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Lactobacillus reuteri ZJ617 Culture Supernatant Attenuates Acute Liver Injury Induced in Mice by Lipopolysaccharide

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ABSTRACT

Background: *Lactobacillus rhamnosus* GG culture supernatant (LGGs) promotes intestinal integrity and ameliorates acute liver injury induced by alcohol in mice.

Objectives: The aim of this study was to investigate the protective effects and molecular mechanisms of *Lactobacillus reuteri* ZJ617 culture supernatant (ZJ617s) on acute liver injury induced by lipopolysaccharide (LPS) in mice.

Methods: Male C57BL/6 mice (20 ± 2 g, 8 wk old) were randomly divided into 4 groups (6 mice/group): oral inoculation with phosphate-buffered saline (control), intraperitoneal injection of LPS (10 mg/kg body weight) (LPS), oral inoculation with ZJ617s 2 wk before intraperitoneal injection of LPS (ZJ617s + LPS), or oral inoculation with LGGs 2 wk before intraperitoneal injection of LPS (LGGs + LPS). Systemic inflammation, intestinal integrity, biomarkers of hepatic function, autophagy, and apoptosis signals in the liver were determined.

Results: Twenty-four hours after LPS injection, the activities of serum alanine transaminase and aspartate transaminase were 32.2% and 30.3% lower in the ZJ617s + LPS group compared with the LPS group, respectively ($P < 0.05$). The ZJ617s + LPS group exhibited higher intestinal expression of claudin 3 (62.5%), occludin (60.1%), and zonula occludens 1 (60.5%) compared with the LPS group ($P < 0.05$). The concentrations of hepatic interleukin-6 and tumor necrosis factor-α were 21.4% and 27.3% lower in the ZJ617s + LPS group compared with the LPS group, respectively ($P < 0.05$). However, the concentration of interleukin-10 was 22.2% higher in the ZJ617s + LPS group. LPS increased the expression of Toll-like receptor 4 (TLR4; by 50.5%), phosphorylation p38 mitogen-activated protein kinase (p38MAPK; by 57.1%), extracellular signal-regulated kinase (by 77.8%), c-Jun N-terminal kinase (by 42.9%), and nuclear factor-κB (NF-κB; by 36.0%) compared with the control group. Supplementation with ZJ617s or LGGs ameliorated these effects ($P < 0.05$). Moreover, the hepatic expression of active caspase-3 and microtubule-associated protein 1 light chain 3 II was 23.8% and 28.6% lower in the ZJ617s + LPS group compared with the LPS group, respectively ($P < 0.05$).

Conclusions: ZJ617s exerts beneficial effects on the mouse liver through suppression of hepatic TLR4/MAPK/NF-κB activation, apoptosis, and autophagy. This trial was registered at Zhejiang University (http://www.lac.zju.edu.cn) as NO.ZJU20170529. *J Nutr* 2019;149:2046–2055.

Keywords: *Lactobacillus reuteri* ZJ617, *Lactobacillus rhamnosus* GG, liver, inflammation, MAPK signaling, autophagy, mice

Introduction

The liver is continuously exposed to gut-derived factors from the blood, including microbial components and their products (1, 2). Pattern recognition receptors, expressed by hepatocytes, recognize bacterial components such as LPSs, which subsequently triggers inflammatory pathways (3). Impairment of the intestinal barrier, termed "leaky gut," facilitates the translocation of bacterial components into the portal vein, leading to systemic inflammation in extraintestinal organs (e.g., the liver). Regulation of the innate immune response in the liver is partly mediated through Toll-like receptor (TLR) signals (4). For example, LPS activates TLR4 in Kupffer cells to produce pro-inflammatory cytokines, such as TNF-α, leading to hepatocyte damage. Therefore, the inflammatory response

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induced by the imbalance of the gut–liver axis contributes to the initiation and development of liver diseases such as alcohol-induced liver disease (5), nonalcoholic steatohepatitis (6), and liver cirrhosis (7).

A growing body of evidence obtained from both animal experiments and human clinical trials suggests that regulation of gut microbiota through the administration of certain probiotics may exert beneficial effects on various hepatic diseases. For instance, *Lactobacillus rhamnosus* GG (LGG) attenuated alcohol-induced hepatic inflammation through inhibition of TLR4-mediated endotoxin activation and TNF-α production (8). Another recent study indicated that probiotic *Lactobacillus paracasei* GMNL-32, *Lactobacillus reuteri* GMNL-89, and *L. reuteri* GMNL-263 mitigated hepatic inflammation and apoptosis by suppressing the MAPK and NF-κB signaling pathways in lupus-prone mice (9). Similarly, in a rat model of acute liver failure, probiotic *Lactobacillus casei* Zhang reduced LPS/D-galactosamine-induced production of TNF-α and nitric oxide in the liver by regulating TLR/MAPK/PPAR-γ signaling and the composition of the gut microbiota (10). Interestingly, Zhao et al. (11) found that *Lactobacillus plantarum* C88 protected against chronic ethanol-induced liver oxidative injury by upregulating nuclear factor erythroid 2-related factor 2 and its downstream antioxidative genes. These findings suggest that probiotics exert health benefits on both animals and humans by modulating the immune and antioxidant system in the liver via the gut–liver axis.

As an alternative to live probiotics, inactivated bacteria and their metabolites have been demonstrated to be effective. The probiotic-mediated restitution of the colonic barrier function and the amelioration of hepatic disease may be the primary result of direct effects on intestinal permeability. Secreted soluble factors are involved in the protective effects on the intestinal barrier (12). The proteins p75 and p40, isolated from LGG culture supernatant (LGGs), have been shown to effectively block the induction of apoptosis and promote proliferation of mouse colon epithelial cells (13). Moreover, LGGs ameliorated liver injury by suppressing the increased intestinal permeability and endotoxemia induced by acute alcohol intake (14). In another study, LGGs prevented chronic alcohol-induced hepatic steatosis and injury by suppression of AMP-activated protein kinase phosphorylation and Bax/Bcl-2-mediated apoptosis (15). Furthermore, a recent study showed that heat-killed *L. reuteri* GMNL-263 was able to decrease high-fat diet-induced metabolic abnormalities by inhibiting the development of hepatic steatosis (16). Therefore, supplementation with certain probiotics and/or their cell-free culture supernatant may confer protective effects to immune function and protect against liver disorders induced by gut-derived factors.

Lactobacillus reuteri ZJ617 is a novel probiotic strain isolated from piglets in our laboratory and selected according to desirable probiotic attributes (17). Subsequently, we found that this probiotic bacterium attenuates LPS-induced intestinal inflammation by suppressing the MAPK/NF-κB signaling pathway (18). In addition, endotoxin-induced autophagy is associated with innate immunity (19). One of the downstream responses to TLR4 signaling following stimulation by LPS is the induction of autophagy (20). However, to date, no studies have investigated the function of the ZJ617 cell-free culture supernatant (ZJ617s). We hypothesized that the culture supernatant obtained from ZJ617 may exert a similar effect to that observed with LGG on acute liver injury. Hepatic abnormality has been strongly associated with an impaired intestinal barrier. Therefore, the current study investigated the mechanisms underlying the protective effect of ZJ617s against LPS-induced acute liver injury, focusing on the link between intestinal barrier, hepatic inflammation, and autophagy.

