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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Administration of *Lactobacillus salivarius* LI01 or *Pediococcus pentosaceus* LI05 improves acute liver injury induced by D-galactosamine in rats

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Abstract This work investigated the effect of the intragastric administration of five lactic acid bacteria from healthy people on acute liver failure in rats. Sprague–Dawley rats were given intragastric supplements of *Lactobacillus salivarius* LI01, *Lactobacillus salivarius* LI02, *Lactobacillus paracasei* LI03, *Lactobacillus plantarum* LI04, or *Pediococcus pentosaceus* LI05 for 8 days. Acute liver injury was induced on the eighth day by intraperitoneal injection of 1.1 g/kg body weight D-galactosamine (D-GalN). After 24 h, samples were collected to determine the level of liver enzymes, liver function, histology of the terminal ileum and liver, serum levels of inflammatory cytokines, bacterial translocation, and composition of the gut microbiome. The results indicated that pretreatment with *L. salivarius* LI01 or *P. pentosaceus* LI05 significantly reduced elevated alanine aminotransferase and aspartate aminotransferase levels, prevented the increase in total bilirubin, reduced the histological abnormalities of both the liver and the terminal ileum, decreased bacterial translocation, increased the serum level of interleukin 10 and/or interferon-γ, and resulted in a cecal microbiome that differed from that of the liver injury control. Pretreatment with *L. plantarum* LI04 or *L. salivarius* LI02 demonstrated no significant effects during this process, and pretreatment with *L. paracasei* LI03 aggravated liver injury. To the best of our knowledge, the effects of the three species—*L. paracasei*, *L. salivarius*, and *P. pentosaceus*—on D-GalN-induced liver injury have not been previously studied. The excellent characteristics of *L. salivarius* LI01 and *P. pentosaceus* LI05 enable them to serve as potential probiotics in the prevention or treatment of acute liver failure.

Keywords Acute liver injury · *Lactobacillus* · D-Galactosamine · Cytokines · Bacterial translocation · Microbiome

Introduction

The liver is the largest digestive gland and one of the five vital organs in humans; it has been described as the body's chemical factory because it performs many critical functions, such as the secretion of bile and bile salts; the phagocytosis of bacteria and dead or foreign materials; the metabolism of carbohydrates, proteins, and fats; detoxification; and immune defense. Acute liver failure or fulminant hepatic failure is a drastic, unpredictable clinical syndrome in which the severe deterioration of liver function rapidly occurs and is typically followed by hepatic encephalopathy, coagulopathy and, in many cases, progressive multiorgan failure in patients with no reported underlying chronic liver disease (Bernal et al. 2010; Wlodzimirow et al. 2012). Acute liver failure is a life-threatening event with a high mortality ranging between 60 and 80 % (Wlodzimirow et al. 2012). The incidence of acute liver failure is between one and six cases per million people every year in the developed world but is higher in locations where infective hepatitis is prevalent (Bernal et al. 2010).

Many factors, such as viral hepatitis, excessive alcohol intake, idiosyncratic reactions to medication, and acute fatty

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liver during pregnancy, can cause acute liver failure (Bernal et al. 2010). Typically, liver transplantation is the most efficient salvage therapy, which has improved survival from less than 20 % to approximately 60 %; however, this option is strongly limited by the availability of donor livers (O'Grady 2012). Consequently, an artificial liver support system is useful to bridge the gap between the onset of acute liver failure and liver transplantation in addition to the regeneration of patient livers (Du et al. 2005). In addition, prevention or adjuvant treatment through the modulation of the gut microbiome composition has drawn increasing attention because several signs and symptoms such as infection, inflammation, sepsis, and hepatic encephalopathy demonstrate strong correlations between acute liver failure and the gut microbiome (Festi et al. 2006; Tranah et al. 2013). Patients are vulnerable to enteric infections by both gut microbes (particularly Gram-negative bacteria such as *Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa*, and other *Enterobacteriaceae*) and their products (Schnabl 2013) and thereby develop immune dysfunction. Pretreatment with *Lactobacillus plantarum* DSM 9843 (Adawi et al. 1997, 1998; Kasravi et al. 1997), *Lactobacillus rhamnosus* ATCC 53103 (Adawi et al. 2001; Osman et al. 2007), or *Lactobacillus casei* Zhang (Wang et al. 2013) was found to significantly improve D-galactosamine (D-GalN)-induced liver injury in rats. A recent review indicates that some probiotics appear to reduce the plasma ammonia concentration in comparison to placebo or no intervention, thus relieving hepatic encephalopathy in patients (McGee et al. 2011). However, because the gut hosts up to 100 trillion (10^{14}) microbes from 500 to 1,000 different species, the effects of most gut microbes on acute liver failure are unclear.

In this study, the effects of five lactic acid bacteria from healthy people on liver enzymes, liver injury, and liver function were evaluated using a D-galactosamine-induced liver injury model in rats. Additionally, the effects of these bacteria on the serum levels of inflammatory cytokines, intestinal barriers, bacterial translocation, and the composition of the cecal microbiome were investigated to determine the possible mechanism of their effects on acute liver failure.

Materials and methods

Strain and culture conditions

Five strains were used in this study: *Lactobacillus salivarius* LI01 (CGMCC 7045), *Lactobacillus salivarius* LI02 (CGMCC 7046), *Lactobacillus paracasei* LI03 (CGMCC 7047), *Lactobacillus plantarum* LI04 (CGMCC 7048), and *Pediococcus pentosaceus* LI05 (CGMCC 7049). All strains were isolated in our laboratory from healthy volunteers, stored at −80 °C in Man–Rogosa–Sharpe (MRS, Oxoid, Thermo Fisher Biochemicals Ltd., Beijing, China) broth with 20 % glycerol, and deposited in the China General Microbiological Culture Collection Center (CGMCC). These strains exhibited tolerance property to bile, acid, and strong antimicrobial activity against tested enteropathogens (data not shown). After revival using standard methods, the bacterial strains were anaerobically cultured in MRS broth for 18 h at 37 °C. Cells were obtained by centrifugation at 8,000*g* for 10 min at 4 °C. Subsequently, these cells were washed twice with physiological saline at 4 °C and resuspended to a concentration of 3×10^9 colony-forming units (CFU)/ml in physiological saline for further use.

Animals and experimental design

Forty-two male Sprague–Dawley rats (from the Experimental Animal Center of Zhejiang Province, Zhejiang, China), with a weight range of 200 to 300 g, were divided into seven groups of six rats: a normal group treated with physiological saline (saline + saline), a control group with acute liver injury (ALI) alone (GalN + saline), and five groups of liver injury treated with each of the following strains: *L. salivarius* LI01 (GalN + *L. salivarius* LI01), *L. salivarius* LI02 (GalN + *L. salivarius* LI02), *L. paracasei* LI03 (GalN + *L. paracasei* LI03), *L. plantarum* LI04 (GalN + *L. plantarum* LI04), and *P. pentosaceus* LI05 (GalN + *P. pentosaceus* LI05). The animals were fed normal food and were maintained at room temperature (22 °C) with a controlled 12-h:12-h light–dark cycle. The animals were treated with oral administration through an orogastric tube (once daily for 8 days) of 1 ml of normal saline in the liver injury control or 1 ml (3×10^9 CFU/ml) of bacterial cells that were freshly prepared as previously described. Acute liver injury was induced on the eighth day by an intraperitoneal injection of 1.1 g/kg body weight D-galactosamine (G0500, Sigma, Saint Louis, MO, USA). Twenty-four hours after the induction of liver injury, the rats were anesthetized by an intraperitoneal injection of 400 mg/kg body weight chloral hydrate (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) to collect the samples for analysis.

Liver enzymes and bilirubin

The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, globulin, and bilirubin were assessed by standard methods using a 7600-210 automatic analyzer (Hitachi 7600-210; Hitachi, Tokyo, Japan).

