Lactobacillus acidophilus and vitamin C attenuate ethanol-induced intestinal and liver injury in mice

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Abstract. Ethanol exposure frequently induces intestinal and liver injury, dysbiosis of the gut microbiota and vitamin C (VC) deficiency. Gut microbiota-targeted therapy is emerging as an important adjuvant method for protecting the body against ethanol-induced injury, particularly probiotics containing *Lactobacillus acidophilus* (LA). However, the feasibility and efficiency of using synbiotics containing LA and VC against ethanol-induced injury remained largely undetermined. To examine the advantages of LA+VC, their effect was evaluated in an ethanol-fed mouse model. The results suggested that LA+VC restored gut microbiota homeostasis and reinstated the immune balance of colonic T-regulatory cells (CD4$^+$CD45$^+$forkhead box p3$^+$). In addition, intestinal barrier disorders were improved via upregulating tight junction proteins (claudin-2, zona occludens-1 and occludin) and mucus secretion, which prevented the translocation of lipopolysaccharide into circulatory systems and subsequently reduced the expression of Toll-like receptor 4 in liver tissues. In this context, LA+VC treatment reduced the inflammatory response in the liver, which was likely responsible for the improved liver function in ethanol-challenged mice. Collectively, these results indicated that LA+VC treatment significantly protected the intestine and liver from ethanol damage by enhancing intestinal barrier function and reducing systemic inflammation.

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The present study paved the way for further exploration of synbiotics based on *Lactobacillus* species and VC.

Introduction

Excessive alcohol consumption has become one of the major causes of diverse diseases (1), and alcohol abuse results in ~3 million deaths per year and is accountable for ~5.1% of the global burden of disease according to the report of the World Health Organization for the year 2018 (2). The toxic effects of alcohol on the human body, particularly intestine and liver tissues, are largely attributed to alcohol-induced metabolic disorders and oxidative stress, and damage to energy homeostasis (3). Of note, studies have revealed that alcohol disturbs the gut microbiota imbalance and damages the gut barrier to cause translocation of microbial products such as lipopolysaccharide (LPS) (4-6). Alcohol-associated endotoxemia also contributes to the development and progression of liver inflammation and activation of the innate immune response (7,8).

The mammalian gastrointestinal tract is colonized by trillions of microorganisms that coevolved with their hosts (9). It is established that the gut microbiota is dynamic and it is pertinent to a wide variety of diseases, including inflammatory bowel disease, obesity, diabetes, Parkinson's disease and even cancer (10-14). The gut microbiota dysbiosis induced by alcohol exposure has been well-characterized, with increased abundance of Proteobacteria and Actinobacteria and decreased abundance of Lachnospiraceae, Ruminococcaceae and *Bifidobacterium* (4,5,15,16). Of these, the overgrowth of Proteobacteria, particularly Enterobacteriaceae, leads to disorders of the healthy gut microbiota and promotes its pathogenic potential by producing endotoxins, such as LPS. In addition, the damaged intestinal barrier enables LPS translocation from the intestinal lumen into the portal circulation (17), which is subsequently recognized by the toll-like receptor 4 (TLR4) complex and induces specific intracellular signalling pathways affecting inflammation (18).

It is well recognized that temperance is the best way to prevent the damage of alcohol to human body (19). However, the alcohol addiction and drinking culture often allow easy exposure of humans to alcohol, at least in the Chinese and Russian population (20,21). Therefore, there is in urgent need for exploring novel approaches to alleviate the alcoholic injury in humans. Yogurt containing *Lactobacillus* or other

probiotics has gradually become a popular beverage in China (https://marketingtochina.com/yogurt-health-supplement-for-chinese/).Probiotics have been demonstrated to be able to prevent the occurrence and progression of ethanol-induced injury. Specifically, supplementation with *Lactobacillus* species, particularly *Lactobacillus acidophilus* (LA), is able to restore the gut microbiota homeostasis and effectively attenuate alcohol-induced liver injury via reducing the accumulation of plasma endotoxin (22). Furthermore, administration of *Lactobacillus* species is able to prevent harmful bacteria from residing in the intestine due to their product, lactic acid (23), and increase the abundance of other healthy bacteria, such as *Bifidobacteria* (24), which likely contributes to the maintenance of the immune balance of the intestine (25). On the other hand, a variety of studies suggested that alcohol consumption leads to vitamin C (VC) deficiency and VC supplementation was able to alleviate ethanol-induced impairment (26,27). While treatment with single LA or VC is capable of improving alcohol-induced injury in mice to a certain extent, this level of improvement may not be sufficient to support their further application, particularly in the clinic.

Therefore, the aim of the present study was to determine whether symbiotic supplementation of LA plus VC is able to reduce ethanol-induced intestine and liver injury by modulating gut microbiota dysbiosis and restoring intestinal barrier function in mice.

Materials and methods

Bacterial strains and VC preparation. *Lactobacillus acidophilus* (cat. no. AS1.3342; Biobw Biotechnology Co., Ltd.) was used in the present study. Separate colonies of LA were cultured in 5 ml MRS broth (cat. no. M8540; Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 48 h with shaking (200 rpm) under aerobic conditions. Subsequently, the cultures were centrifuged at 12,000 x g for 5 min at 4°C, diluted in sterile normal saline solution and mixed thoroughly to obtain the appropriate bacterial density [LA: $5x10^8$ colony-forming units (CFU)/ml]. VC (cat. no. A8100; Solarbio Life Sciences) was dissolved in sterile water and gavage-fed to mice daily at a dose of 100 mg/kg.

Construction of an ethanol-fed mouse model. The animal experiments of the present study were approved by the Ethics and Clinical Research Committee of Tianjin Medical University (Tianjin, China). Forty male C57BL/6J mice (age, 7-8 weeks; weight, 21-23 g) were obtained from Huafukang Biological Technology Co., Ltd. and maintained in a specific pathogen-free environment at 23°C and 40-60% humidity with a 12-h light/12-h dark cycle. The ethanol feeding mouse model was constructed based on the Lieber-DeCarli diet (cat. no. TP 4030C/TP 4030A; TROPHIC Animal Feed High-Tech Co. Ltd.) (28). All of the mice were randomly divided (10 mice per group) into 5 groups: Control group (Ctrl), ethanol-fed group (EH), ethanol-fed and LA supplementation group (LA), ethanol-fed and VC supplementation group (VC), ethanol-fed and LA plus VC supplementation group (LA+VC). All of the mice were allowed to adapt to the laboratory environment for 2 days and to the ethanol-free liquid diet for another 3 days, and then, the diet containing ethanol (5% vol/vol) was fed to these ethanol-fed mice for 10 days, while the control mice received an isocaloric amount of maltodextrin. LA (~$1x10^8$ CFU per mouse) in saline solution, VC (100 mg/kg body weight) in saline solution or vehicle alone (saline solution) were gavage-fed to mice daily after 3 days of ethanol-free liquid diet acclimatization. On the last day of the experiment, the groups of EH, LA, VC and LA+VC received a single dose of ethanol via gavage (5 g/kg body weight), and the control mice received a single dose of maltodextrin (9 g/kg body weight). After 9 h, all mice were anaesthetised (4% isoflurane by inhalation) until loss of paw reflex, exsanguinated and then euthanized by cervical dislocation. Subsequently, distal colon tissues (~1 cm) were collected for H&E staining and fixed in 4% paraformaldehyde. The proximal colon tissues (~1 cm) were collected for reverse transcription-quantitative (RT-q) PCR (stored at -80°C), and the remaining middle part of the colon was used for flow cytometry.

