the BB18+LA100 group. Members of the *Eubacterium* genus are known butyrate producers and participate in bile acid and cholesterol metabolism, thereby contributing to intestinal and metabolic homeostasis [25]. *Oscillibacter* species have been associated with reduced blood and fecal cholesterol levels, and functional analyses have revealed conserved cholesterol-metabolizing capabilities, including glycosylation and dehydrogenation pathways [26]. In addition, several *Clostridium* species are known to exert anti-inflammatory effects through the production of butyrate, secondary bile acids, and indole-3-propionic acid, which collectively enhance epithelial barrier integrity and modulate host immune responses [27]. Conversely, BB18+LA100 supplementation significantly reduced the abundance of opportunistic pathogens such as *D. muricolitica* compared with the Alc group. This species has been

previously linked to intestinal inflammation and body-weight loss in mice models of inflammatory bowel disease [28]. Furthermore, functional analyses revealed a significant upregulation of pathways related to amino acid metabolism in the BB18+LA100 group. This finding is consistent with previous research demonstrating that alleviation of lipid metabolic disorders can be mediated through gut microbiota regulation and modulation of amino acid metabolism [29]. Collectively, these results align with prior research, that probiotic therapy improves liver cirrhosis primarily through restoration of gut microbial homeostasis by promoting beneficial bacteria and suppressing pathogenic taxa [9]. It should be noted that the functional pathway alterations identified in this study are primarily predictive from metagenomic and transcriptomic enrichment analyses. Nevertheless, the prediction is supported by experimental evidence, including suppression of liver TLR4/NF- κ B signaling, reduced circulating LPS and inflammatory cytokines, and improved biochemical indicators of liver injury.

In line with previous studies showing that probiotic mixtures can reverse alcohol-induced gut dysbiosis and enrich SCFAs-producing bacteria in ALD models [16,18], BB18+LA100-induced microbial remodeling in the present study was closely associated with the restoration of SCFAs production, particularly acetate, propionate, butyrate, and isovalerate. SCFAs are pivotal microbial metabolites that reinforce intestinal barrier integrity, suppress inflammatory signaling, and regulate host lipid and energy metabolism [30]. Accordingly, the recovery of SCFAs levels provides a mechanistic link between gut microbiota restructuring and the observed improvements in intestinal morphology, reduced endotoxemia, and attenuation of liver inflammation [31]. Among individual SCFAs, acetate has been reported to reduce lipid accumulation, enhance mitochondrial efficiency, and promote browning of white adipose tissue, thereby increasing thermogenesis and reducing adiposity [32]. Propionate supplementation has been shown to ameliorate ethanol-induced liver injury by restoring gut-liver axis homeostasis, suppressing TLR4-mediated inflammatory signaling, improving intestinal barrier integrity, and limiting LPS translocation in ALD models [33]. Similarly, butyrate alleviates liver steatosis through activation of adenosine monophosphate-activated protein kinase (AMPK) and modulation of macrophage activity via prostaglandin E₂ (PGE₂) signaling, ultimately enhancing liver lipid metabolism [34]. Collectively, these findings highlight SCFAs as key mediators linking BB18+LA100-induced gut microbiota remodeling to improved intestinal barrier function, reduced endotoxemia, and attenuation of liver inflammation, thereby contributing to the protective effects against alcohol-induced liver injury.

In parallel with gut-derived inflammatory triggers, accumulating evidence indicates that ALD initiates a variety of biochemical and physiological disruptions, including persistent oxidative stress, mitochondrial malfunction, and hepatocellular death, ultimately leading to liver injury [35]. Ethanol metabolism is inherently oxidative, leading to the overproduction of reactive oxygen species (ROS), relative hypoxia in the tissues, and mitochondrial dysfunction [36]. Sustained ROS overproduction disrupts hepatocellular redox homeostasis and promotes inflammatory and fibrogenic responses [37]. Increasing evidence suggests that probiotics can effectively scavenge excess ROS generated during metabolic imbalance, thereby contributing to the maintenance of cellular redox equilibrium [38]. Consistent with these mechanisms, chronic alcohol feeding in

the present study markedly depleted liver GSH levels while elevating MDA content, reflecting impaired antioxidant defenses and enhanced lipid peroxidation. Notably, BB18+LA100 supplementation effectively restored liver GSH levels and reduced MDA accumulation, indicating reinforcement of non-enzymatic antioxidant defenses and attenuation of oxidative damage.