Methods

Preparation of bacterial culture supernatants

LGG was a gift from Prof. Jinru Chen at the University of Georgia. ZJ617 was previously isolated in our laboratory from the intestine of piglets (17). LGG and ZJ617 were anaerobically cultured at 37°C in de Man, Rogosa, and Sharpe medium (MRS; Britania) for 18 h. The viability of the micro-organisms was determined by CFUs. These were counted by diluting and streaking on MRS agar plates (Difco), followed by overnight culture at 37°C. ZJ617 and LGG were cultured in liquid MRS medium for 18 h until they reached a bacterial density of 8×10^9 CFU/mL. The cell-free culture supernatant (ZJ617s and LGGs) was obtained from the culture suspension through centrifugation at 10,000 × *g* for 10 min. Subsequently, the supernatant was sterilized via filtering through a 0.22-mm filter. ZJ617s and LGGs were stored at −80°C before being orally administered to mice.

Animals and treatments

All procedures involving animals were performed in full accordance with the "Regulation for the Use of Experimental Animals" in Zhejiang Province, China. This research was specifically approved by the Animal Care and Use Committee of Zhejiang University (Ethics Code Permit ZJU20170529). Male C57BL/6 mice (20 ± 2 g body weight, aged 8 wk) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). They were maintained at 25°C with 12-h light–dark cycles and provided with ad libitum access to food and water. A commercial feed composed of fish meal, wheat, corn, soybean meal, wheat bran, vitamins, minerals, and amino acids was used (P1101F; Slacom). The ration contained at least 20.5% crude protein, 4% crude fat, 1.32% lysine, and 0.78% methionine + cysteine, along with ≤5% crude fiber and ≤8% crude ash. Mice were randomly assigned to 4 groups (6 mice/group). PBS was administered to the control and LPS groups through oral gavage. The ZJ617s + LPS and LGGs + LPS groups were orally inoculated with 0.2 mL of ZJ617s or LGGs daily for 2 wk (equivalent to supernatant from 10^9 CFU bacteria). At 14 d after the initiation of the oral gavage, the LPS, ZJ617s + LPS, and LGGs + LPS groups were intraperitoneally injected with 10 mg/kg LPS of *Escherichia coli* serotype 055:B5 (Sigma). The control group received an intraperitoneal injection of sterile PBS. Blood samples and intestine and liver tissues were collected 24 h after the administration of LPS. To exclude the potential effects of MRS present in the supernatant on the outcome of the experiment, the following 4 groups (6 mice/group) were included in the study as a supplement trial: mice orally inoculated with ZJ617s alone for 2 wk (ZJ617s), mice orally inoculated with

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Supplemental Figures 1–4 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
YC and SQ contributed equally to this work and share first authorship.
Address correspondence to HW (e-mail: haifengwang@zju.edu.cn)
Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DAO, diamine oxidase; ERK, extracellular signal-regulated kinase; GalN, D-galactosamine; JNK, c-Jun N-terminal kinase; LC3, microtubule-associated protein 1 light chain 3; LGGs, *Lactobacillus rhamnosus* GG culture supernatant; MRS, de Man, Rogosa, and Sharpe medium; TJ, tight junction; TLR, Toll-like receptor; ZJ617s, *Lactobacillus reuteri* ZJ617 culture supernatant; ZO; zonula occludens.