FITC assays. To determine intestinal permeability, FITC-dextran (cat. no. 68059; Sigma-Aldrich; Merck KGaA) was orally administered to mice (600 mg/kg body weight) at 4 h prior to sacrifice. These blood samples were collected from the isoflurane-anesthetised mice and blood samples were centrifuged (2,000 x g, 4°C) for 10 min to obtain serum (200 μl). The fluorescence of these serum samples was immediately recorded by a spectrophotometer (Tecan) at an excitation wavelength of 485 nm and emission wavelength of 528 nm.

Isolation of lymphocytes and flow cytometry. For all mice, the middle part of colon tissues was collected and the colon lamina propria lymphocytes (LPMCs) were prepared as described previously (29). In brief, after clearance of feces, residual mesenteric fat tissue and Peyer's patches of the colon were resected, cut into 1-cm pieces and washed in ice-cold PBS. After digestion with predigestion solution (Hank's balanced salt solution and 5 mM EDTA) and digestion solution [collagenase D (cat. no. DH073-2; Beijing Dingguo Changsheng Biotechnology Co., Ltd.), DNase I (cat. no. DH113-5; Beijing Dingguo Changsheng Biotechnology Co., Ltd.) and dispase (cat. no. S10013; Shanghai Yuanye Biotechnology Co., Ltd.)], the collected cells were further purified by a Percoll gradient (40/80%) and the LPMCs were collected and washed with PBS supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd.) for flow cytometric analysis. The cells were then stained with anti-CD45-peridinin chlorophyll (1:500; cat. no. 103130; Biolegend) and anti-CD4-FITC (1:500; cat. no. 100510; Biolegend) diluted in 1X PBS at 4°C in the dark for 45 min, and then fixed and permeabilized with the forkhead box (Fox)P3/True-Nuclear™ Transcription factor buffer set (cat. no. 424401; Biolegend) and stained with anti-FoxP3-phycoerythrin (1:500; cat. no. 320007; Biolegend) diluted in DMEM medium (cat. no. 31600; Beijing Solarbio Science & Technology Co., Ltd.) at 4°C in the dark for 45 min.

Biochemical analysis. Colon samples (100 mg) were homogenized with a mini-bead beater (cat. no. KZ-II) and glass beads (cat. no. G0101-200G; both from Wuhan Servicebio Technology Co., Ltd.) in 1 ml 1X PBS buffer and 700 μl supernatant was transferred to a new centrifuge tube

after centrifugation. The superoxide dismutase (SOD) kit (cat. no. BC0170; Solarbio Life Sciences), myeloperoxidase (MPO) kit (cat. no. ab105136; Abcam) and glutathione peroxidase (GSH-PX) kit (cat. no. BC1190; Solarbio Life Sciences) were used to determine the SOD activity, MPO activity and GSH-PX activity according to the manufacturer's protocols. Serum aspartate transaminase (AST) and alanine transaminase (ALT) were determined by a blood biochemical analyser (Fujifilm DRI-CHEM 3500s; Fujifilm) according to the manufacturer's protocol. Mouse ELISA Kits were used to determine the serum levels of LPS (cat. no. JL20691-96T; Jiang Lai Biological), TNF-α (cat. no. ml002095; Enzyme Link Biotechnology Co., Ltd.), IL-1β (cat. no. ml301814; Enzyme Link Biotechnology Co., Ltd.) and IL-6 (cat. no. M*ml002301; Enzyme Link Biotechnology Co., Ltd.), and spectrophotometric methods were used to measure hepatic triglyceride (cat. no. JL46662-96T; Jiang Lai Biological) and hepatic malonaldehyde (MDA; cat. no. JL13329-96T; Jiang Lai Biological) via a spectrophotometer (Infinite F50; Tecan Group, Ltd.). All the assays were performed in triplicate and all the experiments were performed according to the manufacturer's protocol.

Histopathological observation. For the histological analysis, the liver and colonic tissues were stained with hematoxylin and eosin (H&E). In brief, the tissues were fixed in 10% formalin for 48 h at room temperature, and paraffin-embedded sections (5 μM) were stained with H&E. For the evaluation of mucins (Muc), Alcian Blue-Periodic acid-Schiff (AB-PAS) Stain Kit (cat. no. G1285; Beijing Solarbio Science & Technology Co., Ltd.) was used to stain the paraffin-embedded intestinal tissue sections according to the manufacturer's protocol. H&E- and AB-PAS-stained colonic sections were observed under an optical microscope (IX73; Olympus Corporation) at x100 magnification.

RNA isolation and gene expression analysis. Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the RNA concentration was quantified using the NanoPhotometer® N50 (Implen) and RT was performed with the PrimeScript RT reagent kit with gDNA Eraser (cat. no. RR047Q; Takara Biotechnology Co. Ltd.) according to the manufacturer's protocol. RT-qPCR was performed on a LightCycler 96 System (Roche) using TB Green Premix Ex Taq II (Tli RNaseH Plus; cat. no. RR820A; Takara Biotechnology Co. Ltd.) and a cycling program of initial denaturation for 10 min at 95℃, then 40 cycles of 10 sec at 95℃, 10 sec at 62℃ and 10 sec at 72℃, followed by 95℃ for 60 sec and a dissociation curve analysis. The primer sequences are listed in Table SI and the relative gene expression was normalized to 18S and calculated by the $2^{-\Delta\Delta Cq}$ method (30).

DNA extraction and 16S ribosomal RNA amplification sequencing. Faecal genomic DNA was collected from 150-200 mg of fecal samples by the QIAamp PowerFecal DNA Kit (cat. no. 51804; Qiagen GmbH). The hypervariable V3-V4 region (341F and 805R) was amplified and purified. Sequencing was performed on the paired-end Illumina MiSeq PE300 (2x300 bp) platform (Illumina, Inc.) at Novogene Corp. according to the manufacturer's protocol. These raw sequences were processed using the QIIME (v1.9.1) pipeline (31) and the gut microbiota diversity and composition of fecal samples were determined.

Statistical analysis. All experimental results were obtained from at least three independent experiments. Values are expressed as the mean ± standard deviation. Statistical comparisons were performed by one-way ANOVA and Tukey's post-hoc test. GraphPad Prism 8.0 (GraphPad Software, Inc.) was used for statistical analysis and R (version 3.6.3) was used for plotting the graphs. P<0.05 was considered to indicate a statistically significant difference.

Results

LA plus VC treatment ameliorates ethanol-induced injury in mice. The effects of LA plus VC to reduce ethanol-induced injury were explored using the NIAAA model (mouse model of chronic and binge ethanol feeding) as described previously (28) (Fig. 1A). In the present study, ethanol treatment significantly lowered the body weight gain of the mice (Ctrl: 2.30±0.97 g; EH: 0.45±0.52 g; P<0.01; Fig. 1B) and obviously increased the liver/body weight ratio (P<0.01; Fig. 1C). Supplementation with LA or VC slightly alleviated the decline in body weight gain [LA: 0.80±0.25 g, P<0.05 (LA vs. EH); VC: 0.86±0.21 g, P<0.05 (VC vs. EH); Fig. 1B] and supplementation with LA partially reduced the liver/body weight ratio (Fig. 1C). Of note, the efficiency of alleviating the decline in body weight gain [LA+VC: 1.48±0.27 g; P<0.01 (LA+VC vs. EH); Fig. 1B] and reducing the liver/body weight ratio [P<0.01 (LA+VC vs. EH); Fig. 1C] of LA+VC was greater than that of single administration of LA or VC. Furthermore, the efficiency of attenuating the IL-6 levels by supplementation with LA+VC [P<0.01 (LA+VC vs. EH); Fig. 1D] was greater than that of the single treatments. In conclusion, treatment of mice with LA+VC attenuated ethanol-induced injury more effectively than single LA or VC supplementation.