Serum cytokines

The serum concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), and interferon-γ (IFN-γ) were measured

using commercially available enzyme-linked immunosorbent assay kits following the manufacturer's recommendations (eBioscience Inc., San Diego, CA, USA).

Bacterial translocation

Samples from the caudate lobe of the liver and mesenteric lymph nodes (MLN) were collected, weighed, and milled under aseptic conditions in glass homogenizers that had been autoclaved at 121 °C for 15 min. After appropriate dilution with physiological saline, these milled samples and samples from arterial blood or portal blood were separately incubated under aerobic and anaerobic conditions on brain heart infusion agar (BHI, Oxoid, Thermo Fisher Biochemicals Ltd., Beijing, China) at 37 °C for 48 to 72 h. The number of colonies from each plate was counted. The bacterial translocation of tissue samples was expressed per gram of tissue, whereas that of blood samples was expressed per milliliter of blood.

DNA extraction, PCR amplification, and denaturing gradient gel electrophoresis (DGGE) analysis of the intestinal microbiome

DNA extraction

Cecal contents were collected from sacrificed rats. DNA was extracted from the cecal contents using a Qiagen Stool Kit (Qiagen, Hilden, Germany). The quality of the DNA was determined using agarose gel electrophoresis analysis and UV light visualization with ethidium bromide staining.

PCR amplification for DGGE

The V3 variable region of 16S ribosomal DNA (rDNA) was amplified using a hot start touchdown approach. The nucleotide sequences of the primers were as follows: primer 1, 5′-CCTACGGGAGGCAGCAG-3′; primer 2, 5′-ATTACCGCGGCTGCTGG-3′; and primer 3, 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′ (Muyzer et al. 1993). A combination of primers 1 and 2 or primers 3 and 2 was used to amplify the V3 variable region of 16S rDNA. PCR was performed in a final volume of 50 μl containing 2 μl of template DNA, 25 pmol of each primer, 200 μM of each dNTP mixture, 5 μl of 10×Ex Taq buffer, and 0.5 μl of TaKaRa Ex Taq polymerase (TaKaRa, Dalian, China). Initial denaturation was performed at 95 °C for 3 min, and amplification was performed using 24 cycles of denaturation at 94 °C for 30 s; annealing at 64 °C for 30 s, which was decreased by 1 °C every 3 cycles; and extension at 72 °C for 30 s. Subsequently, ten additional cycles of denaturation at 94 °C for 20 s, annealing at 56 °C for 30 s and extension at 72 °C for 20 s were performed, with a final extension at 72 °C for 5 min. Concentrations were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation). All amplified products were stored at −20 °C before DGGE analysis.

Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed using the DCode universal mutation detection system (Bio-Rad, Hercules, CA, USA) with 16 cm×18 cm×1.5 mm gels. For each sample, 20 μl of PCR fragment was loaded onto the gels. An identical standard reference (a randomly selected sample) was applied in the middle and at both ends of each gel for digital gel normalization and for comparison between gels. Sequence-specific separation of the PCR fragments was obtained in 8 % (wt/vol) polyacrylamide gels in 1×TAE buffer. Electrophoresis was performed for 16 h at 60 °C and 70 V in a linear 35 to 75 % denaturant gradient (100 % denaturant is defined as 7 M urea and 40 % deionized formamide). After electrophoresis, the gels were stained using SYBR green I (Sigma-Aldrich, Castle Hill, Australia) and photographed.

Comparative analyses of the DGGE profiles

DGGE profiles were digitally processed using BioNumerics software version 6.01 (Applied Maths, St-Martens-Latem, Belgium) in a multistep procedure following the manufacturer's instructions. All profiles were compared using the band-matching tool, and uncertain bands were included in the position tolerance settings. Parameters for allocating band classes were chosen according to Joossens et al. (2011). A cluster analysis of DGGE pattern profiles used the unweighted pair group method with the arithmetic means (UPGMA) method based on the Dice similarity coefficient (band based) (Vanhoutte et al. 2004). Multidimensional scaling (MDS) and principal components analysis (PCA) were performed according to the BioNumerics software manuals. MDS is an optimized three-dimensional representation of the similarity matrix, and the Euclidean distance between two points (lanes in the gel profiles) reflects the similarity between them, providing a convenient visual interpretation. PCA is another method to visualize the relationships among lanes using the lane data (band classes) set itself, which generates new variables called principal components (linear components of the original variables) that explain the highest dispersion of the samples (Ma and Dai 2011).

Sequencing of DGGE bands

Predominant bands were excised from the DGGE gels, washed three times in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0), disrupted, and incubated in 50 μl of TE buffer for 30 min at 80 °C (Lu et al. 2013). Subsequently, 5 μl of the supernatant from each sample was used as a

template for PCR reamplification using the primers described above. Cloned PCR products were subsequently verified and sequenced (Invitrogen, Shanghai, China). Homology searches were performed using BLAST. Reference sequences of phylogenetic neighbor species (up to 98 % similarity) were introduced for clustering analysis using multiple sequence alignment with the K-Lite Mega Codec Pack 5.05 (Tamura et al. 2011).

Histopathology

Samples from the left lobe of the liver and from the terminal ileum were immediately fixed in 10 % neutral buffered formalin at the time of death. Paraffin-embedded samples were cut into 4-μm-thick sections. Subsequently, the sections were stained using hematoxylin and eosin and analyzed by a pathologist who was blind to the groups. At least three slides and 20 random microscopic fields were studied from each specimen. To grade the degree of liver injury, the amount of tissues damaged by necrosis or inflammation was semiquantitatively assessed on a scale of 0 (absent), 1 (mild), 2 (moderate), and 3 (extensive) (Deutschman et al. 2006). To grade the degree of terminal ileum injury, the amount of goblet cell depletion or decreased mucus accumulation (score, 0–3), mucosa thickening (score, 0–3), destruction, or loss of crypts (score, 0–3) was also semiquantitatively assessed (Johansson et al. 2013).

Statistics

The obtained data were analyzed using SPSS 16.0 software. The data were first tested for homogeneity of variances using one-way ANOVA. If the variances were homogeneous ($p>0.1$), the data were evaluated using one-way ANOVA followed by the Student–Newman–Keuls method. The values are presented as the means ± SEM. Otherwise, the data were analyzed using Kruskal–Wallis one-way ANOVA on ranks followed by the Student–Newman–Keuls method. Probability levels of <0.05 were considered significant. The values are presented as the median (25th and 75th percentiles). Probability levels of <0.05 were considered significant (Osman et al. 2007).

Results

L. salivarius LI01 or *P. pentosaceus* LI05 reduces elevated levels of both aspartate transaminase and alanine transaminase during D-GalN-induced acute liver injury

Twenty-four hours after the induction of liver injury, no mortality was observed in the different experimental groups. Serum levels of AST, ALT, and ALP were profoundly increased in the liver injury control group (Table 1). Pretreatment with *L. salivarius* LI01, *L. salivarius* LI02, *L. plantarum* LI04, or *P. pentosaceus* LI05 significantly lowered the increase of serum AST induced by D-GalN, but pretreatment with *L. paracasei* LI03 aggravated the increase of serum AST under identical conditions. Pretreatment with *L. salivarius* LI01 or *P. pentosaceus* LI05 relieved the increase of serum ALT, but pretreatment with *L. plantarum* LI04, *L. salivarius* LI02, or *L. paracasei* LI03 did not demonstrate significant effects. These results indicate that pretreatment with either *L. salivarius* LI01 or *P. pentosaceus* LI05 reduces the inflammation or damage to the liver induced by D-GalN because higher than normal amounts of cellular substances, including liver enzymes, typically leaked from inflamed or injured liver cells into the bloodstream. None of the five evaluated bacterial strains prevented the increase of serum ALP in D-GalN-induced acute liver injury, which likely indicates that the five bacterial strains do not aid in the protection against the destruction of mucosal cells that line the bile system of the liver and serve as the source of ALP.