Increasing evidence indicates that TLR4-gut microbiota interactions constitute a key mechanistic link between innate immunity and chronic inflammation [39]. TLR4 plays a central role in mediating inflammatory responses triggered by LPS derived from gram-negative bacteria. Consistently, probiotic and bioactive compound interventions alleviate liver injury and intestinal barrier dysfunction by modulating gut microbiota composition, reducing circulating LPS levels, and suppressing liver TLR4/NF- κ B signaling, thereby attenuating liver inflammation and oxidative stress [40-41]. Alcohol-triggered impairment of the intestinal barrier permits the translocation of bacterial LPS from the gut into the liver portal circulation. The binding of LPS to TLR4 stimulates the NF- κ B signalling pathway, resulting in the overproduction of pro-inflammatory cytokines and an increase of liver inflammation [42-43]. Consistent with this mechanism, alcohol exposure in the present study markedly increased circulating LPS levels and elevated liver pro-inflammatory cytokines, comprising TNF- α , IL-6, and IL-1 β , while concomitantly suppressing the anti-inflammatory cytokine IL-10. Transcriptomic analysis further revealed broad activation of inflammatory pathways in the livers of alcohol-fed mice. Notably, BB18+LA100 supplementation substantially reduced the number of differentially expressed genes and suppressed key components of the TLR4/NF- κ B pathway at both the transcriptional and proteomic stages. Although modulation of TLR4/NF- κ B signaling has also been reported for other probiotic strains or combinations in ALD models, including *Lactobacillus paracasei* DCRFF018 [44], compound probiotics [17], and the combination of *Clostridium butyricum* JJ100 and *Lactobacillus rhamnosus* LR [45], such anti-inflammatory effects are widely recognized to be strain dependent. In this context, the present findings suggest that the strain-specific protective effects of BB18+LA100 against alcohol-induced liver inflammation are closely associated with its capacity to modulate gut microbiota composition and functionality. Such modulation likely leads to a reduction in microbiota-derived pro-inflammatory signals, particularly LPS, thereby attenuating TLR4 activation and subsequent NF- κ B-mediated inflammatory cascades in the liver.

Nevertheless, chronic inflammation is closely intertwined with dysregulation of liver lipid metabolism, a defining feature of ALD progression from simple steatosis to steatohepatitis, particularly with respect to triglyceride homeostasis [46-47]. In the present study, BB18+LA100 intervention markedly alleviated liver steatosis and normalized both systemic and liver lipid profiles. Specifically, BB18+LA100 supplementation significantly reduced liver lipid accumulation, lowered LDL-C levels, and increased HDL-C concentrations. Continuous ethanol exposure induces abnormal liver histological alterations, including excessive lipid droplet accumulation and macrophage infiltration, primarily through disruption of lipid homeostasis involving fatty acid absorption, de novo lipogenesis, β -oxidation, and very-low-density lipoprotein secretion, thereby promoting steatosis and hyperlipidemia [48-49]. Prolonged alcohol consumption further exacerbates these

effects by enhancing intestinal lipid absorption and liver lipogenesis while suppressing lipolysis and lipophagy, ultimately accelerating the progression toward alcoholic steatohepatitis ^[50]. Elevated HDL-C levels are associated with multiple cardiometabolic benefits, most notably a reduced risk of cardiovascular disease ^[51]. Moreover, probiotics have been reported to modulate host cholesterol metabolism through the production of bile salt hydrolase (BSH), which catalyzes bile acid deconjugation and facilitates cholesterol co-precipitation and fecal excretion ^[52]. Therefore, these mechanisms may partially account for the lipid-lowering and hepatoprotective effects observed following BB18+LA100 supplementation. Collectively, this study demonstrates that alcohol-induced liver injury in mice can be ameliorated through alterations in specific microbial taxa were accompanied by increased levels of microbiota-derived SCFAs, which are closely associated with intestinal barrier function and inflammatory regulation, coinciding with reduced endotoxemia and inflammatory burden. Nevertheless, fecal microbiota transplantation, and specific mechanistic intervention strategies, warrant further investigation in future studies to clarify causal relationships within the gut-liver axis.

5. Conclusion

In summary, BB18+LA100 supplementation provides significant protection against alcohol-induced liver damage by thoroughly maintaining the gut-liver axis. BB18+LA100 reinstated gut microbial equilibrium, enhanced SCFAs-producing bacteria, and strengthened intestinal barrier integrity, thereby restricting endotoxin circulation and inhibiting liver TLR4/NF- κ B activation. These effects collectively diminished liver inflammation and oxidative stress while concurrently restoring lipid metabolism. Collectively, our findings indicate that BB18+LA100 significantly inhibits the progression of ALD by coordinating the control of microbiota-induced metabolic and inflammatory pathways, emphasising its promise as a probiotic-oriented strategy for the prevention and management of ALD.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgments

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