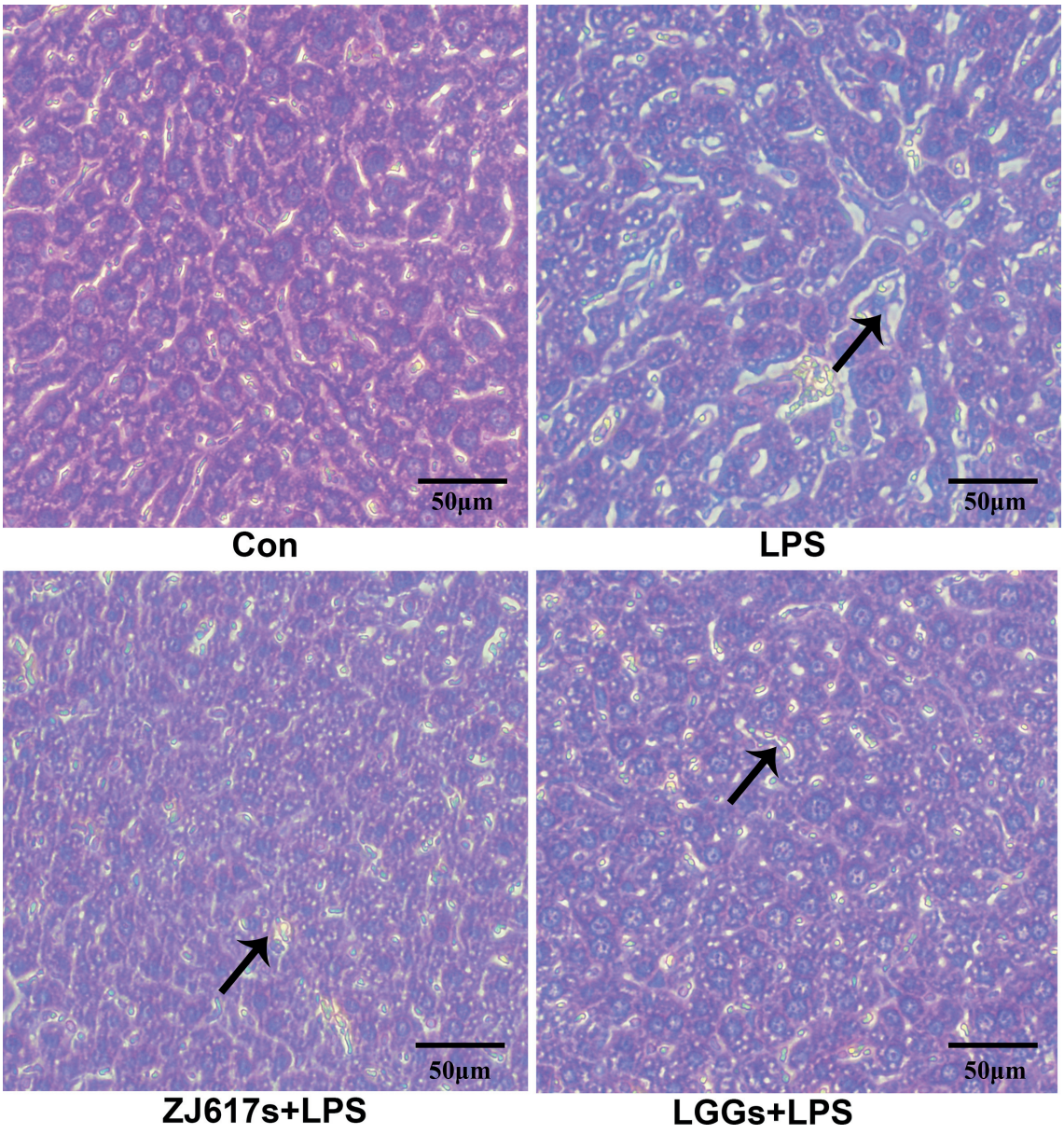

FIGURE 1 Effects of pretreatment with ZJ617s and LGGs on liver histology (original magnification: ×400). C57BL/6 mice were orally inoculated with ZJ617s or LGGs for 2 wk and i.p. injected with LPS (10 mg/kg body weight) for 24 h. Representative photomicrographs of hematoxylin and eosin-stained sections of the liver. Con, control mice orally inoculated with PBS; LPS, mice i.p. injected with LPS (10 mg/kg body weight); ZJ617s + LPS, mice orally inoculated with ZJ617s for 2 wk before i.p. injection of LPS; LGGs + LPS, mice orally inoculated with LGGs for 2 wk before i.p. injection of LPS. LGGs, *Lactobacillus rhamnosus* GG culture supernatant; ZJ617s, *Lactobacillus reuteri* ZJ617 culture supernatant. Arrows in Fig. 1 indicate hepatocyte vaculation.

LGGs alone for 2 wk (LGGs), mice orally inoculated with MRS alone for 2 wk (MRS), and mice orally inoculated with MRS for 2 wk before intraperitoneal injection of LPS (10 mg/kg body weight; MRS + LPS).

Biochemical assays for the serum and liver tissues

The concentrations of TNF-α and IL-6 in the serum were measured using ELISA and commercially available kits (nos. H052 and H007, respectively; Nanjing Jiancheng Bioengineering Institution). The concentrations of TNF-α, IL-6, and IL-10 in liver were quantified using ELISA kits (nos. H052, H007, and H009, respectively; Nanjing Jiancheng Bioengineering Institution). Liver tissue homogenates were prepared as previously described (10), and the supernatant was analyzed according to the instructions provided by the manufacturer. Activities of alanine aminotransferase (ALT; no. C009), aspartate aminotransferase (AST; no. C010), and diamine oxidase (DAO; no. A088) and the concentration of D-xylose (no. A035) in the serum were determined by kinetics-based assays with commercially available kits (Nanjing Jiancheng Bioengineering Institution) using an automatic biochemistry analyzer (Selecta XL; Vital Scientific) according to the protocol provided by the manufacturer.

Hematoxylin and eosin staining

Hematoxylin and eosin staining was performed as previously described (21). Briefly, liver samples from the mice were soaked in 10% neutral paraformaldehyde and covered with wax. The waxed tissue blocks were sliced manually into 3-μm-thick sections and prepared by deparaffinization and dehydration. The sections were soaked in a series of graded alcohols (100%, 95% and 75%) for 15 min each and subsequently stained with hematoxylin and eosin. Photomicrographs were obtained via optical microscopy (Olympus Corporation).

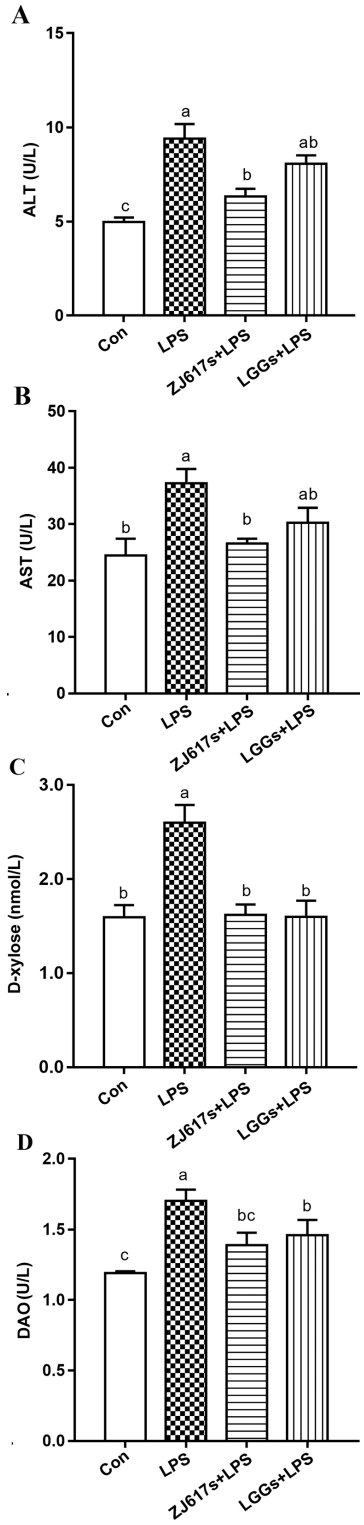

FIGURE 2 Concentrations of ALT (A), AST (B), D-xylose (C), and DAO (D) in the serum of control mice and those orally inoculated with ZJ617s or LGGs for 2 wk and i.p. injected with LPS after 24 h. Data are expressed as means ± SEs ($n = 6$). Means for variables that do not share a common letter differ significantly, $P < 0.05$. Con, control mice orally inoculated with PBS; LPS, mice i.p. injected with LPS (10 mg/kg body weight); ZJ617s + LPS, mice orally inoculated with ZJ617s for 2 wk before i.p. injection of LPS; LGGs + LPS, mice orally inoculated with LGGs for 2 wk before i.p. injection of LPS. ALT, alanine aminotransferase; AST, aspartate aminotransferase; DAO, diamine oxidase; LGGs, *Lactobacillus rhamnosus* GG culture supernatant; ZJ617s, *Lactobacillus reuteri* ZJ617 culture supernatant.

Western blotting analysis

Liver tissues were lysed using a lysis buffer (Sigma). Total protein concentration was determined using the Bradford method (22). Western blotting analysis was performed as previously described (23). The primary antibodies used in this experiment included rabbit anti-claudin 3, anti-occludin, anti-zonula occludens 1 (ZO1), anti-TRL4, anti-p38 and anti-phospho-p38 (p-p38), anti-c-Jun N-terminal kinase (JNK) and anti-p-JNK, anti-extracellular signal-regulated kinase (ERK) and anti-p-ERK, anti-p-p65, anti-IκB, anti-caspase-3, anti-Bax, anti-Beclin1, anti-Atg5, and anti-microtubule-associated protein 1 light chain 3 (LC3; Cell Signaling Technology; 1:1000). The specific proteins were detected by using an enhanced chemiluminescence kit (Perkin Elmer). Protein bands were visualized with a chemiluminescence substrate and a gel-imaging system (Tanon Science and Technology) and analyzed via image analysis software (NIH). In all instances, the density values of bands were corrected through subtraction of background values. GAPDH was used as the internal reference protein. Bands were standardized to the density of GAPDH.