L. salivarius LI01 or *P. pentosaceus* LI05 prevents the increase of bilirubin during D-GalN-induced acute liver injury

As shown in Table 1, the total bilirubin level of the groups supplemented with either *L. salivarius* LI01 or *P. pentosaceus* LI05 was not significantly different from that of the normal control group and, therefore, was significantly lower than that of the liver injury control group. However, pretreatment with *L. paracasei* LI03, *L. plantarum* LI04, or *L. salivarius* LI02 did not significantly prevent this increase in total bilirubin, which indicates that only *L. salivarius* LI01 or *P. pentosaceus* LI05 protects the ability of the liver to take up, process, and secrete bilirubin into the bile from destruction by D-GalN. Compared with the normal control, the serum albumin level decreased in all of the other groups except that supplemented with *L. plantarum* LI04. Changes in the level of albumin were minor in all groups (date not shown), which relates to the relatively long half-life of albumin that can maintain normal concentrations even in the early stages of severe acute liver disease. None of the five microbes prevented the decrease in serum globulin that is induced by D-GalN.

L. salivarius LI01 or *P. pentosaceus* LI05 reduces D-GalN-induced histological abnormalities of both the liver and the terminal ileum

Twenty-four hours after D-GalN administration, all groups, except the normal group, demonstrated histological abnormalities of the liver and terminal ileum. Damaged liver tissues exhibited a loss of hepatocytes, microabscesses, and extensive neutrophil and macrophage accumulation in the parenchyma (Fig. 1b–g). Damaged terminal ileum exhibited the rupture and absence of villi, disintegration of crypts, and perforation of the mucosa (Fig. 2b–g). All strains, except *L. paracasei*

Table 1 Liver enzymes and bilirubin levels in the experimental groups

	AST (U/l)	ALT (U/l)	ALP (U/l)	G (g/l)	TB (μmol/l)
Saline + saline	60±2.8**	33.0 (30.0, 39.0)*	168±27.4**	16.1±1.3**	3.0 (2.3, 3.0)*
GalN + saline	3,267.5±1,503.2	2,224.5 (1,426.3, 3,952.0)	284.8±55.6	10.4±0.6	8.0 (4.5, 10.8)
GalN + *L. paracasei* LI03	4,614.8±335.6*	4,015.5 (3,333.3, 6,209.0)	247.3±43.1	10.4±0.9	13.0 (9.0, 14.0)
GalN + *L. plantarum* LI04	1,336.4±666.7**	2,379.0 (736.5, 8,482.0)	250.8±30.2	10.4±0.9	11.0 (8.0, 15.5)
GalN + *L. salivarius* LI01	675.0±202.3**	530.0 (402.8, 663.3)*	277.3±46.6	9.2±1.7	3.0 (2.3, 3.8)*
GalN + *P. pentosaceus* LI05	652.3±313.9**	606.0 (252.3, 1,160.8)*	268.5±63.5	10.2±1.2	2.5 (2.0, 3.0)*
GalN + *L. salivarius* LI02	1,596.8±1,187.1**	2,140.0 (958.0, 7,942.8)	271.0±35.2	9.4±0.7	6.5 (3.3, 18.0)

Values are expressed as the mean ± SE or median (25th, 75th percentiles). The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), globulin (G), and total bilirubin (TB) were determined after 24 h of D-galactosamine (GalN) administration

*$p<0.05$ compared with the liver injury control; **$p<0.01$ compared with the liver injury control (GalN + saline)

LI03, significantly reduced the histological abnormalities of D-GalN-induced liver injury (Fig. 1, Table 2). However, only pretreatment with *L. salivarius* LI01 or *P. pentosaceus* LI05 reduced the disintegration of the mucosa, villi, and crypts (Fig. 2, Table 2). This result, together with the improved liver function after *L. salivarius* LI01 or *P. pentosaceus* LI05 administration in D-GalN-induced liver injury, indicates that a reduction of the destruction of the intestinal barrier plays a critical role in relieving liver injury.

L. salivarius LI01 or *P. pentosaceus* LI05 increases the serum IL-10 or IFN-γ level during D-GalN-induced acute liver injury

As shown in Fig. 3a, the serum level of TNF-α in the group supplemented with *L. salivarius* LI02 was higher than that in the liver injury control group. No significant difference in serum TNF-α (Fig. 3a), IL-1β (Fig. 3b), or IL-6 (data not shown) was detected among all the other groups supplemented with microbes and the liver injury control group. This finding results from the typical burst of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the early stage of D-GalN treatment. Pretreatment with *L. salivarius* LI01 or *P. pentosaceus* LI05 significantly increases the serum level of IL-10 in rats with liver injury (Fig. 3c), which partially explains the minor liver injury observed in the groups supplemented with these two microbes. Importantly, pretreatment with *L. plantarum* LI04 or *P. pentosaceus* LI05 greatly elevated the serum level of IFN-γ in D-GalN-induced liver injury (Fig. 3d), which is beneficial for innate and adaptive immunity against viral and intracellular bacterial infections.

L. salivarius LI01, *P. pentosaceus* LI05, or *L. paracasei* LI03 decreases the incidence of bacterial translocation during D-GalN-induced acute liver injury

As shown in Table 3, only pretreatment with *L. salivarius* LI01 decreased the incidence of bacterial translocation (BT) in all evaluated tissues. Pretreatment with *P. pentosaceus* LI05 decreased the BT incidence in the portal blood, liver, and MLN. Pretreatment with *L. paracasei* LI03 decreased the BT incidence in the arterial blood, portal blood, and liver. Pretreatment with *L. plantarum* LI04 decreased the BT incidence in arterial blood and portal blood but increased the BT incidence in the MLN. Pretreatment with *L. salivarius* LI02 decreased the BT incidence in the MLN arterial blood and portal blood but increased the BT incidence in the liver. Consequently, *L. salivarius* LI01, *L. paracasei* LI03, and *P. pentosaceus* LI05 efficiently prevented bacterial translocation.

Supplementation with *L. salivarius* LI01 or *P. pentosaceus* LI05 results in a different cecal microbiome compared with that in liver injury

Cecal contents were collected from sacrificed rats and were examined using PCR-denaturing gradient gel electrophoresis (DGGE) of the V3 variable regions of total community 16S rDNA (Fig. 4). Multidimensional scaling (MDS) and principal component analysis (PCA) of the DGGE fingerprinting data were conducted using BioNumerics software version 6.01 to demonstrate the uniqueness and stability of the predominant cecal microbiome composition. Both cluster analysis of samples and the MDS plot (an ordination method that reduces the complex DGGE patterns to one point per sample) indicate that the cecal microbiome composition in the group supplemented with either *L. salivarius* LI01 (Figs. 4 and 5d) or *P. pentosaceus* LI05 (Figs. 4 and 5e) was clearly distinct from that in the liver injury group. Similar results for these two groups were observed using a PCA plot, which is a method based on a linear response model. However, for the groups supplemented with *L. paracasei* LI03 (Fig. 5b), *L. plantarum* LI04 (Fig. 5c), or *L. salivarius* LI02 (Fig. 5f), the cecal microbiome compositions could not be clearly distinguished from that in the liver injury control using either MDS or PCA.

A total of 38 predominant bands were sequenced after excision from the DGGE gels and reamplification (Fig. 4).

