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Gut *Lactobacillus* and Probiotics *Lactobacillus lactis/rhamnosis* Ameliorate Liver Fibrosis in Prevention and Treatment

Sung Min Won[1] · Na Young Lee[1] · Ki-Kwang Oh[1] · Haripriya Gupta[1] · Satya Priya Sharma[1] · Kyung Hwan Kim[2] · Byoung Kook Kim[2] · Hyun Chae Joung[2] · Jin Ju Jeong[1] · Raja Ganesan[1] · Sang Hak Han[3] · Sang Jun Yoon[1] · Dong Joon Kim[1] · Ki Tae Suk[1]

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Abstract

The progression and exacerbation of liver fibrosis are closely related to the gut microbiome. It is hypothesized that some probiotics may slow the progression of liver fibrosis. In human stool analysis [healthy group ($n=44$) and cirrhosis group ($n=18$)], difference in *Lactobacillus* genus between healthy group and cirrhosis group was observed. Based on human data, preventive and therapeutic effect of probiotics *Lactobacillus lactis* and *L. rhamnosus* was evaluated by using four mice fibrosis models. *L. lactis* and *L. rhamnosus* were supplied to 3,5-diethoxycarbonyl-1,4-dihydrocollidine or carbon tetrachloride-induced liver fibrosis C57BL/6 mouse model. Serum biochemical measurements, tissue staining, and mRNA expression in the liver were evaluated. The microbiome was analyzed in mouse cecal contents. In the mouse model, the effects of *Lactobacillus* in preventing and treating liver fibrosis were different for each microbe species. In case of *L. lactis*, all models showed preventive and therapeutic effects against liver fibrosis. In microbiome analysis in mouse models administered *Lactobacillus*, migration and changes in the ratio and composition of the gut microbial community were confirmed. *L. lactis* and *L. rhamnosus* showed preventive and therapeutic effects on the progression of liver fibrosis, suggesting that *Lactobacillus* intake may be a useful strategy for prevention and treatment.

Keywords Liver fibrosis · *Lactobacillus lactis* · *Lactobacillus rhamnosus* · Probiotics · Gut microbiome

Introduction

The gut has approximately 100 trillion microbes that promote normal gastrointestinal tract function, protect the body from infection, and regulate metabolism and the mucosal immune system (Belkaid & Hand, 2014). Irregular lifestyle habits and stress in modern society cause an imbalance of intestinal microbes (Zhang et al., 2015). Recently, probiotics have been shown to modulate the gut microbiome by increasing beneficial bacteria and reducing harmful bacteria in the gut (Azad et al., 2018). Microbes have been found to play important roles in human health and disease (Turnbaugh & Stintzi, 2011). Gut microbiome have been linked to a variety of disorders, from bowel diseases such as colorectal cancer and inflammatory bowel disease to more systemic disease, such as diabetes, metabolic syndrome, and atopy (Walker & Lawley, 2013). The gut microbiota also affects patients with obesity-induced diabetes and related complications (Ortega et al., 2020).

Liver fibrosis is an excessive accumulation of extracellular matrix proteins, including collagen, that occurs in most chronic liver diseases (Bataller & Brenner, 2005). The main causes of liver fibrosis in developed countries are alcohol abuse, chronic hepatitis viral infection, and nonalcoholic fatty hepatitis. Fibrosis is the final and common pathological consequence of many chronic inflammatory diseases (Wynn, 2008). Collagen deposition is essential and is usually a reversible part of wound healing, but if tissue damage is severe or repetitive or if the wound

Sung Min Won and Sang Jun Yoon contributed equally to this work.

✉ Ki Tae Suk
ktsuk@hallym.ac.kr

[1] Institute for Liver and Digestive Diseases, Hallym University, Chuncheon 24252, Republic of Korea

[2] Chong Kun Dang Bio Research Institute, Ansan, Republic of Korea

[3] Department of Pathology, Hallym University College of Medicine, Chuncheon 24252, Republic of Korea

healing response itself becomes dysregulated, normal tissue repair can progressively develop into an irreversible fibrosis reaction (Wynn & Ramalingam, 2012). Liver fibrosis can progress into more severe stages, such as cirrhosis and further to liver cancer (Yanguas et al., 2016). Controlling inflammation can prevent fibrosis progression. The goal of developing antifibrotic medication is to suppress or reverse the progression of fibrosis in chronic liver disease (Trautwein et al., 2015).

Liver and biliary tract disease refer to a related cause of chronic liver disease associated with severe complications (Arroyo et al., 2016). The intake of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) recreates the main pathological features of human liver and biliary tract diseases, such as fibrosis and inflammatory infiltration (Pose et al., 2019). The toxicity of carbon tetrachloride (CCl_4) can induce the physiological changes observed in cirrhosis and liver fibrosis. CCl_4 can be inhaled; administered orally or intragastrically; or subcutaneously or intraperitoneally injected, and its effects can be induced more quickly if added to drinking water (Dong et al., 2016).

Probiotics can live by forming colonies in the gut through various mechanisms in the stomach and intestines. The beneficial effect of lactic acid bacteria via the gut-liver axis is extended to liver function in cirrhosis, nonalcoholic fatty liver diseases and alcoholic liver disease (Sharma et al., 2013). A previous study showed that the administration of beneficial bacterial strains helps to improve harmful interactions and liver disease (Lee et al., 2020a, 2020b). In addition, many studies have shown that probiotics can play a role in controlling harmful bacteria in the body (Kim et al., 2019). To take advantage of the beneficial properties of these probiotics, it is important to determine the effects of microbes in the digestive tract of humans and animals (Markowiak & Slizewska, 2017). Moreover, some studies have reported that the use of probiotics may be an alternative to antibiotic treatments. Therefore, probiotics that restore or improve the gut microbiota can be expected to have a role in the development of treatments for modern diseases (Hemarajata & Versalovic, 2013).

Antifibrotic therapy is aimed at inhibiting the accumulation of fibrotic cells or preventing the deposition of extracellular matrix proteins (Ghiassi-Nejad & Friedman, 2008). Recent advances in microbiome research have shown that the gut microbiome modulates barrier function and affects dysbiosis (Fukui, 2019; Lee et al., 2020a, 2020b). In previous studies, probiotics have been shown to reduce weight and body mass index, improve liver function, lower glucose levels in plasma, relieve inflammation, and restore liver fat penetration. Therefore, these findings suggest a potential role for probiotics in the prevention or treatment of liver disease. This study evaluated preventive and therapeutic role in liver fibrosis of probiotics *L. lactis* and *L. rhamnosus*.