Statistical analysis

Experimental data are presented as means ± SEs. Statistical significance was analyzed through 1-factor ANOVA, followed by Duncan's multiple range test using SAS software (SAS Institute). Significance was determined at $P < 0.05$.

Results

Liver histopathology

Pathological damage was characterized by irregular hepatic lobular architecture, abnormal hepatocytes, and numerous inflammatory accumulation, all of which were not observed in the control, ZJ617s, LGGs, and MRS groups (**Figure 1**, **Supplemental Figure 1**). Compared with the LPS group, there was a remarkable decrease in hepatic inflammatory infiltration in both the ZJ617s + LPS and LGGs + LPS groups, suggesting amelioration of liver histopathology. This beneficial effect was not observed in the MRS + LPS groups (Supplemental Figure 1). These results indicate that supplementation with ZJ617s or LGGs may play a role during endotoxic shock, which seems to not be associated with MRS present in ZJ617s or LGGs.

Serum biomarkers of hepatic function

The activities of ALT and AST in the serum were significantly increased following the administration of LPS compared with the control group (0.85-fold and 0.44-fold, respectively; $P < 0.05$). However, compared with the LPS group, pretreatment with ZJ617s significantly decreased the activities of ALT and AST in the serum by 32.2% and 30.3%, respectively ($P < 0.05$; **Figure 2**A, B). This indicates that pretreatment with ZJ617s ameliorated LPS-induced liver injury.

Permeability of intestines and expression of tight junction proteins

Administration of LPS significantly increased the concentrations of D-xylose (Figure 2C) and DAO (Figure 2D) in the serum. In contrast, pretreatment with ZJ617s and LGGs significantly reduced the concentration of D-xylose versus that observed in the control group ($P < 0.05$). Treatment with LPS resulted in lower expression levels of claudin 3, occludin, and ZO1 ($P < 0.05$). However, pretreatment with ZJ617s significantly increased the expression levels of claudin 3, occludin, and ZO1 ($P < 0.05$) compared with those reported in the control group ($P < 0.05$) (**Figure 3**A–C). Pretreatment with LGGs significantly increased the expression levels of claudin 3 and ZO1

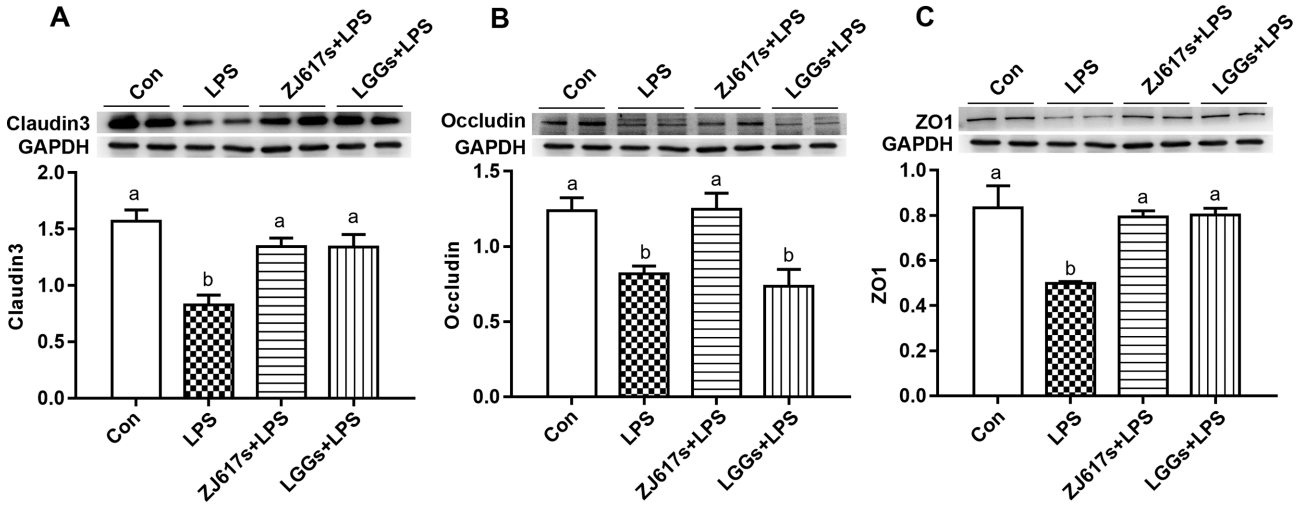

FIGURE 3 Western blotting analysis of the expression of intestinal tight junction proteins claudin 3 (A), occludin (B), and ZO1 (C) in control mice and those orally inoculated with ZJ617s or LGGs for 2 wk and i.p. injected with LPS after 24 h. The protein bands were quantified through densitometry analysis and normalized to the level of GAPDH. Data are expressed as means ± SEs ($n = 6$). Means for variables that do not share a common letter differ significantly, $P < 0.05$. Con, control mice orally inoculated with PBS; LPS, mice i.p. injected with LPS (10 mg/kg body weight); ZJ617s + LPS, mice orally inoculated with ZJ617s for 2 wk before i.p. injection of LPS; LGGs + LPS, mice orally inoculated with LGGs for 2 wk before i.p. injection of LPS. LGGs, *Lactobacillus rhamnosus* GG culture supernatant; ZJ617s, *Lactobacillus reuteri* ZJ617 culture supernatant; ZO1, zonula occludens 1.

compared with those observed in the control group ($P < 0.05$) (Figure 3A, C).

Liver and serum inflammatory factors

In the liver, the LPS group exhibited higher concentrations of IL-6 and TNF-α (by 31.3% and 60.0%, respectively) compared with those reported in the control group (**Figure 4**A, B). In the ZJ617s + LPS group, these concentrations were lower than those observed in the LPS group (by 28.6% and 33.3%, respectively; $P < 0.05$) but did not differ from those reported in the control group (Figure 4A, B). Administration of LPS resulted in a significant decrease in the concentration of hepatic IL-10 (by 43.8%). However, pretreatment with ZJ617s and LGGs significantly increased the concentration of hepatic IL-10 by 22.2% and 38.9%, respectively ($P < 0.05$; Figure 4C). Consistently, treatment with LPS led to significant increases in the concentrations of TNF-α and IL-6 in the serum. In contrast, the probiotic supernatants, particularly ZJ617s, significantly decreased these concentrations ($P < 0.05$; Figure 4D, E; **Supplemental Figure 2**). Notably, no significant difference was observed in the concentration of TNF-α in the serum between the LPS and MRS + LPS groups ($P > 0.05$; Supplemental Figure 2).