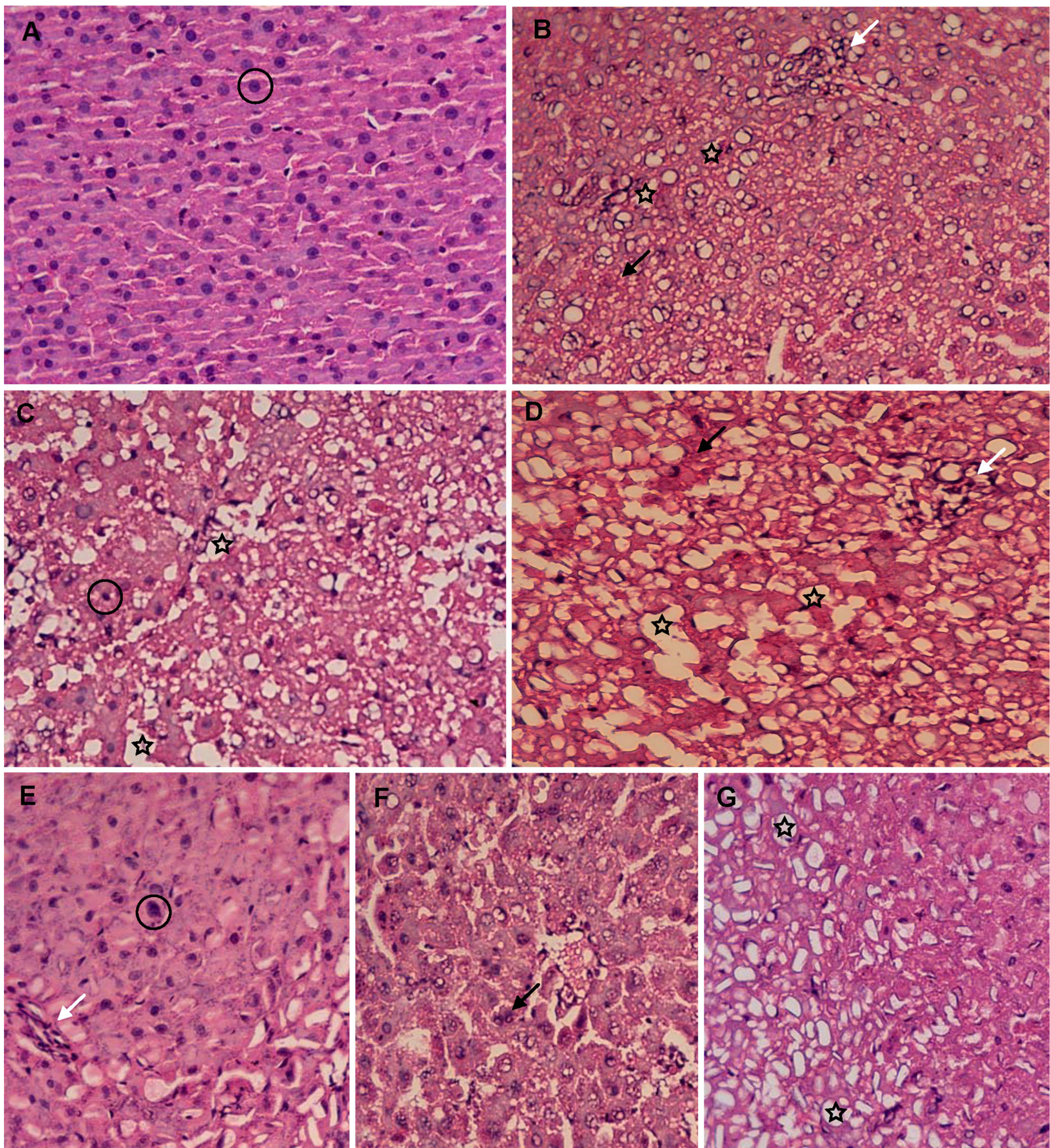

Fig. 1 Effects of pretreatment with different lactic acid bacteria on liver histological alterations during D-GalN-induced acute liver injury. **a** Saline + saline; **b** GalN + saline; **c** GalN + *L. paracasei* LI03; **d** GalN + *L. plantarum* LI04; **e** GalN + *L. salivarius* LI01; **f** GalN + *P. pentosaceus* LI05; **g** GalN + *L. salivarius* LI02. *Circles*, cells with a clear nucleus; *stars*, lipid vacuoles; *black arrows*, microabscesses; *white arrows*, neutrophil and macrophage accumulation

The closest matches (and percentages of similarity) for the sequences retrieved from each band were determined using BLAST searches. The intensity of each band in every group was compared with that in the liver injury control group. As shown in Fig. 6, an uncultured *Bacteroidetes* (band class 66, GenBank accession no. GU959627.1) increased in all groups except the group supplemented with *L. plantarum* LI04, but *Clostridium* sp. (band class 8.6, GenBank accession no. AB809064.1) only increased in the group supplemented with *L. plantarum* LI04. *Clostridiales bacterium* (band class 51.4,

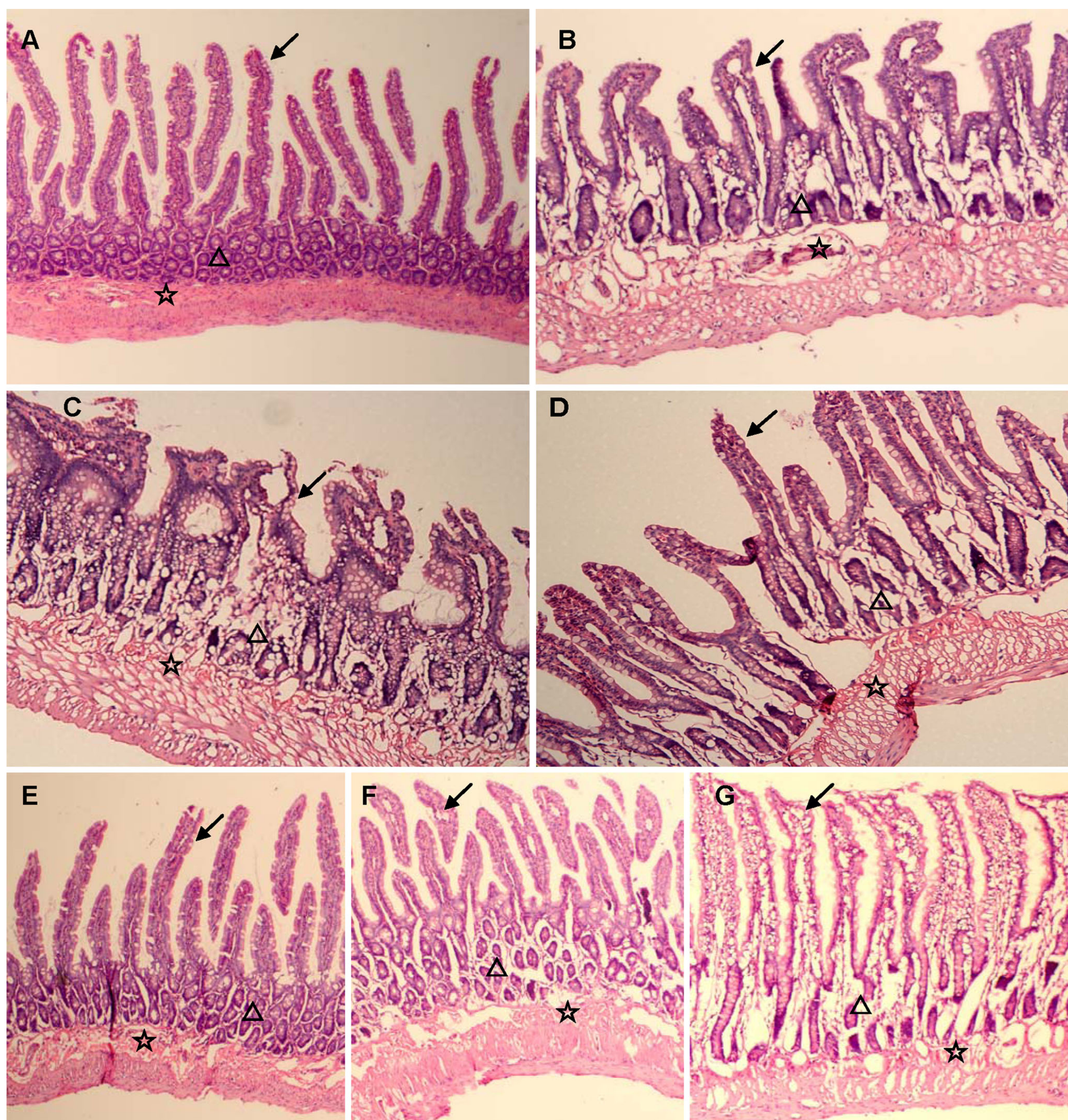

Fig. 2 Effects of pretreatment with different lactic acid bacteria on histological alterations in the distal ileum during D-GalN-induced acute liver injury. **a** Saline + saline; **b** GalN + saline; **c** GalN + *L. paracasei* LI03; **d** GalN + *L. plantarum* LI04; **e** GalN + *L. salivarius* LI01; **f** GalN + *P. pentosaceus* LI05; **g** GalN + *L. salivarius* LI02. *Stars*, mucosa; *triangles*, crypts; *black arrows*, villi