Materials and Methods

Patients

This observational study was carried out between April 2018 and March 2021 (Table 1). A total of 62 patients comprising normal controls ($n=44$) and alcoholic cirrhosis patients ($n=18$) were enrolled. The patients underwent standard treatment for their disease. Stools of the normal control group were collected from the health center in the hospital. The diagnosis of cirrhosis was made through imaging findings, alcohol consumption history, and pathologic examination of liver biopsy. The exclusion criteria were as follows: patients with a history of viral hepatitis, nonalcoholic fatty liver disease, autoimmune disease, tumor presence, or drug-induced liver injury. This study was controlled in accordance with ethical guidelines from the 1975 Helsinki Declaration as reflected by prior approval by the institutional review notice for human research in the hospital participating in the trial (2016-134). Basic information has been registered on ClinicalTrials.gov for registration in the public trial registry (NCT04339725). Informed consent for enrollment was received from each participant.

A baseline evaluation was performed, including a complete blood count, liver function test, and assessment of viral markers. Patients underwent abdominal ultrasound or computed tomography imaging. aspartate amino transferase (AST), alanine amino transferase (ALT), total bilirubin (T-BIL), gamma-glutamyl transferase, albumin, prothrombin time, international normalized ratio creatinine, and α-fetoprotein were included in the serum biochemical parameters. Tests for hepatitis viruses and human

Table 1 Baseline characteristics of patients

Variable	Normal control ($n=44$)	Cirrhosis patients ($n=18$)	*P*-value
Sex (male)	23 (21)	7 (11)	
Age (years)	61.1 ± 8.0	70.6 ± 10.3	< 0.001
AST (IU/l)	23.1 ± 4.6	86.4 ± 126.0	0.001
ALT (IU/l)	19.1 ± 6.8	58.3 ± 86.8	0.004
Creatine (mg/dl)	0.9 ± 0.2	1.0 ± 0.3	N.S
Cholesterol (mg/dl)	177.1 ± 38.8	125.9 ± 76.0	0.001
γGT (IU/l)	25.7 ± 17.2	175.0 ± 229.5	< 0.001
TG (mg/dl)	118.3 ± 109.4	92.3 ± 47.4	N.S
HDL (mg/dl)	54.0 ± 18.6	50.1 ± 20.2	N.S
BMI	23.1 ± 3.3	25.1 ± 7.0	N.S

Data are presented as mean (standard deviation or %)

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *γGT* gamma glutamyl transferase, *TG* triglyceride, *HDL* high density lipoprotein, *BMI* body mass index, *N.S* not significant

immunodeficiency virus were conducted in all subjects. Enrolled patients and control patients underwent stool sampling and clinical analysis. Clinical data were simultaneously matched with metagenomics data. Fecal samples were obtained in a plastic collection kit at various times during the day. All samples were stored at -80 °C. Stool samples were collected from healthy controls at home and kept at -20 °C in a refrigerator. The patients then sent the stool box to the hospital, where the samples were kept at − 80 °C.

Microbiome Analysis

Metagenomic DNA was extracted using a QIAamp stool kit (Qiagen). After the first amplification of the V3-V4 region of the bacterial 16S rRNA gene, the second amplification was performed using Barcoded universal primers. An Agencourt AMPure XP system (Beckman) was used for the purification of amplicons. PicoGreen and quantitative PCR were utilized for quantification of the purified amplicons. After pooling of the barcoded amplicons, a MiSeq sequencer on an Illumina platform (CJ bioscience Inc.) was used for sequencing according to the manufacturer's specifications.

The 16S-based Microbial Taxonomic Profiling platform of EzBioCloud Apps (CJ bioscience Inc.) was used for microbiome profiling. After taxonomic profiling of each sample, a comparative analysis of the samples was performed by comparison with the EzBioCloud database. CJ bioscience's 16S rRNA database (DB ver. PKSSU4.0) (Yoon et al., 2017) was used for the taxonomic assignment of reads. OTU picking was achieved with UCLUST (Edgar, 2010) and CDHIT utilizing a 97% similarity cutoff (Xie et al., 2006). Beta-diversity, which includes PCoA and UPGMA clustering, was displayed in the comparative MTP analyzer.

Strain Preparation

Lactobacillus lactis and *L. rhamnosus* are lactic acid bacteria that have been isolated from various sources, including sour milk and newborn baby feces, respectively. *Lactobacillus* spp. were inoculated into a flask containing de Man, Rogosa and Sharpe medium (BD Difco). The strains were incubated under anaerobic conditions at 37 °C for 24 h. Stocks of each strain were prepared by mixing the culture broth with an equivalent 20% skim milk solution and then stored at -80 °C until use. The seed culture for *Lactobacillus* spp. was grown at 37 °C for 24 h in a flask containing MRS broth. Each culture was inoculated in an optimized medium in a fermenter (Bio Control & Science, MARADO-05D-PS, Daeduk). The fermentation was carried out at a constant pH of 5.5–6.0 via automatic addition of NaOH solution (25% w/v) under the conditions of 120 rpm agitation at 37 °C for 18–20 h. At the end of the fermentation, the cells were harvested by centrifugation at 6000 rpm for 10 min (Hanil, Supra R12, Hanil). Lyophilization of 40 × concentrated cells was accomplished in accordance with the Cooling & Heating System manual (Lab-Mast 10). After lyophilization, the colony-forming units (CFU) per gram of each probiotic powder were measured using a serial dilution method. Probiotics were suspended in 0.1 M PBS and adjusted to a density of 10^9 CFU/ml prior to use. The baseline characteristics of the probiotic strains are reported in Table 2.

Animal Study

Five weeks old pathogen free male C57BL/6J mice were sourced from Doo Yeol Biotech. All mice were housed in individual steel micro isolator cages maintained at 22 °C ± 2 °C having a 12/12-h light/dark cycle. Throughout the experiment, mice had free access to water and food, and were monitored daily. The experiment design included an adaptation period for all groups, during which mice were fed a normal diet for a week for adaptation period. Mice were treated humanely, and all aspects of the animal study was performed in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. This experiment was designed by selecting a candidate for the treatment of liver disease among the probiotics that approved by Ministry of Food and Drug Safety, Korean. All the procedures were licensed by the Institutional Animal Care and Use Committee of the College of Medicine, Hallym University R1(2019-04).

DDC diet (2018S, Doo Yeol Biotech) was prepared using 0.1% of DDC (Sigma-Aldrich) reagent. In the prevention and treatment model, DDC diet was supplied as a chew diet every day for 23 days. CCl4 was mixed 1:4 ratios with corn oil (Sigma-Aldrich) and intraperitoneal injection with 100 µl twice in a week. CCL4 was injected for 9 weeks in prevention and treatment model.