Hepatic TLR4/MAPK/NF-κB signaling

Mice treated with LPS demonstrated a significantly higher abundance of TLR4 compared with those injected with PBS ($P < 0.01$). Compared with the mice that received the LPS injection, those fed ZJ617s or LGGs exhibited a significantly lower expression level of TLR4 ($P < 0.05$). This level was not different from those reported in control mice (**Figure 5**A). In addition, there was no significant difference in the expression level of TRL4 in the liver between the MRS and MRS + LPS groups ($P > 0.05$; **Supplemental Figure 3**). Moreover, the hepatic concentrations of p-p38 ($P < 0.01$), p-ERK ($P < 0.05$), p-JNK ($P < 0.05$), and p-p65 ($P < 0.01$) were higher in the LPS group than in the control group (Figure 5B–E, respectively). The protein expression levels of p-p38, p-ERK, p-JNK, and p-p65 were lower in the ZJ617s + LPS and LGGs + LPS groups than in the LPS group ($P < 0.05$). The ZJ617s + LPS and LGGs + LPS groups showed lower expression levels of p-p38 and p-p65 compared with the control group (Figure 5B and E, respectively). Furthermore, ZJ617s + LPS exhibited similar expression levels of p-ERK and p-JNK to those reported in the control (Figure 5C and D, respectively).

The apoptotic signaling pathway in the liver

Compared with the control group, administration of LPS led to a significant increase in the ratio of cleaved caspase-3 to caspase-3 ($P < 0.05$) and Bax protein expression ($P < 0.01$). However, the ZJ617s + LPS group exhibited lower expression levels of these proteins compared with the LPS group ($P < 0.05$; **Figure 6**A and B, respectively). Moreover, the ratio of cleaved caspase-3 to caspase-3 in the ZJ617s + LPS group did not differ from that reported in the control group (Figure 6A). The expression of the Bax protein was significantly lower in the LGGs + LPS group than in the LPS group ($P < 0.05$; Figure 6B). Furthermore, no difference was observed in the ratio of cleaved caspase-3 to caspase-3 between the MRS and MRS + LPS groups ($P > 0.05$; Supplemental Figure 3B).

The autophagic signaling pathway in the liver

The LPS group showed higher expression levels of Beclin1, Atg5, and LC3-II compared with the control group. In contrast, the ZJ617s + LPS and LGGs + LPS groups exhibited lower expression levels of autophagic proteins compared with the LPS group, and they exhibited similar levels as those reported in the control group ($P < 0.05$; **Figure 7**A–C). These results suggest that ZJ617s + LPS or LGGs + LPS may normalize LPS-induced autophagy during endotoxic shock. This beneficial effect was not observed in the MRS + LPS group (Supplemental Figure 3C).

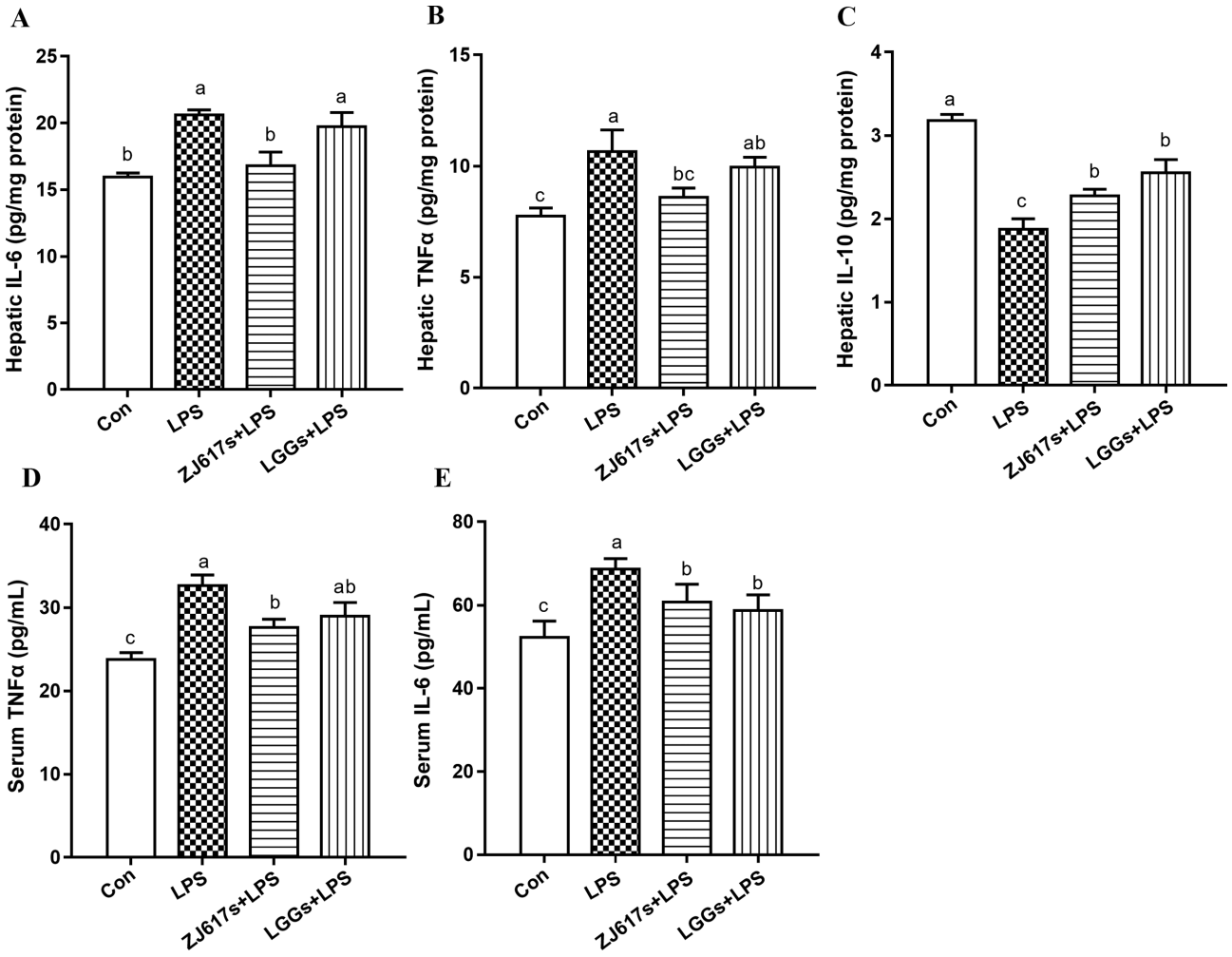

FIGURE 4 Hepatic concentrations of inflammatory cytokines IL-6 (A), TNF-α (B), and IL-10 (C) and serum concentrations of TNF-α (D) and IL-6 (E) in control mice and those orally inoculated with ZJ617s or LGGs for 2 wk and i.p. injected with LPS after 24 h. Data are expressed as means ± SEs ($n = 6$). Means for variables that do not share a common letter differ significantly, $P < 0.05$. Con, control mice orally inoculated with PBS; LPS, mice i.p. injected with LPS (10 mg/kg body weight); ZJ617s + LPS, mice orally inoculated with ZJ617s for 2 wk before i.p. injection of LPS; LGGs + LPS, mice orally inoculated with LGGs for 2 wk before i.p. injection of LPS. LGGs, *Lactobacillus rhamnosus* GG culture supernatant; ZJ617s, *Lactobacillus reuteri* ZJ617 culture supernatant.