GenBank accession no. AB702927.1) and an uncultured rumen bacterium (band class 25, GenBank accession no. JX218354.1) increased in the group supplemented with *L. salivarius* LI01 or *P. pentosaceus* LI05. *Lactobacillus reuteri* (band class 62.2, GenBank accession no. KF267448.1), an uncultured *Bacteroidetes* (band class 62.2, GenBank accession no. GU959253.1), and *Bacteroides chinchilla* (band class 6.4, GenBank accession no. AB547637.1) increased in the group supplemented with *L. salivarius* LI02. An uncultured bacterium (band class 81.9, GenBank accession no. JF245950.1) and another uncultured bacterium (band class 58.2, GenBank accession no. JF794942.1) increased in the group supplemented with *L. salivarius* LI01 or *L. salivarius* LI02. *Corynebacterium*

Table 2 Histological scores of the liver, ileal mucosa, ileal crypts, and ileal villi

Group	Histological scores (0–3)			
	Liver	Mucosa	Crypts	Villi
Saline + saline	0.2±0.1	0.3 (0.2, 0.3)	0.2 (0.1, 0.2)	0.1 (0, 0.1)
GalN + saline	2.7±0.2	2.5 (2.3, 2.5)	1.7 (1.3, 1.7)	1.1 (0.4, 2.0)
GalN + *L. paracasei* LI03	2.7±0.3	2.2 (1.5, 2.3)*	1.9 (1.3, 2.2)	1.1 (0.2, 1.4)
GalN + *L. plantarum* LI04	2.1±0.3**	2.5 (2.3, 2.7)	1.7 (1.6, 1.8)	1.3 (0.9, 1.4)
GalN + *L. salivarius* LI01	2.1±0.2**	1.2 (1.0, 1.3)**	0.5 (0.4, 0.5)**	0.2 (0.2, 0.2)**
GalN + *P. pentosaceus* LI05	1.9±0.2**	1.8 (1.6, 2.0)**	0.9 (0.5, 1.4)*	0.6 (0.2, 0.8)
GalN + *L. salivarius* LI02	2.0±0.1**	2.0 (1.7, 2.5)	1.8 (0.3, 2.1)	1.1 (0.4, 1.8)

Values are expressed as the mean ± SE or median (25th, 75th percentiles). The amount of tissues damaged by necrosis or inflammation was semiquantitatively assessed on a scale of 0 (absent), 1 (mild), 2 (moderate), and 3 (extensive)

*$p<0.05$ compared with the liver injury control; **$p<0.01$ compared with the liver injury control (GalN + saline)

casei (band class 62.7, GenBank accession no. JX966460.1) and an uncultured bacterium (band class 62.7, GenBank accession no. FJ835427.1) increased in the group supplemented with *P. pentosaceus* LI05 or *L. salivarius* LI02. An uncultured bacterium (band class 39.4, GenBank accession no. HQ320396.1) increased, but another uncultured bacterium

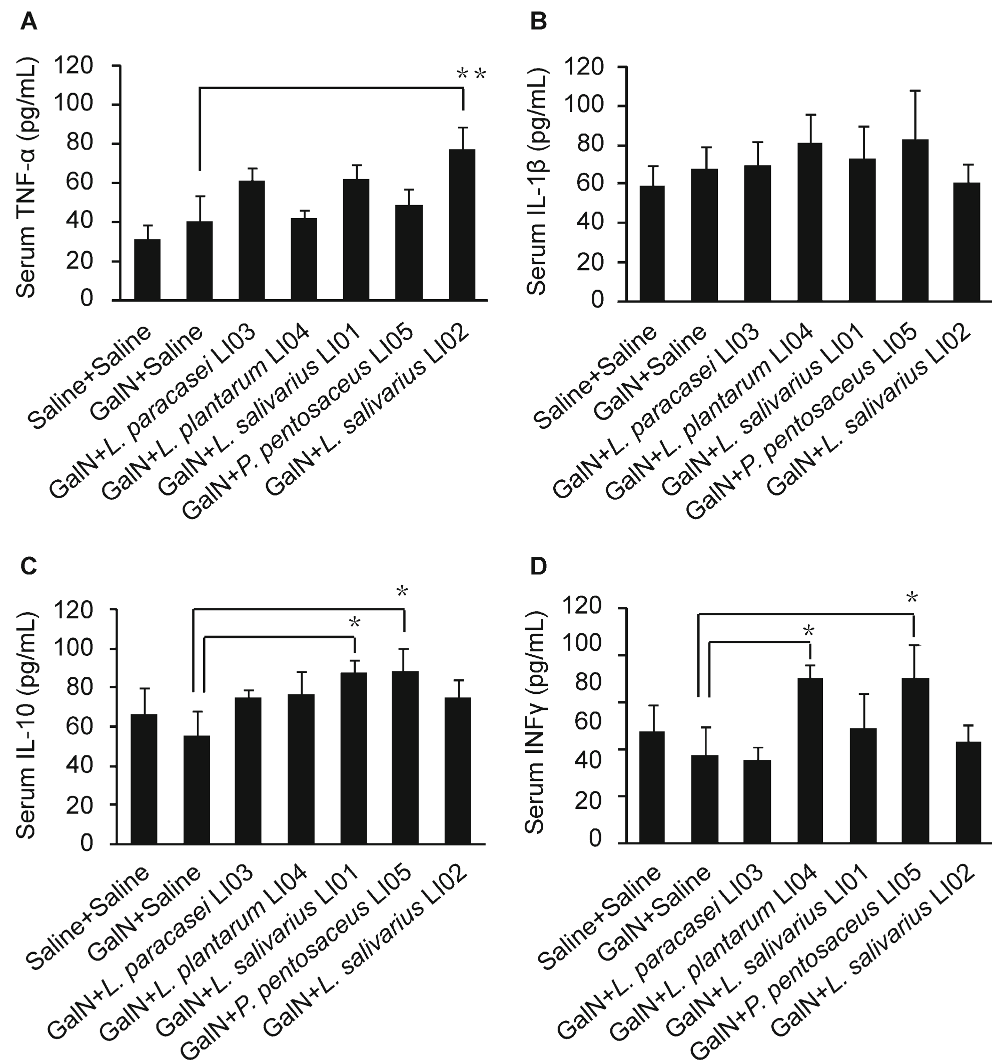

Fig. 3 Effects of pretreatment with different lactic acid bacteria on the serum levels of TNF-α (**a**), IL-1β (**b**), IL-10 (**c**), and IFN-γ (**d**) during D-GalN-induced acute liver injury. *$p<0.05$ compared with the liver injury control; **$p<0.01$ compared with the liver injury control (GalN + saline)