Strains were suspended in distilled water or binge that maintaining concentration of 10^9 CFU/g. Weight was recorded daily from the first day of the experiment. The mice were eventually sacrificed via overdose of inhalation anaesthesia by isoflurane (Hana Pharm) at the conclusion of treatment period. Mice were sacrificed after 1 day starvation at the last day. Following the weighing of the mouse blood, liver, cecum, and small intestine were collected. The

Table 2 Information of used strains

Strain	Number of bacteria (CFU/g)	Characteristics	Origin of strain
L. lactis	1.10E+11	Gram-positive	Sour milk
L. rhamnosus	4.70E+11	Gram-positive	Newborn baby feces

CFU colony forming unit; *L*, *Lactobacillus*

collected blood by cardiac puncture was placed overnight at 4 °C and centrifuged to collect serum. Serum was collected via centrifugation (5 min for 19,000×*g*) from whole blood (800 µl). The liver and cecum were excised and subsequently stored at − 80 °C. Cecal stool was collected for the microbiota analysis and stored at − 80 °C.

Serum Biochemistry Analysis

From animal serum, AST, ALT and T-BIL were quantified utilizing a biochemical blood analyzer (KoneLab 20, Thermo Fisher Scientific).

Pathology Analysis

10% formalin was used in fixation of specimens, which were embedded in paraffin. The tissue sections underwent staining with Hematoxylin and Eosin, Masson trichrome, and Sirius red stain. The liver was categorized in accordance with the clinical research network scoring system for lobular inflammation and necrosis from grade 0 to 4 (0, none; (1) minimal and patchy; (2) mild and involving some or all portal tracts; (3) involving all portal tracts; (4) severe may have bridging necrosis) and criteria from stage 0 to 4 (0, no fibrosis; 1, portal fibrosis; 2, periportal fibrosis; 3, septal fibrosis; 4, cirrhosis). All these biopsy specimen analyses were performed by one pathologist (SHH) who was blinded to the experimental conditions.

RNA Extraction and Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

Liver tissue samples were homogenized in TRIzol reagent (Invitrogen). High Pure RNA Isolation Kit (Roche) was used to isolate RNA from liver tissue. Total RNA isolation from tissue was used a cDNA reverse transcription kit (Applied Biosystems), aliquots of total RNA (2 µg) were transformed into cDNA. The cDNA subsequently underwent amplification for quantitative PCR utilizing Luna® Universal Probe qPCR Master Mix (New England Biolabs Beverly) and target-specific probe-primer (Applied Biosystems).

For the evaluation of fibrosis severity, we used molecular marker of liver fibrosis/injury (Collagen, type I, alpha 1 [Col1a1, major component of type I collagen], tissue inhibitor of metalloproteinases1 [Timp1, degradation of the extracellular matrix], and transforming growth factor beta [TGF-β, multifunctional cytokine]).

Statistical Analysis

Continuous variables were expressed as means and standard deviations. One-way ANOVA and independent sample *T*-test were performed for body weight, liver function test, and histology analyses. For additional statistical analysis, data underwent normalization based on MSTUS18 which is implemented in NOREVA (http://idrb.zju.edu.cn/noreva). Multiple Experiment Viewer (MeV) was employed for hierarchical clustering analysis (HCA) and analysis of variance (ANOVA) with a post hoc test. P value < 0.05 was statistically significant. All statistical analyses were performed via SPSS software (ver. 19, SPSS Inc.).

Results

Baseline Characteristics of Patients

There was a significant difference in the mean levels of AST ($P < 0.001$) and ALT ($P < 0.004$) between the groups, with higher levels in the cirrhosis patient group than in the normal group. The mean cholesterol level was significantly ($P < 0.001$) higher in the normal group than in the cirrhosis patient group. The mean level of γGT was significantly ($P < 0.001$) higher in the cirrhosis patient group than in the normal group. There was no significant difference in triglyceride, High-density lipoprotein, or low-density lipoprotein levels (Table 1).

Human Stool Microbiome Analysis

We performed a taxonomic composition comparison between groups at the phylum level. The proportion of *Bacteroidetes* was decreased in the cirrhosis patient group (24.6%) compared to the normal control group (50.9%), and *Proteobacteria* abundance was increased in the cirrhosis group (35.8%) compared to the normal control group (11.8%) (Fig. 1A). The alpha diversity indices were significantly different between the groups. The ACE and Chao1 indices, which are species richness indices, were significantly decreased ($P < 0.01$) in the cirrhosis patient group compared to the normal group, and a significant decrease ($P < 0.01$) in the cirrhosis patient group was also confirmed by the Shannon index, which is a diversity index (Fig. 1B). A comparative analysis of beta diversity between groups was performed. For principal coordinates analysis (PCoA), UniFrac distances were used to check the differences between groups, and the change in movement between groups was confirmed through region discrimination (Fig. 1C).

We compared and analyzed the relative abundance of specific taxa in the normal control group and cirrhosis patient group (Fig. 1D). *Bacteroidetes* and *Firmicutes* are the dominant taxa accounting for the largest proportion of the intestinal microbiota, and the ratio of *Bacteroidetes* and *Firmicutes* can be used as an indicator of various diseases. The relative abundance of *Bacteroidetes* was significantly decreased ($P < 0.01$) in the cirrhosis patient group compared