Discussion

Following the disruption of gut homeostasis, gut-derived bacterial components are prone to translocation into the portal circulation, triggering systemic, pro-inflammatory immunity. Excessive immune activation may result in acute liver failure (24). The release of bacterial products, such as endotoxin from nonviable bacteria, may result in numerous physiopathological consequences (25). In the current study, we demonstrated that ZJ617s decreased gut permeability and suppressed the release of hepatic pro-inflammatory cytokines and hepatic injury through inhibition of the TLR4/MAPK/NF-κB and autophagy signaling pathways. These findings reveal the beneficial effects of ZJ617s on hepatic inflammation and autophagy during endotoxic shock in mice.

Probiotic culture supernatants are composed of multiple active substances secreted from live bacteria. Certain probiotic factors, including short-chain fatty acids (26), proteins (13), polyamines (27), and proteins p75 and p40, have been identified as main contributors to the beneficial effects of probiotics. The cellular barrier of the gut is composed of a layer of simple columnar epithelial cells interspersed with specialized cells such as goblet cells, lymphocytes, and M cells. Maintenance of normal epithelial cell structure and function, including the preservation of tight junction (TJ) proteins, prevents the transepithelial or paracellular migration of bacteria (28). Studies have shown that probiotic culture supernatants exert therapeutic effects on gut dysbiosis and liver disease through enhancement of immune function and intestinal barrier integrity (14, 15). However, the protective effects of the novel probiotic strain ZJ617 and its culture supernatant against liver injury have not been studied.

This is the first study to demonstrate the beneficial effects of ZJ617s against LPS-induced liver injury. These effects were characterized by decreased concentrations of AST and ALT in the plasma, alleviation of hepatic histological changes, and suppression of pro-inflammatory cytokines. This evidence is consistent with previous research showing that probiotics led to a significant reduction in liver transaminases, TNF-α, and insulin resistance (29). Patients with nonalcoholic fatty liver disease exhibited reduced concentrations of ALT, TNF-α, and leptin in the serum after 1 y of treatment with probiotic VSL#3 (30). Our previous study showed that live ZJ617 may also reduce the production of TNF-α in LPS-challenged mice (18).

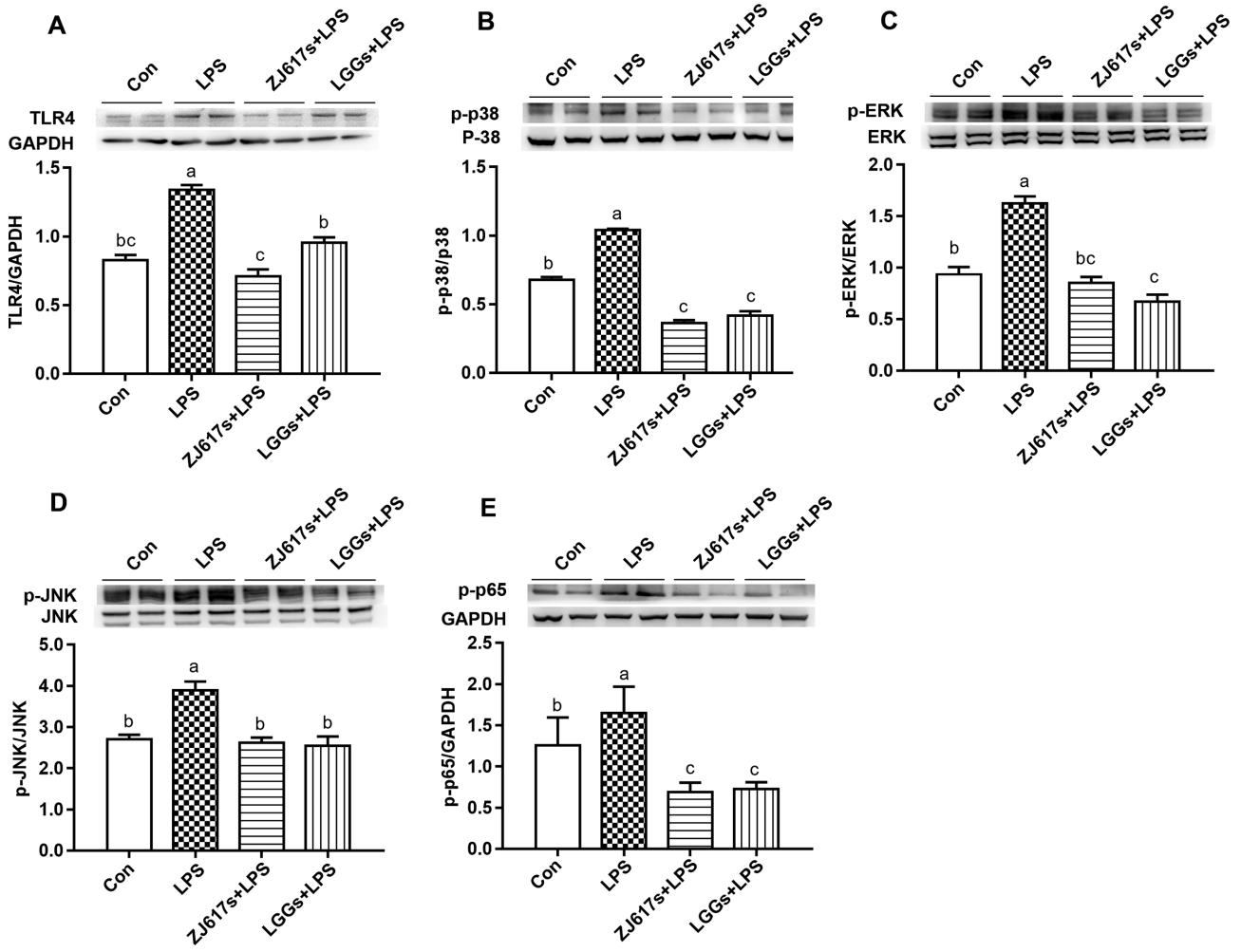

FIGURE 5 Western blotting analysis of the expression of liver TLR4/MAPK/NF-κB signaling proteins TLR4 (A) and p-p38 (B), p-ERK (C), p-JNK (D), and p-p65 (E) in control mice and those orally inoculated with ZJ617s or LGGs for 2 wk and i.p. injected with LPS after 24 h. The protein bands were quantified through densitometry analysis and normalized to the level of GAPDH. Data are expressed as means ± SEs ($n = 6$). Means for variables that do not share a common letter differ significantly, $P < 0.05$. Con, control mice orally inoculated with PBS; LPS, mice i.p. injected with LPS (10 mg/kg body weight); ZJ617s + LPS, mice orally inoculated with ZJ617s for 2 wk before i.p. injection of LPS; LGGs + LPS, mice orally inoculated with LGGs for 2 wk before i.p. injection of LPS. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; LGGs, *Lactobacillus rhamnosus* GG culture supernatant; ZJ617s, *Lactobacillus reuteri* ZJ617 culture supernatant; TLR4, Toll-like receptor 4.