Table 3 Bacterial translocation in the experimental groups (CFU/ml of blood or g tissue)

	Arterial blood (CFU/ml)	Portal blood (CFU/ml)	Liver (log10 CFU/g)	MLN (log10 CFU/g)
GalN + saline	3.3±1.7**	0 (0, 7.5)	0.0 (0.0, 0.0)	2.8 (2.7, 30)
Saline + saline	105.0±10.8	115.0 (110.0, 120.0)	2.5 (2.5, 2.5)	3.9 (3.9, 4.0)
GalN + *L. paracasei* LI03	68.8±7.5**	72.5 (66.3, 82.5)*	0.0 (0.0, 0.0)*	3.9 (3.8, 3.9)
GalN + *L. plantarum* LI04	65.0±3.5**	65.0 (55.0, 65.0)*	2.7 (2.5, 3.5)	4.2 (4.2, 4.2)*
GalN + *L. salivarius* LI01	52.5±6.5**	102.5 (100.0, 105.0)*	1.7 (1.6, 1.7)*	3.8 (3.7, 3.8)*
GalN + *P. pentosaceus* LI05	103.8±16.5	30.0 (26.3, 30.0)*	1.9 (1.9, 1.9)*	3.7 (3.6, 3.7)*
GalN + *L. salivarius* LI02	52.5±5.0**	47.5 (40.0, 58.8)*	2.6 (2.6, 2.6)*	3.6 (3.5, 3.6)*

Values are expressed as the mean ± SE or median (25th, 75th percentiles)

MLN mesenteric lymph nodes

*$p<0.05$ compared with the liver injury control; **$p<0.01$ compared with the liver injury control (GalN + saline)

(band class 46.4, GenBank accession no. JF794942.1) decreased in the normal control. The other 27 bands were not significantly different between the liver injury control group and each of the other groups. These results offer potential directions for further studies on the impact of specific microbes on liver injury.

Discussion

Acute liver failure is often accompanied by certain complications, such as sepsis, bacterial peritonitis, and hepatic encephalopathy, that are related to the gut microbiome (Tranah et al. 2013). However, the molecular mechanism for the interaction between acute liver failure and the gut microbiome, particularly the roles of most gut microbes during this process, is not fully understood. Furthermore, although modulations of the gut microbiome with probiotics or prebiotics such as lactulose have been applied in treatment, no probiotics have been developed for the clinical prevention and treatment of liver failure (Kirpich and McClain 2012). Lactic acid bacteria are excellent sources of probiotics (Capozzi et al. 2012; Hynonen and Palva 2013; Tsai et al. 2012). In this study, we examined the effects of five lactic acid bacteria from healthy people on acute liver failure using a D-galactosamine-induced rat model. All strains exhibited tolerance property to bile, acid, and strong antimicrobial activity against tested enteropathogens (data not shown). To the best of our knowledge, three important species—*L. salivarius*, *P. pentosaceus*, and *L. paracasei*—have not been previously investigated in this field. *L. salivarius* has been frequently isolated from the mammalian digestive tract and has been studied as a candidate probiotic (Messaoudi et al. 2013). In addition to mammalian sources, *L. paracasei* is naturally found in fermented products and natural dairy products such as raw milk, and *P. pentosaceus* is used as a starter culture to ferment foods such as various meats, vegetables, and cheeses (Bengmark 2009). We found that *L. salivarius* LI01 and *P. pentosaceus* LI05 were beneficial in the prevention of acute liver failure. *L. plantarum* LI04 and *L. salivarius* LI02 did not demonstrate significant beneficial effects. *L. paracasei* LI03 aggravated liver injury. Two strains from the same species, *L. salivarius* LI01 and *L. salivarius* LI02, demonstrated differential effects. Our *L. plantarum* LI04 did not prevent acute liver injury, as was reported for *L. plantarum* 9843 (Adawi et al. 1998; Kasravi et al. 1997; Osman et al. 2007). These conflicting findings may be explained by confounding variables but are likely caused by different sources of lactic acid bacteria and differences in their characterization.

The levels of AST and ALT typically indicate hepatocyte damage or liver inflammation. Supplementation with the two lactic acid bacteria—*L. salivarius* LI01 or *P. pentosaceus* LI05—significantly reduced elevated levels of both AST and ALT during D-GalN-induced acute liver injury. It was reported that pretreatment with *L. plantarum* DSM 9843 (Adawi et al. 1997, 1998; Kasravi et al. 1997), *L. rhamnosus* ATCC 53103 (Adawi et al. 2001; Osman et al. 2007), or *L. casei* Zhang (Wang et al. 2013) decreased both AST and ALT levels. Pretreatment with *Bifidobacterium infantis* DSM 15159 (=CURE21) (Osman et al. 2007) or *L. casei* CRL 431 (Haro et al. 2009) decreased the levels of ALT but not those of AST. Pretreatment with *Lactobacillus acidophilus* NM1 decreased ALT levels (Adawi et al. 2001), whereas the effect of this strain on AST has not been reported. However, pretreatment with *L. reuteri* R2LC (Adawi et al. 1997; Kasravi et al. 1997), *L. rhamnosus* DSM 6594 (Adawi et al. 1997), *L. fermentum* 8704:3 (Adawi et al. 1997), *L. reuteri* 108 (Adawi et al. 1997), *Bifidobacterium animalis* NM2 (Adawi et al. 2001), or *L. plantarum* DSM 15313 (Osman et al. 2007) did not demonstrate significant effects.

Bilirubin, ALP, albumin, and globulin are important factors that reflect liver function. None of the five evaluated microbes significantly decreased elevated ALP levels in D-GalN-induced liver injury, which is consistent with other studies under identical conditions (Adawi et al. 2001; Kasravi et al. 1997; Wang et al. 2013). This finding likely indicates that

Fig. 4 Cluster analysis of the total V3 variable regions of 16S rDNA of cecal microbiome in different groups using Dice's coefficient and UPGMA. Predominant bands were excised from the DGGE gels and were numbered from 1 to 38

pretreatment with probiotics does not aid in the protection against the D-GalN-induced destruction of mucosal cells of the liver and biliary system. The administration of D-GalN decreased the serum level of albumin and globulin, thereby decreasing the total serum protein levels. Only pretreatment with *L. plantarum* LI04 slightly prevented the decrease in albumin and total protein. No significant modifications in the serum concentrations of total proteins were observed after the administration of 800 mg/kg body weight D-GalN (Haro et al. 2009). However, most studies have not reported the effects on serum proteins. Different results on the serum total protein level are most likely caused by different doses of D-GalN. Both *L. salivarius* LI01 and *P. pentosaceus* LI05 significantly reduced elevated total bilirubin in liver injury, which indicates the ability of these two probiotics to improve liver function. In other reports, *L. plantarum* DSM 9843