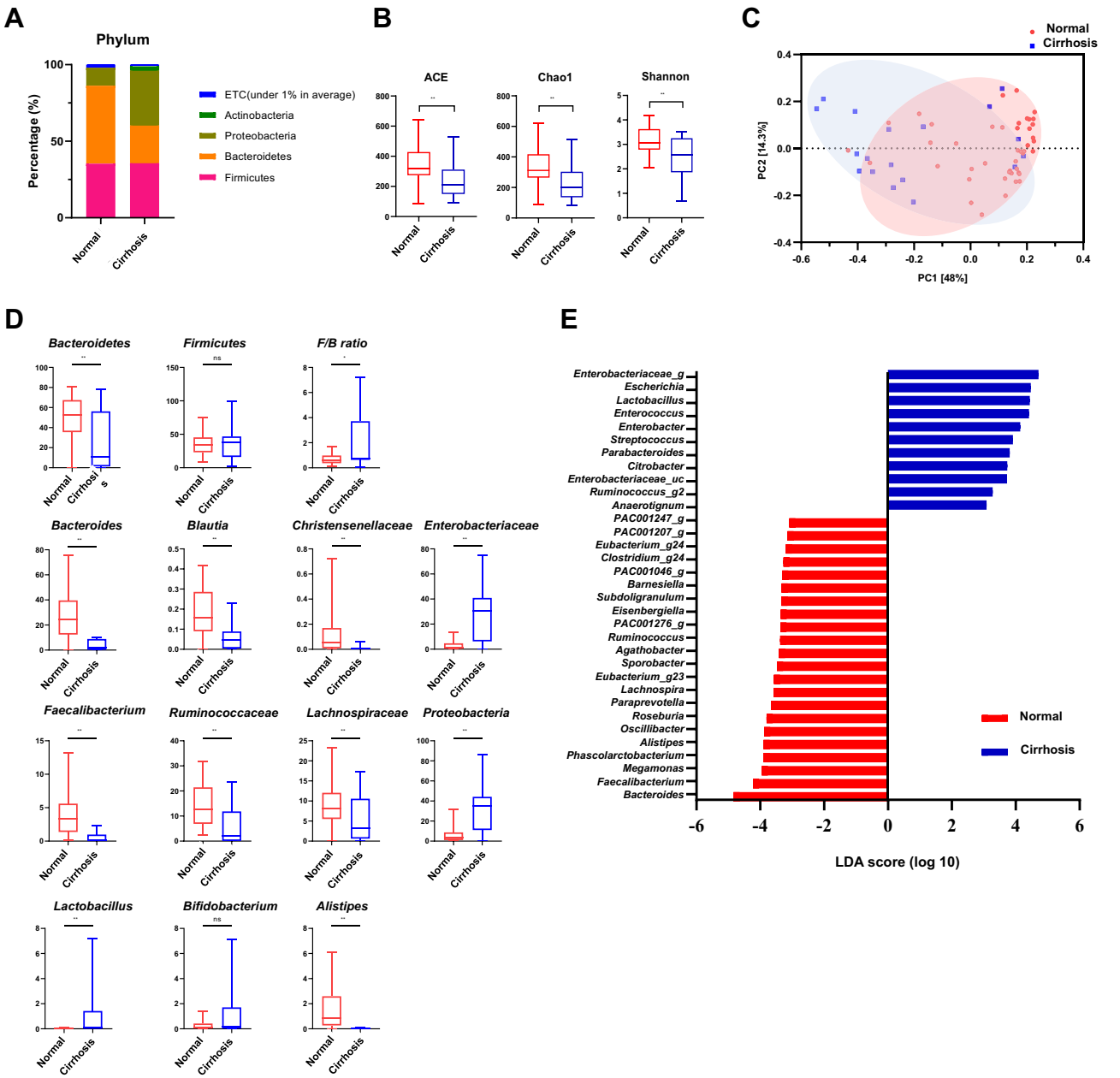

Fig. 1 The human gut microbiome analysis. **A** Phylum composition of normal control and cirrhosis patients group. **B** Alpha diversity in normal control and cirrhosis patients group. **C** Beta diversity: PCoA in normal control and cirrhosis patients group. **D** The relative abundance of important taxa in human gut. **E** LEfSe analysis between normal control and cirrhosis patients group. $*P<0.05$, $**P<0.01$

with the normal control group. There was no significant difference in *Firmicutes*. However, the *Firmicutes/Bacteroidetes* (F/B) ratio was significantly increased ($P<0.05$) in the cirrhosis patient group compared to the normal control group. We analyzed the relative abundance between groups of major taxa known to be important in the human gut. *Bacteroides*, *Blautia*, *Christensenellaceae*, *Faecalibacterium*, *Ruminococcaceae*, *Lachnospiraceae* and *Alistipes* were significantly decreased ($P<0.01$) in the cirrhosis patient group compared to the normal control group. *Enterobacteriaceae*, *Proteobacteria* and *Lactobacillus* were significantly increased ($P<0.01$) in the cirrhosis patient group compared to the normal control group. There was no significant difference between the two groups in *Bifidobacterium*.

We analyzed the difference between the normal control group and the cirrhosis patient group using linear discriminant analysis effect size (LEfSe) analysis in the gut microbial community at the genus level (Fig. 1E). In the cirrhosis

patient group, at the genus level, taxa belonging to the Enterobacteriaceae family, such as the *Enterobacteriaceae_g*, *Escherichia*, *Enterobacter*, and *Enterobacteriaceae_uc*, and *Lactobacillus* and *Streptococcus* genera, were dominant. In the normal control group, the predominance of the *Bacteroides*, *Faecalibacterium*, *Megamonas*, *Alisipes*, *Oscillibacter* and *Roseburia* genera was confirmed.

Preventive Effect in Fibrosis Mouse Model

To confirm a preventive effect, *Lactobacillus* spp. were administered while liver fibrosis was induced by feeding with a DDC diet for 3 weeks (Fig. 2A). Body weight decreased in all the groups due to the DDC diet feeding but not in the normal diet group (Fig. 2B). There was no significant difference in the body and liver weights after 3 weeks in the probiotics groups compared with the DDC group. In serum biochemical analysis, the strain-administered groups showed a tendency toward decreased AST compared to the DDC diet group, and AST was significantly decreased in the *L. rhamnosus* group ($P < 0.05$) (Fig. 2C). There was no significant difference in AST and T-BIL due to strain administration (Fig. S1A). A significant decrease in the fibrotic area ($P < 0.01$) was confirmed in the group administered *L. lactis* and *L. rhamnosus*, as shown by Sirius red staining (Fig. 2D). In the measurement of fibrosis-related gene expression levels, *L. lactis* administration significantly reduced *Col1a*, *Timp1*, and *TGF-β* expression levels ($P < 0.05$) (Fig. 2E).