LA plus VC treatment restores the gut microbiota homeostasis. Chronic ethanol consumption is a major cause of gut microbiota dysbiosis, which may support the pathophysiology of ethanol-related morbidity (5,32). In the present study, ethanol treatment markedly reduced the gut microbiota abundance and diversity compared with that in the control group (Fig. 2A-C). The LA, VC and LA+VC treatments obviously increased the Chao1 and Shannon index and reduced the Simpson index in comparison to the EH group (Fig. 2A), which suggested that these treatments markedly increased the community richness and diversity of the gut microbiota. In addition, β-diversity analysis based on the Bray-Curtis distance indicated that the gut microbiota of different groups clustered separately and treatment with LA+VC obviously restored the gut microbiota composition and diversity (Fig. 2B).

Subsequently, the taxonomic changes in the bacterial community were explored. At the phylum level, Firmicutes and Bacteroidetes were dominant in the faecal microbiota of the control group, whereas Firmicutes, Proteobacteria and Bacteroidetes were dominant in the EH group (Fig. 2C). Of note, LA+VC treatment significantly elevated the proportion of Firmicutes and reduced the Proteobacteria abundance in ethanol-treated mice (P<0.01; Fig. 2D). However, single
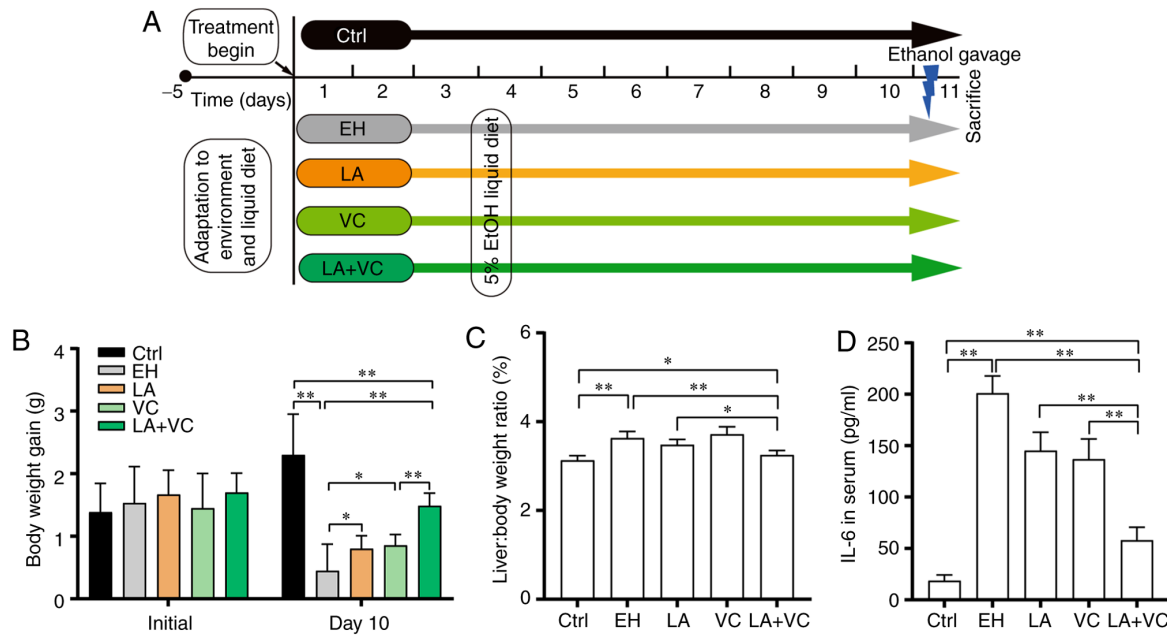
Figure 1. Effects of LA plus VC on an ethanol-fed mouse model. (A) Treatment schedule: The mice were divided into 5 groups: Ctrl, EH, LA, VC and LA+VC. LA (1x10^8 colony-forming units/mouse) and VC (100 mg/kg body weight) were administered to mice by gavage daily. (B) Body weight gain (initial, from day -5 to day 0). (C) Liver/body weight ratio (%). (D) IL-6 levels in serum. Values are expressed as the mean ± standard deviation of at least three independent experiments. *P<0.05; **P<0.01. VC, vitamin C; LA, *Lactobacillus acidophilus*; Ctrl, control; EH; ethanol.

LA or VC treatment exhibited this effect to a lesser extent. After treatment with LA+VC, the family of Lachnospiraceae (Firmicutes phyla), which is able to ferment diverse polysaccharides to short-chain fatty acids (5), was significantly enriched compared with the EH group (P<0.01; Fig. 2E). Furthermore, ethanol exposure markedly increased the abundance of Enterbacteriaceae, which are closely associated with intestinal diseases (33), and this was significantly suppressed by the treatments of LA, VC and LA+VC (P<0.01; Fig. 2E). Collectively, the LA+VC treatment suppressed the potentially pathogenic ethanol-associated changes of the gut microbiota and restored the gut microbiota perturbances caused by ethanol treatment.

LA plus VC treatment attenuates ethanol damage to the intestinal barrier. Ethanol and ethanol-associated gut microbiota dysbiosis directly influence the physiological status of the intestine (34). To determine whether LA+VC has a beneficial effect on ethanol-induced intestinal injury, changes in gut permeability were first explored. It was observed that ethanol exposure markedly increased the serum FITC concentration; however, the LA+VC treatment significantly reduced the serum FITC levels compared with those in ethanol-fed mice (P<0.01; Fig. 3A), suggesting recovery of intestinal integrity. The beneficial effects were further confirmed by the results of the serum LPS concentration (P<0.01; Fig. 3B), which also demonstrated that the LA+VC treatment reduced the translocation of microbial products via restoring intestinal permeability. In addition, the intestinal injury induced by ethanol was visualized in the colon sections stained by HE (Fig. 3C), and LA+VC obviously attenuated the ethanol-induced injury and restored the crypt structure and length (P<0.01; Fig. 3C and D). These beneficial effects were further confirmed by the results of the relative expression of the genes Claudin-2, zona occludens (ZO)-1

and occludin (P<0.01; Fig. 3E), which suggested that LA+VC reversed the ethanol-associated injury on the intercellular tight junction of the intestine. On the other hand, ethanol treatment significantly reduced the number of goblet cells in colonic crypts that are responsible for the Muc excretion; however, treatment with LA, VC and LA+VC markedly increased the number of goblet cells (Fig. 3F and G; P<0.01). Furthermore, the relative expression of mucus secretion-related genes, such as Muc2/3/4 and Kruppel-like factor (Klf)4 was examined (Fig. 3H). The RT-qPCR results indicated that the expression of Muc2/3/4 and Klf4 were markedly decreased in the ethanol-treated mice and the decreased expression of these genes was significantly improved by treatment of LA, VC and LA+VC (P<0.01; Fig. 3H). Collectively, treatment with LA+VC achieved better results in improving the intestinal tight junction and restoring mucus secretion than single treatment with LA or VC in the ethanol-challenged mice.