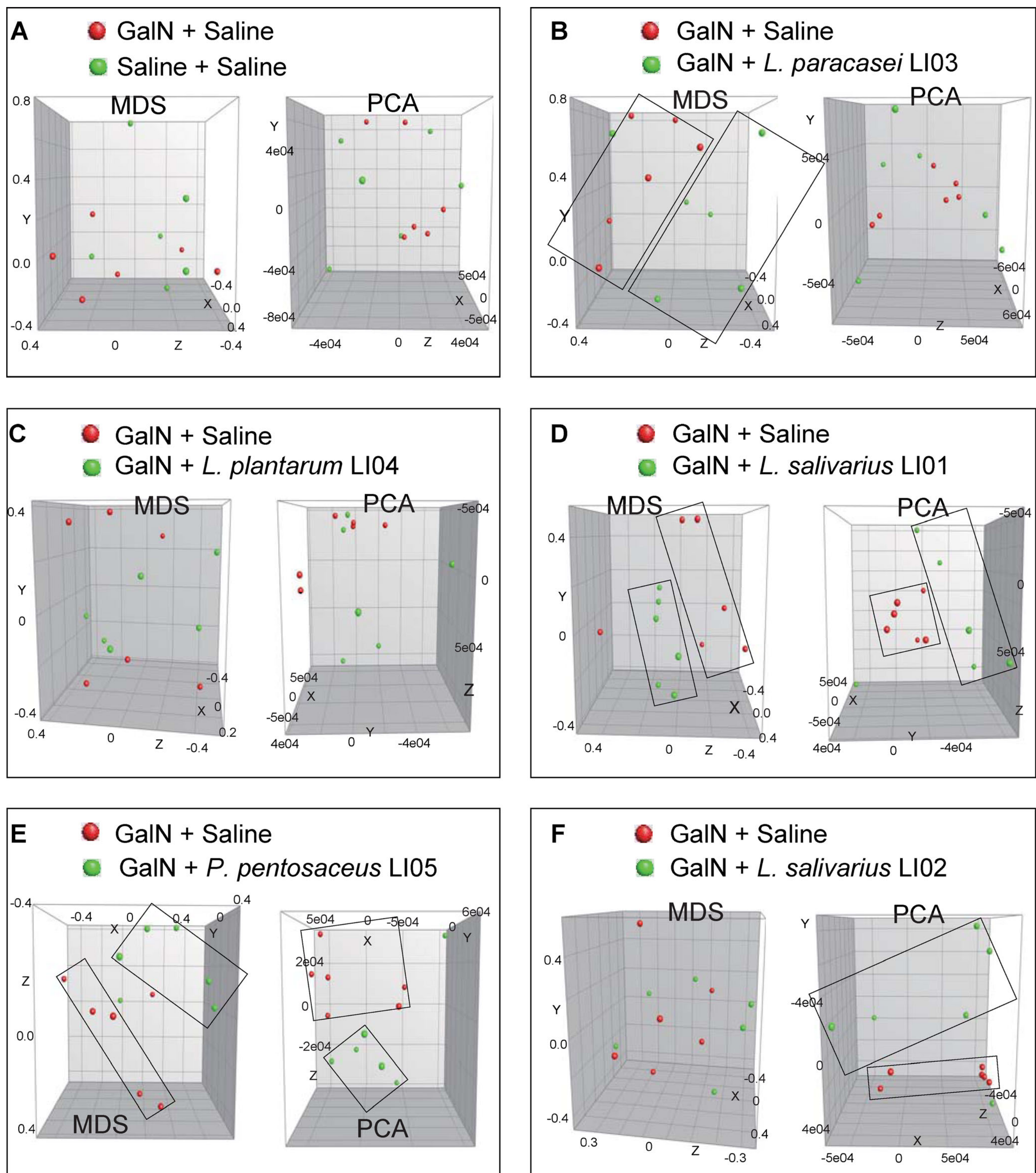

Fig. 5 Effects of pretreatment with different lactic acid bacteria on the composition profiles of the cecal microbiome during D-GalN-induced acute liver injury. *MDS* multidimensional scaling analysis, *PCA* principal component analysis. Composition profiles of the cecal microbiome in the liver injury control group were compared with those in the groups supplemented with saline + saline (**a**), GalN + *L. paracasei* LI03 (**b**), GalN + *L. plantarum* LI04 (**c**), GalN + *L. salivarius* LI01 (**d**), GalN + *P. pentosaceus* LI05 (**e**), or GalN + *L. salivarius* LI02 (**f**)

(Adawi et al. 1997, 1998), *L. plantarum* DSM 15313 (Osman et al. 2007), and *B. infantis* DSM 15159 (Osman et al. 2007) also significantly decreased total bilirubin levels.

Cytokines are critical pathogenic factors involved in liver injury and can be broadly divided into proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and anti-inflammatory

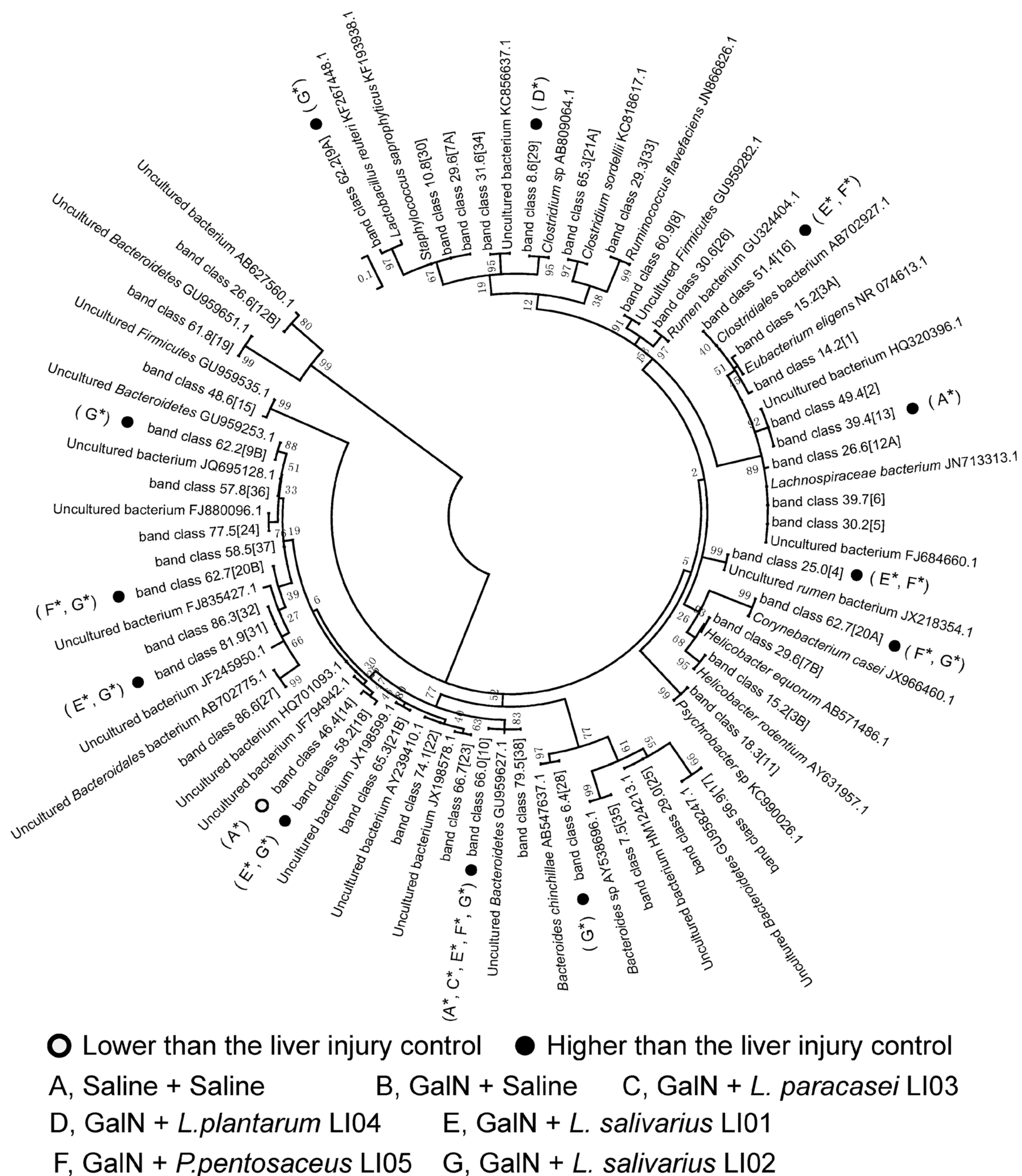

Fig. 6 Effects of pretreatment with different lactic acid bacteria on the predominant bacteria of the cecal microbiome during D-GalN-induced acute liver injury. The phylogenetic tree was based on the sequences of the V3 variable regions of 16S rDNA of selected bands. *$p<0.05$ compared with the liver injury control; **$p<0.01$ compared with the liver injury control (GalN + saline)