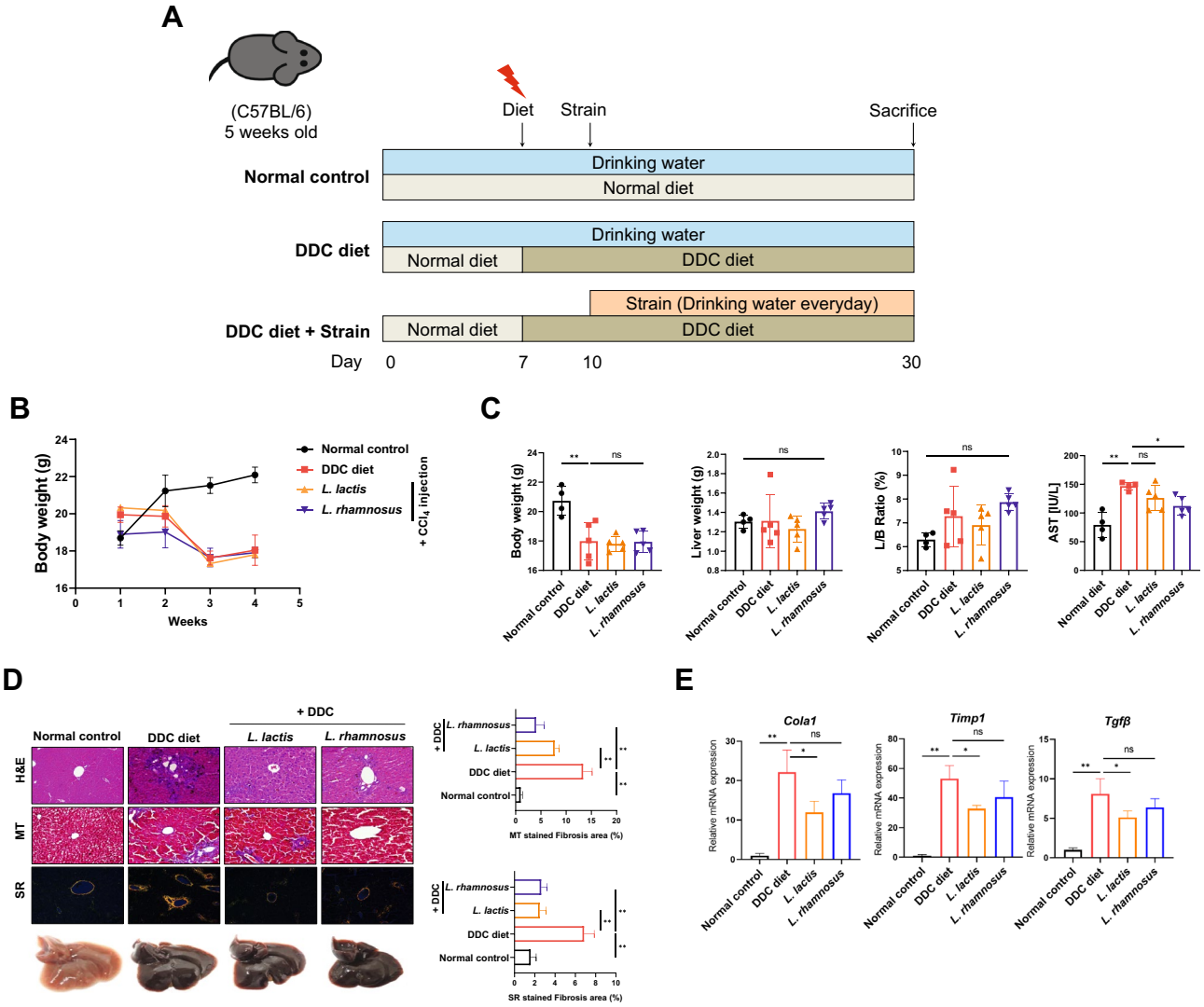

Fig. 2 Prevention effect of *Lactobacillus* in DDC diet-induced liver fibrosis model. **A** Flow chart of the DDC diet-induced liver fibrosis model. **B** The body weight change of DDC diet-induced liver fibrosis model for 4 weeks. **C** The body weight, Liver weight, Liver/Body weight ratio and serum biochemistry AST. **D** Effects of strain on the liver tissue: H&E. MT, and Sirius red staining. × 200. **E** mRNA expression of the liver tissue. $^{*}P < 0.05$, $^{**}P < 0.01$

To confirm the preventive effect of *Lactobacillus* in the liver fibrosis, it was induced by CCl_4 injection for 9 weeks. CCl_4 was diluted 1:4 with corn oil and injected by intraperitoneal injection (Fig. 3A). For 9 weeks, *L. lactis* group was protect-ed from weight loss, but the *L. rhamnosus* group was not (Fig. 3B). There was no significant difference in weight and L/B ratio between groups (Fig. 3C). In blood biochemical analysis, AST and ALP were not significantly different between the CCl_4 group and the probiotics groups (Figs. 3C, S1B). However, T-BIL was significantly decreased in the *L. lactis* group ($P < 0.05$) and *L. rhamnosus* group ($P < 0.01$) compared with the CCl_4 group (Fig. S1B). There was a significant decrease ($P < 0.01$) in fibrosis area by probiotics administration (Fig. 3D). The *L. lactis* reduced the expression of *Col1a* and *Timp1* significantly in the liver tissue ($P < 0.01$) (Fig. 3E).

Therapeutic Effect on Fibrosis Model

To confirm the therapeutic effect of *Lactobacillus* in the DDC diet-induced liver fibrosis model, a 1 week intake period of *Lactobacillus* was added after liver fibrosis induction for 3 weeks with the DDC diet (Fig. 4A). After being fed the DDC diet, the body weight of the mice in all groups decreased and then increased again when the mice were switched to a normal diet (Fig. 4B). However, weights