LA plus VC treatment alleviates ethanol-induced intestinal inflammation. Since the immune imbalance and intestinal inflammation are essential for the onset and progression of ethanol-associated intestinal injury (35), it was investigated whether LA+VC treatment has any influence on intestinal immunity and inflammation. The results indicated that ethanol considerably reduced the proportion of T-regulatory (Treg) cells (CD4+CD45+Foxp3+) in colon lamina propria and promoted the mRNA expression of pro-inflammatory genes such as IL-1β, IL-6 and TNF-α, along with reducing the expression of the anti-inflammation gene IL-10 in the colonic tissues of ethanol-challenged mice (P<0.01; Fig. 4A-C). Of note, treatment with LA+VC significantly increased the proportion of Treg cells from 14.30±2.58 to 22.60±1.06% (P<0.01; Fig. 4A and B), which indicated that the LA+VC treatment was able to reinstate the immune balance of colonic
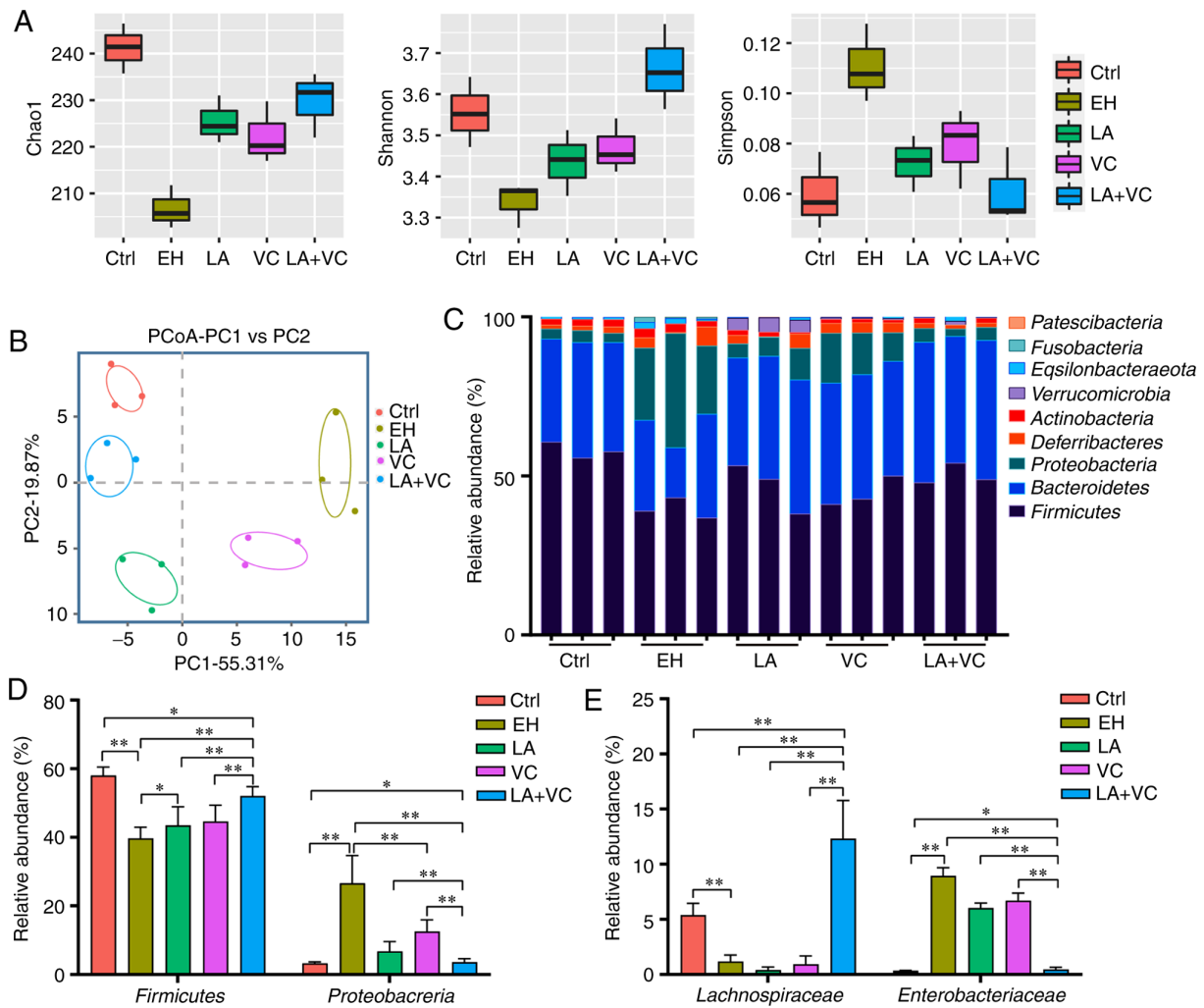
Figure 2. LA plus VC improve ethanol-induced gut microbiota dysbiosis. (A) Effects of LA plus VC on the α-diversity (Chao1, Shannon and Simpson index) of the fecal microbiota. LA+VC treatment increased the Chao1 and Shannon index and reduced the Simpson index in comparison to the EH group. (B) Effects of LA plus VC on the β-diversity of the fecal microbiota as assessed by PCoA. (C) Effects of LA plus VC on the microbiota composition at the phylum level. (D) Relative abundance of Firmicutes and Proteobacteria. LA+VC treatment decreased the abundance of proteobacteria in comparison to the EH group. (E) Relative abundance of Lachnospiraceae and Enterobacteriaceae at the family level. The markedly increased abundance of Enterbacteriaceae induced by ethanol was significantly suppressed by the LA+VC treatment. Values are expressed as the mean ± standard deviation of at least three independent experiments. *P<0.05; **P<0.01. VC, vitamin C; LA, *Lactobacillus acidophilus*; Ctrl, control; EH; ethanol; PC, principal component; PCoA, principal coordinate analysis.

Treg cells. Consistently with this, LA+VC treatment significantly inhibited the mRNA expression of IL-1β, IL-6 and TNF-α, and promoted the mRNA expression of IL-10 (P<0.01; Fig. 4C). In addition, the ethanol-induced alterations in the production of IL-10 and IL-17A were partially abrogated by treatment with LA+VC (P<0.01; Fig. 4D and E). Furthermore, the LA+VC treatment obviously decreased the activity of MPO (marker of inflammation) induced by ethanol from 5.27±0.35 to 4.17±0.40 U/g protein (P<0.05; Fig. 4F). Overall, these results revealed that the LA+VC restored the Treg cells' immune balance that was perturbed by ethanol and inhibited the inflammatory responses induced by ethanol.

To evaluate the effects of LA plus VC on oxidative stress, SOD activity and GSH-Px activity in the colon were determined. The results indicated that ethanol exposure significantly reduced the activity of SOD from 20.80±1.29 to 17.57±1.14 U/mg protein (P<0.05; Fig. 4G). Treatment with LA+VC led to a significant increment of SOD activity (28.46%) compared with ethanol treatment (P<0.01; Fig. 4G).

However, there was no significant difference among the LA, VC and EH groups (Fig. 4G). In addition, treatment with LA+VC slightly increased the GSH-Px activity damaged by alcohol from 21.70±2.33 to 26.98±1.59 U/mg protein (P<0.01; Fig. 4H), while the results of the LA and VC groups were not significantly from those in the EH group (P>0.05; Fig. 4H).