These findings suggest that ZJ617 and its cell-free culture supernatant are effective in suppressing systemic inflammation.

Pro-inflammatory cytokines increase epithelial permeability, allowing the diffusion of gut-derived endotoxin into the blood and liver and exacerbating hepatic inflammation (31). Notably, ZJ617s attenuated the LPS-induced increase in the concentrations of D-xylose and DAO, suggesting restoration of intestinal integrity. Previous studies indicated that disruption of the TJ between intestinal epithelial cells is a key reason for the dysfunction of the intestinal barrier (32–34). The current study showed that LPS significantly decreased the abundance of barrier-forming TJ proteins, and ZJ617s normalized these alterations. Therefore, it is reasonable to speculate that the decreased inflammatory responses in response to pretreatment with ZJ617s are partly attributed to the maintenance of intestinal barrier function, which is dependent on TJ integrity. Consistent with our findings, a previous study showed that the LGG culture supernatant ameliorates acute alcohol-induced intestinal permeability and liver injury (14). Moreover, mice injected with the combination of D-galactosamine (GalN) and LPS exhibited a breakdown in the function of the colonic barrier. This effect was correlated with enhanced secretion of pro-inflammatory cytokines, bacterial translocation, and significant hepatic injury. Treatment with *Lactobacillus fermentum* and *Lactobacillus salivarius* was linked to the preservation of intestinal barrier function in dextran sulfate sodium-induced mouse colitis (35). Pretreatment with the oral probiotic compound VSL#3 for 7 d prevented the observed breakdown in intestinal barrier function, reduced bacterial translocation, and significantly attenuated liver injury (36).

Translocation of bacterial endotoxin into the portal circulation activates inflammatory response and leads to liver injury through the activation of various pattern recognition receptors (e.g., TLR4) on the surface of hepatic Kupffer cells (37). The binding of LPS to TLR4 induces a cascade of MAPK (p38, ERK, and JNK) phosphorylation, activation of NF-κB signaling, and subsequently the overexpression of pro-inflammatory cytokines (38). Downstream mediators generate a pro-tumorigenic milieu in a pre-existing inflammatory microenvironment leading to the development of hepatic injury. LPS-induced TLR4 signaling promotes hepatic carcinogenesis in a mouse model of liver damage induced by a combination

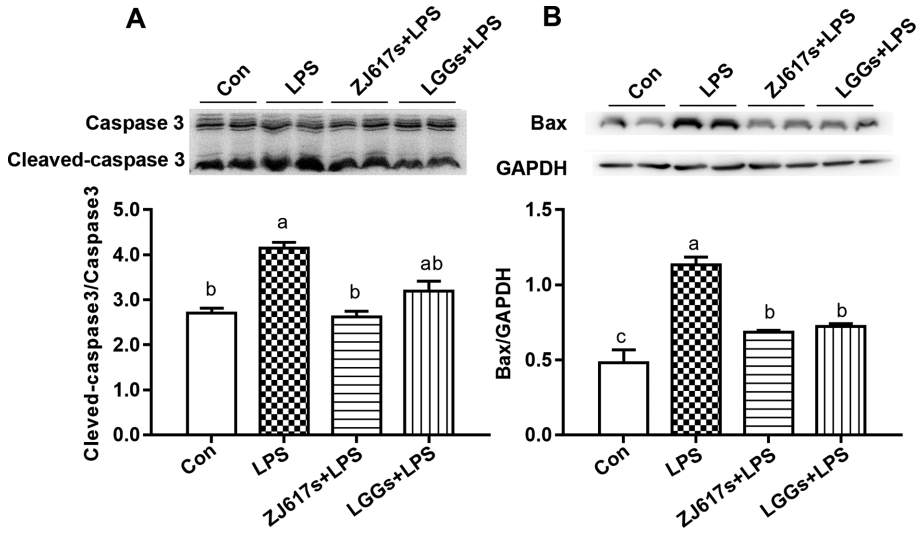

FIGURE 6 Western blotting analysis of the expression of hepatic apoptosis proteins cleaved caspase-3/caspase-3 (A) and Bax (B) in control mice and those orally inoculated with ZJ617s or LGGs for 2 wk and i.p. injected with LPS after 24 h. The protein bands were quantified through densitometry analysis and normalized to the level of GAPDH. Data are expressed as means ± SEs ($n = 6$). Means for variables that do not share a common letter differ significantly, $P < 0.05$. Con, control mice orally inoculated with PBS; LPS, mice i.p. injected with LPS (10 mg/kg body weight); ZJ617s + LPS, mice orally inoculated with ZJ617s for 2 wk before i.p. injection of LPS; LGGs + LPS, mice orally inoculated with LGGs for 2 wk before i.p. injection of LPS. LGGs, *Lactobacillus rhamnosus* GG culture supernatant; ZJ617s, *Lactobacillus reuteri* ZJ617 culture supernatant.

of diethylnitrosamine and hepatotoxin carbon tetrachloride (39). Therefore, we hypothesize that the decreased production of inflammatory cytokines TNF-α and IL-6 in the liver of mice treated with ZJ617s may be related to the regulation of the TLR4/MAPK/NF-κB signaling pathway. In support of this hypothesis, previous studies have demonstrated that probiotic *L. casei* Zhang attenuated LPS/GalN-induced liver inflammation through inhibition of ERK, JNK, and p38 MAPK phosphorylation and inflammatory mediators (i.e., myeloperoxidase and IL-1β) (10). Likewise, *L. plantarum* C88 downregulated the expression of NF-κB in a LPS/GalN-induced acute liver injury mouse model (40). Furthermore, treatment with a probiotic mixture containing *Bifidobacterium* and *Lactobacillus* species prevented LPS/GalN-induced liver injury through the activation of peroxisome proliferator-activated receptor that modulates NF-κB activity (36).