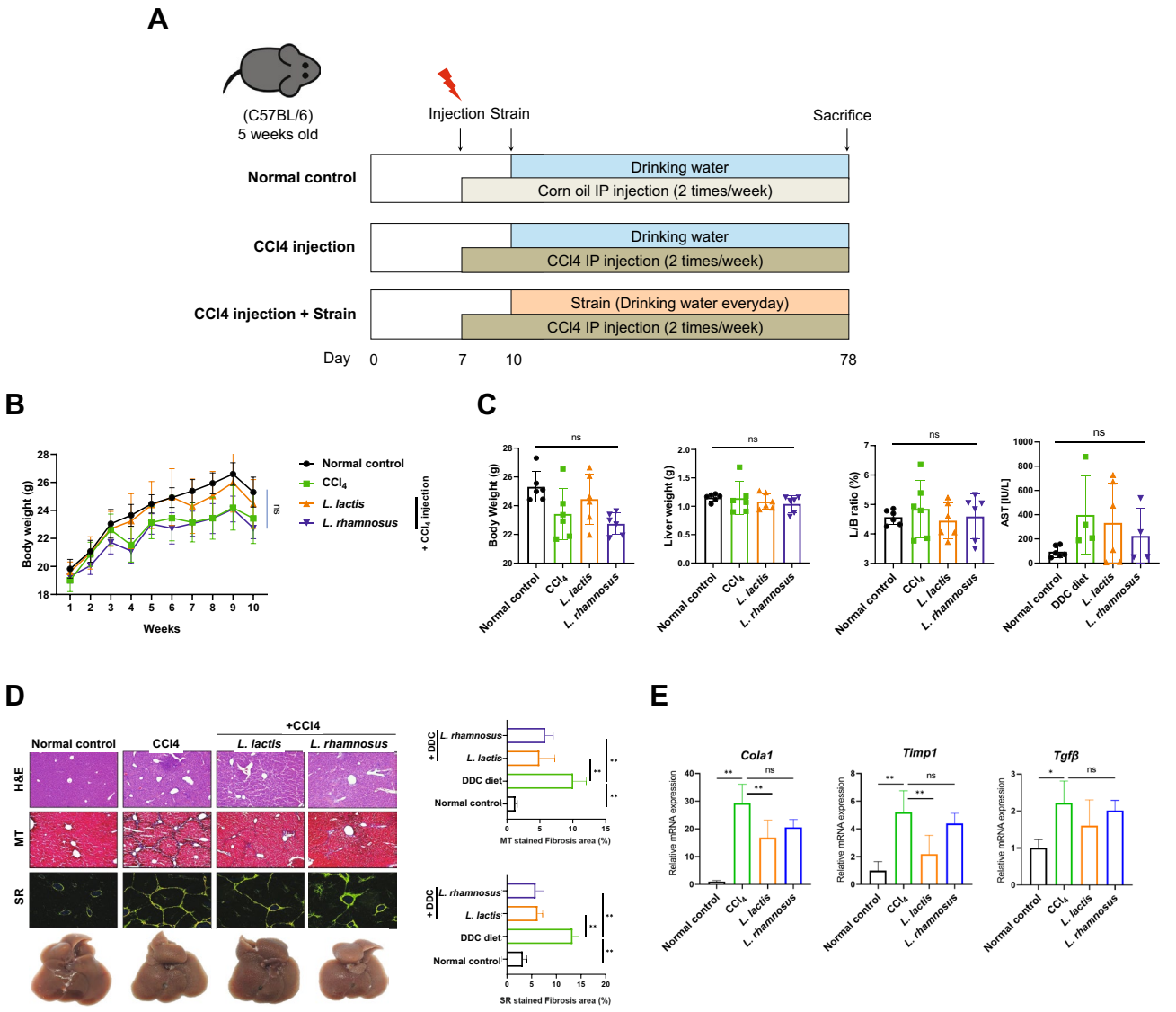

Fig. 3 Prevention effect of *Lactobacillus* in CCl_4 injection-induced liver fibrosis model. **A** Flow chart of the CCl_4 injection-induced liver fibrosis model. **B** The body weight change of CCl_4-induced liver fibrosis model for 10 weeks. **C** The body weight, Liver weight, Liver/Body weight ratio and serum biochemistry AST. **D** Effects of strain on the liver tissue: H&E. MT, and Sirius red staining. × 200. **F** mRNA expression of the liver tissue. $^*P < 0.05$, $^{**}P < 0.01$

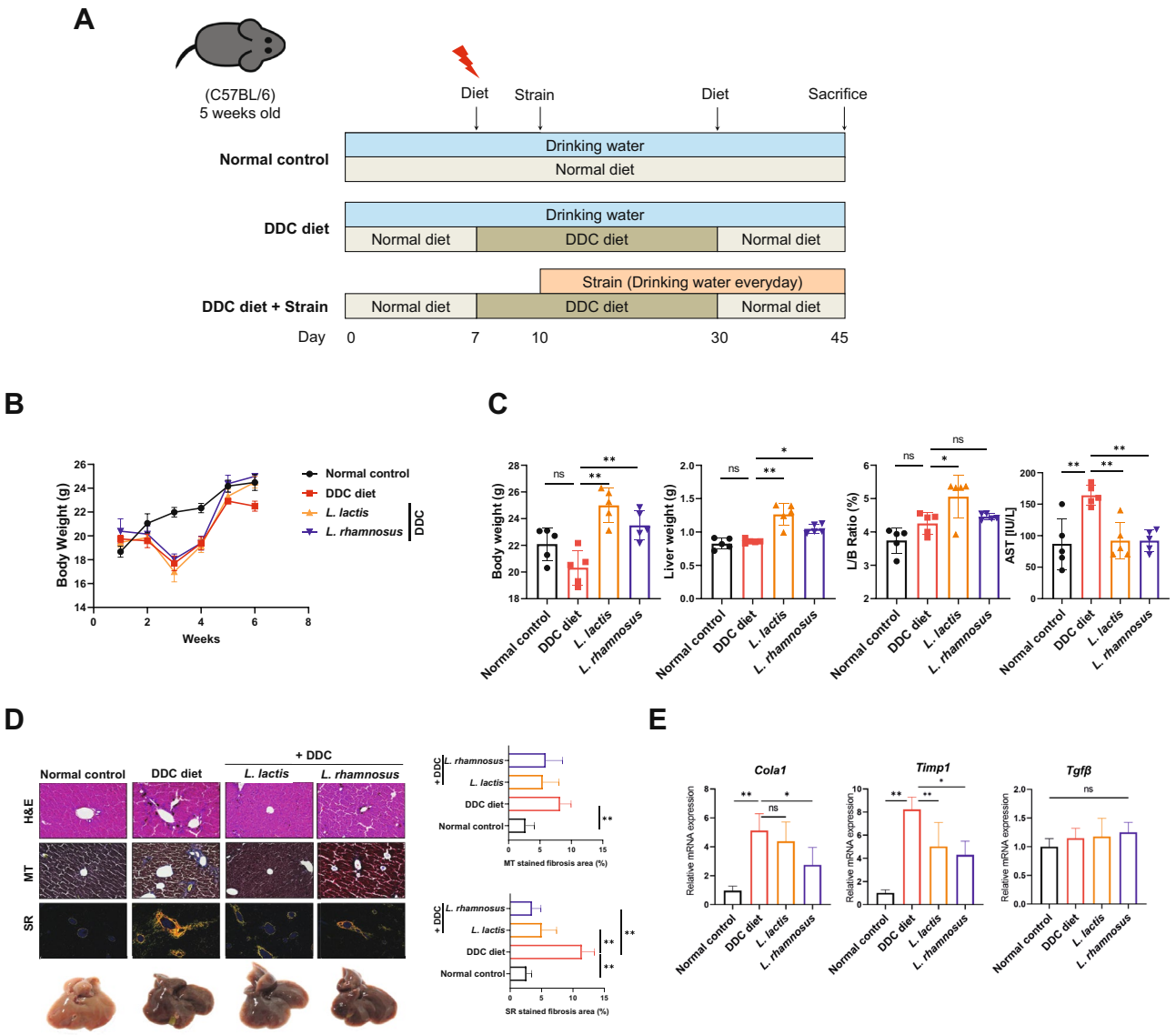

Fig. 4 Treatment effect of *Lactobacillus* in DDC Diet-induced liver fibrosis model. **A** Flow chart of the DDC diet-induced liver fibrosis model. **B** The body weight change of DDC diet-induced liver fibrosis model for 4 weeks. **C** The body weight, Liver weight, Liver/Body weight ratio and serum biochemistry about AST. **D** Effects of strain on the liver tissue: H&E. MT, and Sirius red staining. × 200. **F** mRNA expression of the liver tissue. $^{*}P < 0.05$, $^{**}P < 0.01$

decreased again in the DDC diet group. With the additional intake of *Lactobacillus* after induction of liver fibrosis, mice in the *L. lactis* (25 ± 1.1 g) and *L. rhamnosus* (23.5 ± 1 g) groups recovered body weight compared with mice in the DDC diet group (20.3 ± 1.2) ($P < 0.01$) (Fig. 4C). In addition, in all groups, *L. lactis* (92 ± 26.4) and *L. rhamnosus* (92 ± 15) significantly reduced AST levels compared to levels in the DDC diet group (164 ± 14.3) ($P < 0.01$) (Fig. 4C). In both the *L. lactis* group and *L. rhamnosus* group, the fibrotic area was significantly reduced compared to that in the DDC group ($P < 0.01$) (Fig. 4D). The *L. rhamnosus* group showed significantly reduced expression of *Col1a* ($P < 0.05$) and *Timp1* ($P < 0.01$) compared to the DDC group. The *L. lactis* group exhibited significantly reduced expression of *Timp1* ($P < 0.05$) compared to the DDC group (Fig. 4E). The *TGF-β* level in all groups recovered to a normal level after the DDC diet was stopped.