LA plus VC treatment attenuates ethanol-induced liver injury. To determine whether LA+VC protects the liver against ethanol-induced damage, HE staining of liver sections was performed. The results indicated distinct pathological alterations upon ethanol exposure, including neutrophil infiltration and steatosis, whereas LA+VC led to improvement of these pathological alterations (Fig. 5A). Next, it was observed that ethanol exposure markedly increased the serum levels of ALT and AST, and that LA+VC significantly improved the liver function in ethanol-treated mice with reduced serum levels of ALT and AST (P<0.01; Fig. 5B and C). Additionally, ethanol exposure dramatically increased the hepatic triglyceride and
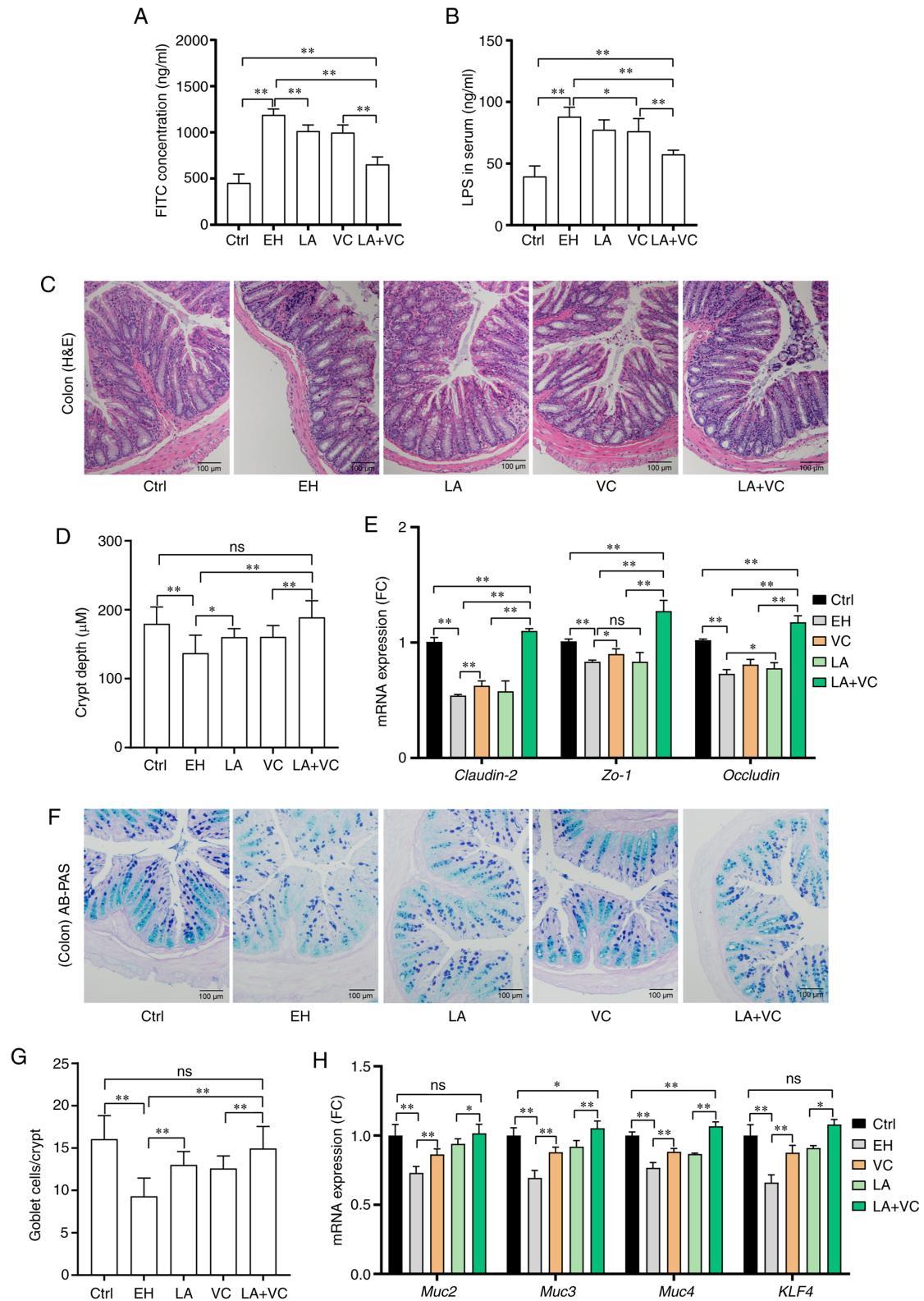
Figure 3. LA plus VC improves ethanol-induced intestinal barrier dysfunction. (A) Serum concentration of FITC-dextran. (B) LPS levels in serum. (C) Representative histology images of colon sections (magnification, x100; scale bar, 100 mm; H&E). (D) Quantified crypt depth in colon tissues. (E) Relative mRNA expression of Muc2, ZO-1 and occludin in colon tissues. (F) Representative colon sections with AB-PAS staining (magnification, x100; scale bar, 100 mm). (G) Quantified goblet cells per crypt. (H) Relative mRNA expression of mucus secretion-associated genes and Klf4 gene in colon tissues. The markedly decreased expression of Muc2/3/4 and Klf4 in the EH mice was significantly improved by the LA+VC treatment. Values are expressed as the mean ± standard deviation of at least three independent experiments. *P<0.05; **P<0.01. ns, no significance; VC, vitamin C; LA, *Lactobacillus acidophilus*; Ctrl, control; EH; ethanol; AB-PAS, Alcian blue-periodic acid-Schiff; ZO-1, zona occludens-1; Muc, mucin; KLF, kruppel-like factor; LPS, lipopolysaccharide.

MDA levels, which was significantly inhibited by LA+VC (P<0.01; Fig. 5D and E).

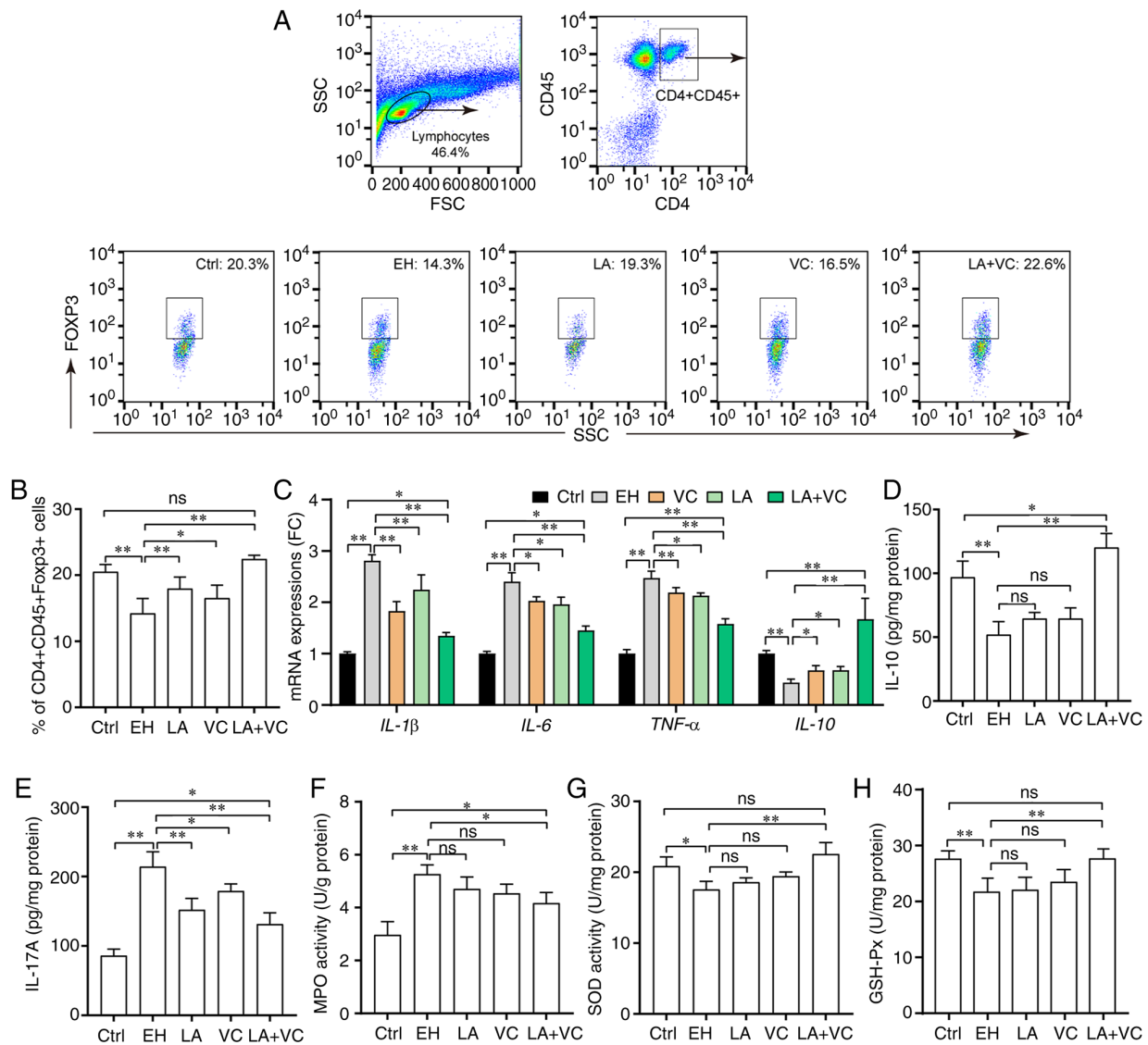Furthermore, the mRNA expression of genes related to steatosis was determined. The RT-qPCR results revealed