Our previous studies have shown that live ZJ617 suppressed LPS-induced MAPK activation in the ilea of mice (18). Notably, in the current study, we found that the expression of TLR4, p-ERK, p-JNK, p-p38 MAPK, and p-p65 NF-κB was reduced following pretreatment with ZJ617s and LGGs in LPS-treated mice. Collectively, our results indicate that pretreatment with

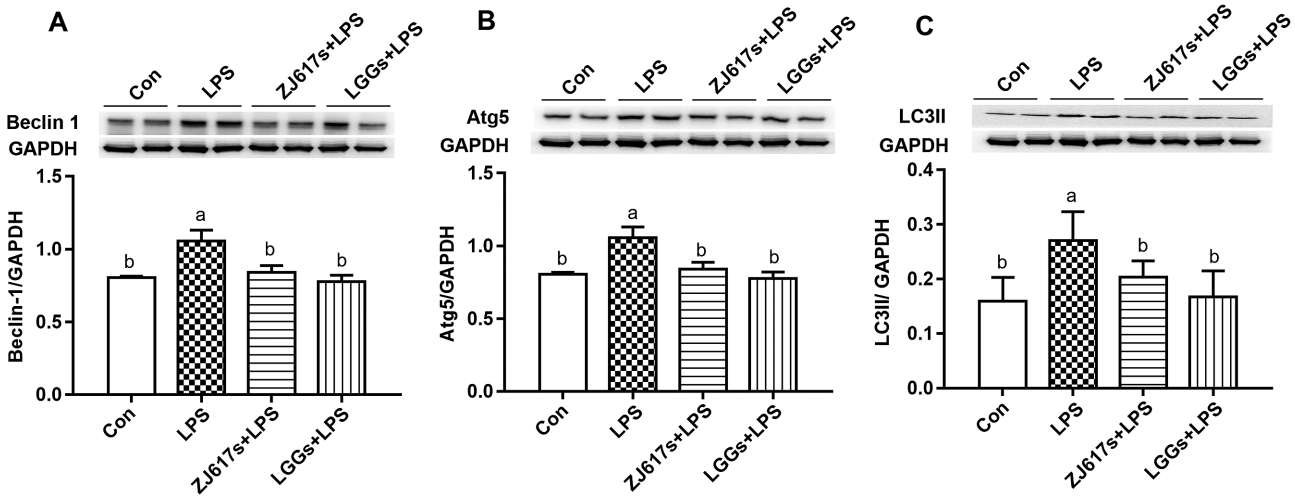

FIGURE 7 Western blotting analysis of the expression of hepatic autophagy proteins Beclin1 (A), Atg5 (B), and LC3-II (C) in control mice and those orally inoculated with ZJ617s or LGGs for 2 wk and i.p. injected with LPS after 24 h. The protein bands were quantified through densitometry analysis and normalized to the level of GAPDH. Data are expressed as means ± SEs ($n = 6$). Means for variables that do not share a common letter differ significantly, $P < 0.05$. Con, control mice orally inoculated with PBS; LPS, mice i.p. injected with LPS (10 mg/kg body weight); ZJ617s + LPS, mice orally inoculated with ZJ617s for 2 wk before i.p. injection of LPS; LGGs + LPS, mice orally inoculated with LGGs for 2 wk before i.p. injection of LPS. LC3-II, microtubule-associated protein 1 light chain 3 II; LGGs, *Lactobacillus rhamnosus* GG culture supernatant; ZJ617s, *Lactobacillus reuteri* ZJ617 culture supernatant.

ZJ617s or LGGs may reduce TLR4/MAPK/NF-κB signaling, thus inhibiting the secretion of inflammatory cytokines and blunting hepatic injury.

A major finding of this study was that ZJ617s and LGGs reduced liver apoptosis and, consequently, attenuated LPS-induced hepatic injury. TLR4 mediates cell apoptosis through the activation of the MAPK signaling pathway (41). Indeed, administration of LPS contributed to an increase in the expression of cleaved caspase-3, which is indicative of apoptosis. Thus, the underlying mechanism of the antiapoptotic effects exerted by ZJ617s and LGGs may involve the deactivation of the TLR4/MAPK signaling pathway. Our results are consistent with those obtained from a previous in vitro study, showing that LGG prevented cytokine-induced apoptosis in both human and mouse intestinal epithelial cells through inhibition of p38 MAPK activation (13). In addition, we showed that ZJ617s and LGGs mitigated liver apoptosis by downregulating the proapoptotic factor Bax. Bax directly opens the mitochondrial permeability transition pore, thereby facilitating the release of cytochrome c into the cytosol and activating apoptotic signaling pathways (42). Collectively, these results indicate that ZJ617s and LGGs robustly reduce LPS-induced liver apoptosis.

Autophagy plays a crucial role in cellular homeostasis and adaption to environmental stimuli such as serum starvation, oxidative stress, and pathogen invasion (43, 44). Recent studies demonstrated that disruption of autophagy induces various hepatic diseases, such as viral hepatitis, fatty liver, and steatosis. In contrast, excessive autophagy is responsible for pathological conditions and even autophagic cell death (type II programmed cell death); notably, inhibition of this process reduces hepatocytotoxicity (45). In the current study, we showed that ZJ617s and LGGs reduced LPS-induced liver autophagy and alleviated liver injury. LPS can directly induce autophagy in immune cells (e.g., the murine macrophage RAW264.7 cell line) and hepatocytes (e.g., AC2F rat liver hepatocytes and the HepG2 human hepatoma cell line) through activation of the TLR4/p38MAPK signaling pathway (19, 46). Indeed, LPS-induced autophagy may be dependent on the combination of TLR4/p38MAPK and Beclin1 complex signaling, evidenced by the increased expression of TLR4, p-p38, Beclin1, Atg5, and LC3-II. Pretreatment with ZJ617s and LGGs suppressed the TLR4/p38MAPK signaling pathway and its downstream autophagic cascade. Consistent with these results, we and others have shown that probiotic *Lactobacillus* and *Bifidobacterium* species suppress intestinal epithelial cell autophagy after treatment with LPS in vivo and in vitro (23, 47). In addition, a recent study reported that probiotic LGG inhibited autophagy in the intestines of piglets challenged with *Salmonella* infantis (48). Collectively, our findings suggest that ZJ617s and LGGs reduce LPS-induced autophagy by suppressing the TLR4-mediated p38MAPK signaling pathway and downstream Beclin1-mediated autophagic cascade, thus protecting against liver injury.

In conclusion, further research regarding the precise mechanism through which ZJ617s and LGGs act on liver injuries during endotoxic shock is warranted. However, based on the current evidence, we suggest that the hepatoprotection conferred by ZJ617s and LGGs in mice may involve anti-inflammatory, antiapoptotic, and anti-autophagic effects. As shown in **Supplemental Figure 4**, ZJ617s and LGGs maintain intestinal integrity, minimizing the diffusion of gut-derived endotoxin into the liver. In addition, they significantly mitigate liver inflammation by reducing the production of IL-6 and TNF-α via inhibition of TLR4/MAPK/NF-κB inflammatory signaling. Moreover, they significantly mitigate liver apoptosis by downregulating the TLR4/MAPK signal and proapoptotic factor Bax. Furthermore, they mitigate excessive autophagy by suppressing the TLR4/p38MAPK signaling pathway and its downstream autophagic cascade events. These findings indicate that ZJ617s is similar to LGGs in ameliorating LPS-induced liver injury in mice. Future studies are required to identify the specific active ingredients present in ZJ617s and LGGs that may yield alternative strategies for the prevention of acute liver injury in mice.

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