cytokines, such as IL-4, IL-10, and IL-13. Compared with the liver injury control, the serum level of TNF-α was higher in the group supplemented with *L. salivarius* LI02, but serum TNF-α levels in other groups and serum levels of IL-1β or IL-6 in all groups were not significantly different. Although the evaluated strains did not decrease the levels of proinflammatory cytokines, this result likely reflects the typical increase in TNF-α, IL-1β, or IL-6 in the early stage of D-GalN-induced liver injury, which then quickly decreases. Therefore, a combination of D-galactosamine and endotoxin has also been widely used to induce liver failure in rats, which triggers high levels of proinflammatory cytokines (Tunon et al. 2009). The increase of these cytokines varied greatly with the dose of D-GalN, the type of animals, and other drugs such as endotoxin that were used together with D-GalN. Because some probiotics function by reducing the endotoxin of Gram-negative bacteria in the gut, we did not use endotoxin in this study. In a rat model induced by 1,100 mg/kg body weight D-GalN together

with 10 μl of endotoxin, *B. infantis* DSM 15159 (=CURE21) (Osman et al. 2007) or *Lactobacillus plantarum* DSM 15313 (Osman et al. 2007) decreased TNF-α and IL-1β levels in liver tissue. When 800 mg/kg body weight D-galactosamine (Haro et al. 2009) or 50 μg/kg body weight endotoxin together with 300 mg/kg body weight D-GalN (Wang et al. 2013) was used, *L. casei* Zhang decreased serum TNF-α levels. In the present study, we also found that the level of the anti-inflammatory cytokine IL-10 increased in the group supplemented with *L. salivarius* LI01 or *P. pentosaceus* LI05, which partially explains the reduction of liver injury caused by these two strains. Pretreatment with *L. plantarum* LI04 or *P. pentosaceus* LI05 significantly increased IFN-γ, which indicates the activation of circulating T cells against infection. Other studies using D-GalN-induced rat models supplemented with probiotics have not reported on the effects on IL-10 and IFN-γ.

Bacterial translocation has been postulated as the major mechanism in the pathogenesis of acute liver failure. We found that *L. salivarius* LI01, *L. paracasei* LI03, and *P. pentosaceus* LI05 efficiently prevented bacterial translocation in liver injury. Pretreatment with *L. plantarum* DSM 9843 (Adawi et al. 1997, 1998; Kasravi et al. 1997), *L. plantarum* DSM 15313 (Osman et al. 2007), or *L. reuteri* 108 (Adawi et al. 1997; Kasravi et al. 1997) decreased BT in the liver and MLN. Pretreatment with *L. acidophilus* NM1 or *L. rhamnosus* ATCC 53103 decreased BT in portal blood, liver, and MLN (Adawi et al. 2001). Intestinal bacterial overgrowth (IBO), increased intestinal permeability, and impaired immunity are important factors in the development of BT (Schnabl 2013). Therefore, the mechanism of BT prevention by probiotics is very complex. In this study, pretreatment with *L. salivarius* LI01 protected the intestinal mucosa, crypts, and villi; pretreatment with *P. pentosaceus* LI05 protected the intestinal mucosa and crypts; and pretreatment with *L. paracasei* LI03 protected the intestinal mucosa. These results partially explain the ability of these three strains to decrease the incidence of BT. Moreover, MDS analysis indicated that the composition of the cecal microbiome in the groups supplemented with each of these three strains differed from that in the liver injury control, indicating that the microbiome plays an important role in preventing BT.

Because most probiotics were applied through the gastrointestinal tract, their effects on liver disease were intensively related to their modulation of gut flora. We studied the effects of the five microbes on gut flora using PCR-DGGE. In the groups supplemented with *L. salivarius* LI01 or *P. pentosaceus* LI05, the microbiome compositions were clearly distinct from that of the liver injury control using both MDS and PCA analysis. This result is consistent with the ability of the two strains to decrease BT, reduce liver injury, and improve liver function. In the group supplemented with *L. paracasei* LI03, the composition of the microbiome in the MDS analysis was distinct from that in the liver injury control, but the principal components of the microbiome in PCA analysis were not significantly different. Although BT decreased in this group, liver injury was more serious, indicating that the alterations of the gut microbiome caused by *L. paracasei* LI03 were most likely only beneficial in the prevention of BT. In the group supplemented with *L. plantarum* LI04 or *L. salivarius* LI02, the principal components of the microbiome in PCA analysis were different from those of the liver injury control, but the composition of the microbiome in MDS analysis was not clearly distinguished from the control. No improvements in liver injury or decreases in BT were observed in the two groups. This result indicates that alterations in the profile of the microbiome likely contribute more than changes in only its principal components. In other studies, a culture-dependent method to determine bacterial counts was used to analyze alterations in the microbiome. No significant differences were found in the counts of total aerobic, total anaerobic, Gram-negative anaerobic, *Enterobacteriaceae*, or *Lactobacillus* between groups without or rectally supplemented with 3×10^9 colony-forming units of *L. plantarum* 9843 once daily for 8 days in D-GalN-induced liver injury (Adawi et al. 1997). Oral pretreatment with $2.5–5.0 \times 10^9$ colony-forming units of *L. plantarum* 9843 for 7 days decreased *Enterobacteriaceae* counts and increased *Lactobacillus* counts in the cecum, but pretreatment with *L. reuteri* R2LC did not significantly influence any species in an identical liver injury model (Kasravi et al. 1997). When 3×10^9 colony-forming units of probiotics were daily rectally administered for 8 days and compared with the liver injury control, pretreatment with *B. animalis* NM2 decreased *Enterobacteriaceae* counts in both the cecum and colon, pretreatment with *L. acidophilus* NM1 or *L. rhamnosus* ATCC 53103 decreased *Enterobacteriaceae* counts in the colon, and pretreatment with *L. rhamnosus* ATCC 53103 increased *Lactobacillus* counts in the colon (Adawi et al. 2001). Pretreatment with 3 ml of probiotics at a concentration of approximately 10^8 cells/ml through an orogastric tube twice daily for 8 days decreased aerobic counts in the group with *L. plantarum* DSM 15313 or *B. infantis* DSM 15159 in comparison to the liver injury control (Osman et al. 2007). Although the determination of the intestinal microbiome by bacterial counts is cheap, rapid, and simple, this method is limited by the fact that most gut bacteria are uncultivable. A metagenomic approach will provide more accurate information, but it is expensive and not generally available. PCR-DGGE is less expensive and can briefly reflect the change of microbiota. In this study, we did not observe the increase of any of the five strains in cecal microbiome. This reflects resolution and sensitivity limitations of PCR-DGGE, but is most probably because of no significant increases in the amount of these strains in the experimental rat cecum.

In conclusion, this study describes the effects of five microbes, which were isolated from healthy people, on D-GalN-induced liver injury in rats. Pretreatment with *L. plantarum* LI04 or *L. salivarius* LI02 demonstrated no significant effects during this process. Pretreatment with *L. paracasei* LI03 aggravated liver injury. Pretreatment with *L. salivarius* LI01 or *P. pentosaceus* LI05 significantly reduced elevated ALT and AST levels, prevented the increase in total bilirubin, reduced the histological abnormalities of both the liver and the terminal ileum, decreased bacterial translocation, increased the serum level of IL-10 and/or IFN-γ, and resulted in a cecal microbiome that differed from that of the liver injury control. To the best of our knowledge, the effects of the three species, *L. paracasei*, *L. salivarius*, and *P. pentosaceus*, on D-GalN-induced liver injury have not been previously reported. The excellent characteristics of *L. salivarius* LI01 and *P. pentosaceus* LI05 enable them to serve as potential probiotics in the prevention and treatment of acute liver failure.

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