To confirm the therapeutic effect of *Lactobacillus* in the liver fibrosis model induced by CCl_4 injection, a 1 week *Lactobacillus* intake period was added after induction of liver fibrosis for 9 weeks via CCl_4 injection (Fig. S2A). Intake of *L. lactis* was not shown to protect against body weight loss or liver weight loss (Fig. S2C). There was no significant difference in the ALP level, but treatment with *L. lactis*

intake increased the ALT concentration, and the *Lactobacillus* intake treatment increased the T-BIL concentration (Fig. S2D). *Lactobacillus* treatment led to a significant decrease in the fibrosis area, measured by Sirius red staining (Fig. S2E). *L. lactis* significantly decreased the expression of *Col1a* ($P < 0.01$) (Fig. S2F). In the *L. rhamnosus* group, the *TGF-β* level recovered to the normal control level within 1 week after the CCl_4 injections were stopped.

Mouse Cecal Microbiome Analysis

At the phylum level, the microbiome composition in each mouse model group showed differences. In the DDC prevention model, a decrease in the proportion of *Proteobacteria* was confirmed in the *Lactobacillus*-treated group compared to the DDC diet group. Similarly, in the DDC treatment model, the proportion of *Proteobacteria* was decreased by treatment with *Lactobacillus*. There was no significant change in the ratio of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* in the CCl_4 prevention model and the CCL_4 treatment model. In the CCl_4 treatment model, an increase in the rate of *Verrucomicrobia* (21.6%) through *L. lactis* administration was confirmed (Fig. 5A).

We analyzed alpha diversity by group in four types of mouse models, and no significant difference in the species richness index Chao1 and the Shannon diversity index were observed (Fig. 5B). We confirmed beta diversity by model through PCoA. As the movement in each group was clearly distinct in the DDC prevention model and the DDC treatment model, changes in the microbiome through *Lactobacillus* treatment were confirmed. In the CCl_4 prevention model, there was a change in the distance in each group, but a clear distinction was not made. In particular, distinction and distance changes induced by *L. lactis* and *L. rhamnosus* treatment were insignificant in the CCl_4 treatment model (Fig. 5C).

The genera showing differences in each group were selected and compared through a heatmap (Fig. 5D). In the DDC prevention group, the *Eubacterium_g17, Eubacterium_g23* and Eubacterium_g6 genera were increased through *Lactobacillus* treatment. In the DDC treatment group, a marked increase in the *Muribaculum*, *Eubacterium_g17* and *Akkermansia* genera was confirmed to be induced by *Lactobacillus* treatment. In the CCl_4 prevention group, the change caused by *L. lactis* treatment was clear, and increases in the *Alisipes*, *Muribaculum*, *Parabacteroides* and *Mucispirillum* genera were confirmed. In the CCl_4 treatment group, *Lactobacillus* treatment increased the *Bacteroides* genus, and in particular, *L. lactis* treatment increased the *Eubacterium_g23* and *Akkermansia* genera. We confirmed the abundance of major *Lactobacillus* species that changed after *L. lactis* and *L. rhamnosus* administration (Fig. S3). When the mice were treated with *L. lactis* and *L. rhamnosus*, the abundance of the *Lactobacillus reuteri*, *Lactobacillus murinus* and *Lactobacillus gasseri* and *Lactobacillus* genera in the intestine, which were commonly distributed in the four models, was confirmed. In the DDC prevention model and the DDC treatment model, an increase in intestinal *Lactobacillus* abundance induced by *Lactobacillus* treatment was confirmed, but in the CCl_4 prevention model and CCl_4 treatment model, a change in intestinal *Lactobacillus* abundance induced by *Lactobacillus* treatment was not confirmed.

Discussion

Hepatic fibrosis is a response to chronic liver damage, in which some hepatocytes undergo apoptosis and progressive loss of liver function occurs (Schuppan & Afdhal, 2008). Cirrhosis patients have a high probability of progressing to liver cancer and liver failure in the late stage of liver fibrosis (Wiegand & Berg, 2013). There are several causes for the onset and progression of liver fibrosis, and recent studies on its association with the microbiome have been extensively conducted (Ray, 2017). Through portal vein connections, the liver and microbiome interact in a bidirectional manner, and dysbiosis of the gut microbiome may contribute to the early stages of the hepatic fibrosis phase (Schnabl & Brenner, 2014). In our human stool microbiome data, the differences between the normal and cirrhosis patient groups are evident, supporting a bidirectional relationship between the liver and the microbiome. Excessive proliferation of the *Proteobacteria* taxa containing many harmful bacteria and the *Enterobacteriaceae* (Acharya & Bajaj, 2019) taxa containing mainly ammonia-producing bacteria is consistent with previous cirrhotic microbiome studies, suggesting a negative effect through dysbiosis. The data we collected showing decreased microbial diversity and species richness and changes in *Firmicutes/Bacteroidetes* ratios in the microbiome of mice in the normal and cirrhotic groups indicate distinct signs of dysbiosis. In addition, the pattern of changes in the abundance of important taxa in the human gut observed in these results suggests a link between disease and dysbiosis.