Figure 4. LA plus VC alleviates ethanol-induced inflammatory responses. (A) Flow cytometry plots and quantification of Treg cells (CD4$^+$CD45$^+$Foxp3$^+$) in the colon lamina propria. (B) Quantified percentage of Treg cells in the colon lamina propria in the different groups. (C) Relative mRNA expression of pro-inflammatory and anti-inflammatory genes. (D) Liver IL-10 levels in colon tissues. (E) Liver IL-17A levels in colon tissues. (F) MPO activity (U/g protein) in colon tissues. (G) SOD activity (U/mg protein) in colon tissues. (H) GSH-Px activity (U/mg protein) in colon tissues. Values are expressed as the mean ± standard deviation of at least three independent experiments. *P<0.05; **P<0.01. ns, no significance; VC, vitamin C; LA, *Lactobacillus acidophilus*; Ctrl, control; EH; ethanol; Treg, regulatory T; Foxp3, forkhead box p3; SOD, superoxide dismutase; MPO, myeloperoxidase; GSH-Px, glutathione peroxidase; FC, fold change; SSC, side scatter; FSC, forward scatter.

that ethanol exposure markedly increased the expression of genes encoding peroxisome proliferator activated receptor-γ (PPAR-γ) and transporter CD36 for fatty acids (P<0.01; Fig. 5F and G), which demonstrated that ethanol led to disorders of the liver functions of triglyceride synthesis and fatty acid uptake. However, LA+VC treatment significantly improved the liver function of triglyceride synthesis and fatty acid uptake with obviously reduced mRNA expression of PPAR-γ and CD36 (P<0.01; Fig. 5F and G). In addition, the decreased expression of Fas, stearoyl-CoA desaturase-1 (Scd1) and sterol regulatory element-binding transcription protein 1c (Srebp-1c) induced by ethanol exposure were improved via LA+VC treatment, suggesting that the LA+VC treatment likely accelerated fatty acid metabolism and attenuated the impairment of hepatic function induced by ethanol exposure. Treatment with LA and VC alone also alleviated

alcohol-induced liver injury, but the combined effects of LA+VC were stronger.

LA plus VC treatment ameliorates ethanol-induced liver inflammation. Ethanol exposure markedly increased the gut permeability and caused LPS translocation into the bloodstream, which contributed to ethanol-associated liver inflammation (36). To evaluate the effects of LA plus VC on liver inflammation states of ethanol-challenged mice, the TNF-α and IL-1β concentration in liver tissues was examined. The results revealed that ethanol exposure markedly stimulated the inflammatory response with increased TNF-α and IL-1β levels in liver tissues, which was significantly reduced by LA+VC (P<0.01; Fig. 6A and B). Next, the mRNA expression of genes in the LPS/TLR4-associated pathway was determined. The RT-qPCR results revealed that ethanol exposure obviously activated the myeloid differentiation
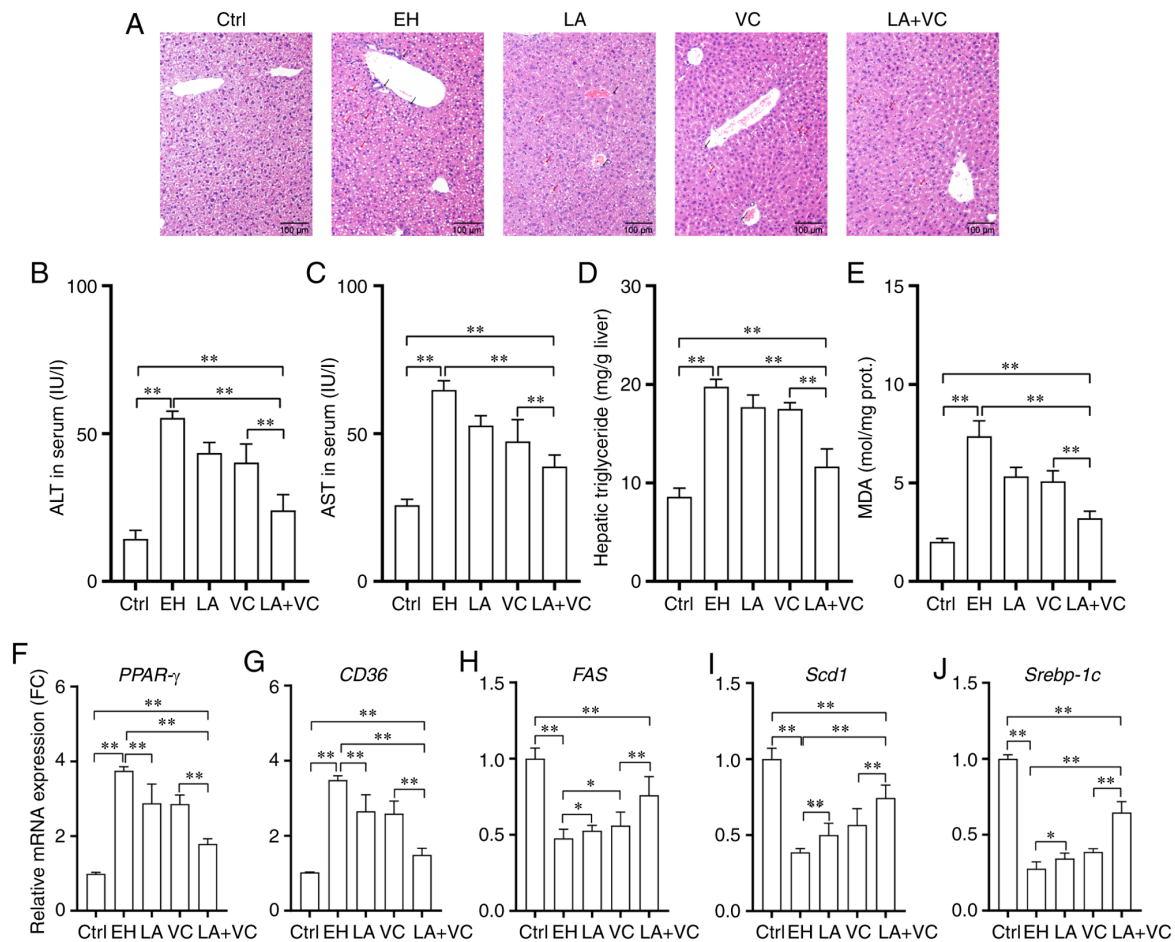
Figure 5. LA plus VC restores ethanol-induced liver function disorders. (A) Representative histological sections of the liver (H&E; magnification, x100; scale bar, 100 μm; red arrows indicate areas of steatosis and the black arrows indicate the sites of neutrophil infiltration in liver tissues). (B) Serum ALT levels. (C) Serum AST levels. (D) Hepatic triglyceride levels. (E) Hepatic MDA levels. Relative mRNA expression of (F) PPAR-γ, (G) CD36, (H) Fas, (I) Scd1 and (J) Srebp-1c in liver tissues. Values are expressed as the mean ± standard deviation of at least three independent experiments. *P<0.05; **P<0.01. VC, vitamin C; LA, *Lactobacillus acidophilus*; Ctrl, control; EH; ethanol; PPAR-γ, peroxisome proliferator activated receptor-γ; ALT, alanine aminotransferase; AST, aspartate aminotransferase; MDA malondialdehyde; FC, fold change; prot., protein; Scd1, stearoyl-CoA desaturase-1; Srebp-1c, sterol regulatory element-binding transcription protein 1c.

primary response 88 (Myd88)-dependent TLR4 signaling pathway with increased mRNA expression levels of TLR4, Myd88, IL-1 receptor associated kinase 4 (IRAK4) and TNF receptor associated factor 6 (TRAF6) (P<0.01; Fig. 6C). Since the LA+VC treatment obviously reduced the serum LPS accumulation in the ethanol-challenged mice (P<0.01; Fig. 3B), these mice had reduced mRNA expression of TLR4, Myd88, IRAK4 and TRAF6 (P<0.01; Fig. 6C). Furthermore, the mRNA expression of pro-inflammatory markers was examined and the results indicated that ethanol exposure significantly stimulated the mRNA expression of TNF-α, NF-κB, IL-1β and monocyte chemoattractant protein (MCP-1) (P<0.01; Fig. 6D). Of note, the LA+VC treatment significantly reduced the mRNA levels of TNF-α, NF-κB, IL-1β and MCP-1 in the ethanol-challenged mice (P<0.01; Fig. 6D). Taken together, LA+VC treatment alleviated the inflammatory response in the liver via the MyD88-dependent TLR4 signalling pathway.

Discussion

Mounting evidence revealed that gut microbiota dysbiosis has a crucial role in ethanol-associated organ injury and gut

microbiota-targeted therapy is emerging as an important adjuvant therapy for protecting the body against ethanol-induced damage (34,37-39). Previous studies have reported that single *Lactobacillus* species or VC treatment had beneficial effects by protecting against ethanol damage in murine models (40,41). However, to the best of our knowledge, no previous study has explored the efficiency and possibility of compatibility of *Lactobacillus* species and VC in the reduction of ethanol damage. The results of the present study indicated that LA plus VC restored gut microbiota homeostasis and improved gut barrier dysfunction via upregulating the tight junction proteins and mucus secretion, which prevented the translocation of LPS into circulatory systems and thus reduced the inflammatory responses induced by TLR4 in liver tissues. In this context, LA plus VC attenuated liver injury in ethanol-challenged mice.

Ethanol exposure leads to significant gut microbiota dysbiosis and reduces the abundance of *Lactobacillus* species (6,42). Of note, *Lactobacillus* species as probiotics inhibit pathogens within the Enterobacteriaceae family by producing bacteriocins and protect the intestine against invasive bacteria via adhering to intestinal epithelial cells (43,44). On the other hand, ethanol intake generally leads to the deficiency of VC in
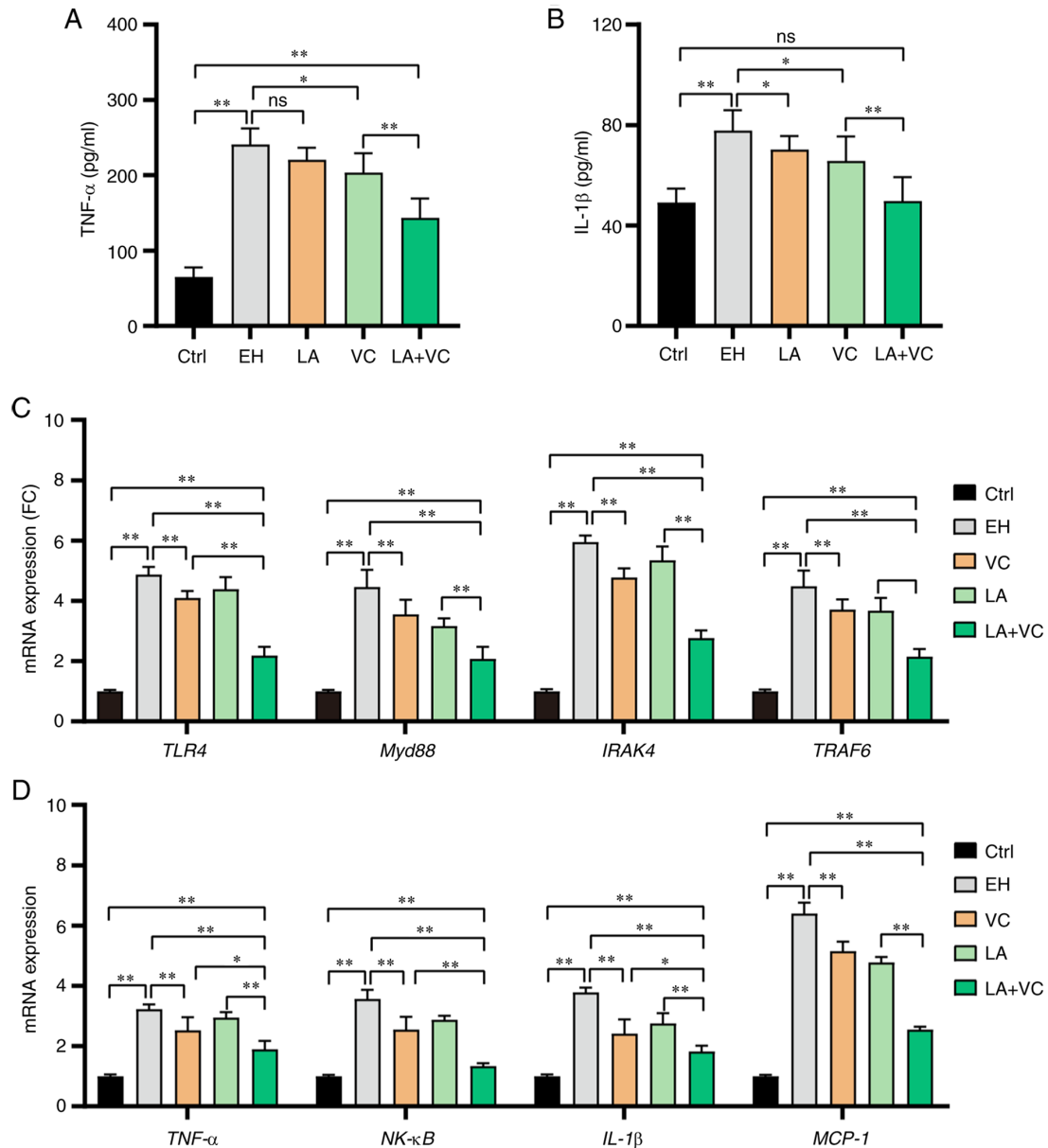
Figure 6. LA plus VC alleviates ethanol-induced liver inflammation. (A) TNF-α levels in liver tissues. (B) IL-1β levels in liver tissues. (C) Relative mRNA expression of TLR4, Myd88, IRAK4 and TRAF6 in liver tissues. (D) Relative mRNA expression of the proinflammatory cytokines TNF-α, NF-κB and IL-1β and the chemokine MCP-1 in liver tissues. Values are expressed as the mean ± standard deviation of at least three independent experiments. *P<0.05; **P<0.01. ns, no significance; MCP, monocyte chemoattractant protein; TLR4, Toll-like receptor 4; IRAK4, IL-1 receptor associated kinase 4; TRAF6, TNF receptor associated factor 6; Myd88, myeloid differentiation primary response 88; VC, vitamin C; LA, *Lactobacillus acidophilus*; Ctrl, control; EH; ethanol; FC, fold change.

the gut, which contributes to the overgrowth and transcytosis of enteric bacteria and causes accumulation of circulating LPS (45). The gut microbiota dysbiosis (reduced diversity and overgrowth of enteric pathogenic bacteria) exaggerates the intestinal inflammation and gut leakage induced by ethanol, which likely poses a great threat to the liver. In agreement with prior studies, single treatment with LA or VC partially restored the gut microbiota dysbiosis and slightly reduced the abundance of the enteric bacteria within the Enterobacteriaceae family. Specifically, treatment with LA+VC markedly increased the abundance of Firmicutes and Bacteroides and suppressed the overgrowth of Enterobacteriaceae, which suggests that LA+VC is able to relieve the ethanol damage to the gut microbiota. A balanced gut microbiota and microbial

metabolites contribute to regulating the proportion of Foxp3+ Treg cells in the intestine (46,47), which are critical in maintaining immune tolerance and homeostasis of the immune system (48). In terms of cytokines, Foxp3+ Treg cells express the immunosuppressive cytokine IL-10, which is important for the control of the inflammatory response. As indicated in previous studies, elevated levels of IL-10 may participate in suppressing the secretion of Th17 cytokines (49); thus, the inflammatory responses in alcohol-challenged mice were markedly attenuated by LA+VC.

Previous studies have revealed that alcohol consumption results in depletion of GSH levels and declined antioxidant activity (50,51). In the present study, single treatment with LA or VC was not able to significantly restore the SOD and

GSH-Px enzyme activity in the intestine. However, combined treatment of LA+VC significantly improved the SOD and GSH-Px enzyme activity in ethanol-challenged mice, which markedly attenuated the ethanol-induced oxidative stress in the intestine. In addition, activation of neutrophil granulocytes facilitates secretion of MPO and generation of oxidants (hypochlorous acid and tyrosyl radicals) which have an important role in the body's inflammatory response (52,53). In the present study, LA+VC treatment reduced the MPO activity in the colon tissues of ethanol-challenged mice, which contributed to alleviating ethanol-associated oxidative stress and inflammatory response in tissues. The improved intestinal oxidative stress and inflammatory response were associated with the restoration of intestinal barrier function. The reduced intestinal permeability in the mice treated with LA+VC was further confirmed by the reduced FITC and LPS concentrations in serum. In addition, ethanol exposure considerably reduced the excretion of mucus as well as the mRNA expression of mucus secretion-related proteins (Muc2/3/4 and Klf4) and tight junction-related components (claudin-2, ZO-1 and occludin), which was significantly restored by the treatment with LA+VC. Collectively, LA+VC treatment attenuated alcohol-induced intestinal injury, enhanced the intestinal barrier function and reduced the translocation of LPS from the gut into the circulation.

Increased LPS in the circulatory system triggers the innate immune response and leads to inflammatory response via the TLR4 pathway (54). The activated TLR4 receptor then stimulates the expression of inflammatory cytokines such as TNF-α, as well as NF-κB (55), which maintains a constant low-grade inflammatory state and has a negative influence on the liver (56). LA+VC treatment markedly reduced steatosis and neutrophil infiltration, as well as reducing oxidative stress in the liver of ethanol-challenged mice. In addition, LA+VC treatment improved triglyceride synthesis and fatty acid uptake via regulating PPAR-γ and CD36, and accelerated fatty acid metabolism through upregulating Fas, Scd1 and Srebp-1c in the liver of ethanol-challenged mice. Excessive ethanol intake leads to lipid metabolism disorders and perturbs fatty-acid transport and oxidation. Ethanol exposure activates the PPAR-γ receptor and inactivates the PPAR-α receptor in liver tissues (57-59), which promotes dysbiosis of fatty-acid metabolism via the retinoid X receptor (60). Ethanol exposure also decreases the mitochondrial membrane potential and causes mitochondrial dysfunction in liver tissues, and mitochondrial dysfunction and lipid metabolism disorders may lead to steatosis in the liver (61,62). Furthermore, inflammatory responses induced by ethanol exposure are frequently associated with an abnormal redox state in liver tissues, which promotes the phosphorylation of the p65 subunit of NF-κB and its nuclear translocation (63), and this inflammatory pathway may be interrupted by antioxidants (64,65). Furthermore, we hypothesized that nuclear factor erythroid 2 like 2, as the master regulator of the intracellular adaptive antioxidant response to oxidative stress, probably participated in the protective effect of LA+VC treatment against ethanol exposure via regulating the antioxidant response and impacting on ethanol metabolism (66-68). In the present study, the improved liver function was likely attributed to the alleviation of the inflammatory response, amelioration of the redox state and reduced mitochondrial dysfunction in liver tissues. Further studies should be performed to confirm the potential role of LA+VC in ethanol-challenged mice.

The primary mechanisms by which LA+VC significantly attenuated alcohol-induced intestinal injury involved restoring the gut microbiota, reinstating the immune balance, inhibiting pro-inflammatory cytokines, reducing oxidative stress and maintaining gut barrier function. Based on all of these results, it may be concluded that LA+VC attenuated intestinal inflammatory responses and oxidative stress, and restored the intestinal tight junction and mucus excretion, which markedly alleviated the translocation of gut-derived LPS. In addition, the decrease of LPS in serum contributed to the relief of inflammatory cytokine expression in the Myd88-dependent TLR4 pathway, which was responsible for the amelioration of liver function in ethanol-challenged mice. These results provide mechanisms by which LA+VC attenuated ethanol-induced intestinal and liver injury, and hence, guide the further exploration of synbiotics based on *Lactobacillus* species and VC. However, there were some limitations in the present study. The specific modulation of LA+VC treatment on gut microbiota and intestine of ethanol-treated mice were not clearly identified. In the examination of gene expression in intestine and liver tissues, three samples from each group were randomly selected, which may result in different error bar values.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Raw sequencing reads of 16S rRNA sequencing have been deposited in the NCBI Sequence Read Archive (accession nos. PRJNA732292 and SRX10973300; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA732292, https://www.ncbi.nlm.nih.gov/sra/?term=SRX10973300).

Authors' contributions

FW designed the experiments; XL performed experimental experiments and data analysis under the supervision of FW. XL and FW wrote the manuscript, and confirmed the authenticity of the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments of the present study were approved by the Ethics and Clinical Research Committee of Tianjin Medical University (Tianjin, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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