Several studies have been conducted on the ability of probiotics to regulate the intestinal microbial community and suppress harmful bacteria in the gut (Mulaw et al., 2019). In addition, probiotics aid the growth of beneficial bacteria in the intestine, and an immune enhancing effect of the substances produced by these beneficial bacteria has also been observed (Famouri et al., 2017). Many studies have been conducted on the effects of lactic acid bacteria treatment on liver cirrhosis and liver fibrosis, and an inhibitory effect of certain probiotics on fibrosis has been observed (Liu et al., 2020; Shi et al., 2017). However, although probiotics are

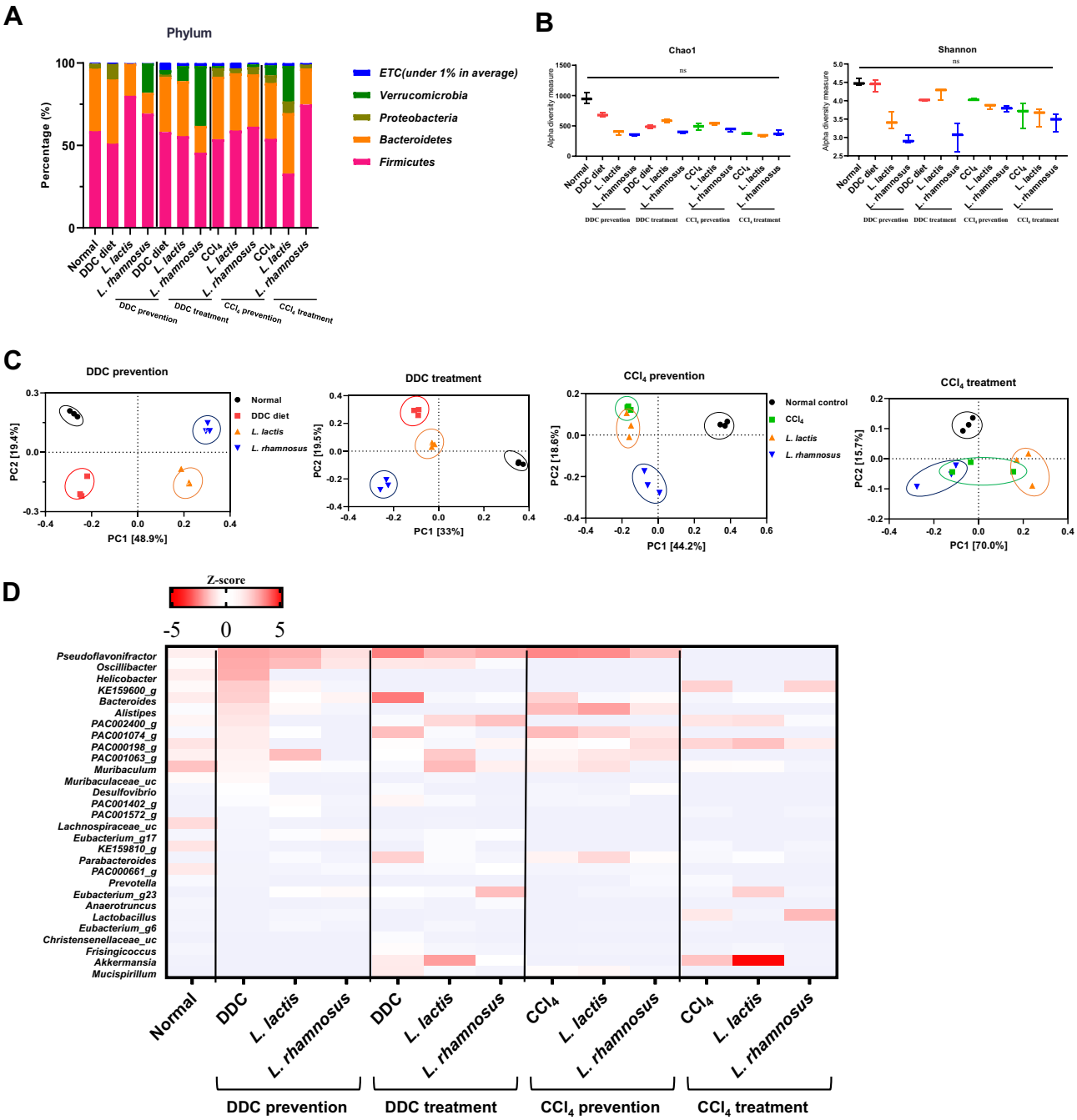

Fig. 5 The animal gut microbiome analysis. **A** Phylum composition of 4 types of models. **B** Alpha diversity between groups. **C** Beta diversity: PCoA of 4 types of models. **D** The heatmap of composition of genus diversity in groups. *N.S.* not significant

attracting attention as a promising option for treatment of liver disease, the mechanism of action remains unclear.

Based on previous research evidence, the experiments in the present study were performed with animal models, and the results suggest that lactobacillus strains might be a treatment option for liver fibrosis. A model of liver fibrosis caused by various factors was induced by a DDC diet and CCl_4 intraperitoneal injection. In our study, when DDC diet-induced liver fibrosis was present, simultaneously ingested *Lactobacillus*, *L. lactis* showed the most effective preventive effect against liver fibrosis by lowering the expression of *Col1a, Timp1*, and *TGF-β*. When *Lactobacillus* was ingested even after the induction of liver fibrosis, body weight was recovered in all the *L. lactis* and *L. rhamnosus* groups compared to the DDC diet group. In addition, all groups showed significantly reduced AST levels in serum

compared to the DDC diet group. Moreover, all groups had significantly reduced cirrhosis scores. In the DDC diet model, *Lactobacillus* showed a greater effect in the treatment of induced liver fibrosis.

Both *Col1a* and *Timp1* mRNA expression was decreased, but the decrease was most significant in the *L. rhamnosus* group. In the case of *TGF-β*, all groups recovered to normal levels. However, the *TGF-β* level appears to have recovered due to cessation of the DDC diet. In a previous report, *L. plantarum* and *L. brevis* counteracted the TGF-β-induced fibrotic marker by modulating SMAD-assocoated TGF-β signaling (Kanmani & Kim, 2022). It is supposed that *L. lactis* and *L. rhamnosus* are not related with SMAD-assocoated TGF-β signaling.

In our previous study, *L. lactis* alleviated inflammation and steatosis in nonalcoholic fatty liver disease (NAFLD) through regulation of the gut microbiome (Lee et al., 2020a, 2020b). This *L. lactis* strain, which was the same strain used in the present study, induced an effective reduction in AST, ALT, and cholesterol levels in the NAFLD model. Additionally, in this study, *L. lactis* showed positive effects on liver fibrosis in both the DDC diet and CCl_4 injection models. *L. lactis* showed a particularly prophylactic effect against liver fibrosis in both models.

Lactobacillus rhamnosus is considered a subspecies of *L. casei* but has now been identified as its own species (Huang et al., 2018). *L. rhamnosus* is a probiotic that has been extensively studied. In a recent study, *L. rhamnosus GG* was shown to prevent fibrosis in a liver cirrhosis model induced by bile duct ligation (Liu et al., 2020). *Lactobacillus rhamnosus GG* treatment significantly attenuated liver inflammation, damage, and fibrosis and led to a reduction in hepatic bile acids (BA) in BDL mice. Additionally, this treatment altered the gut microbiota, which was associated with increased BA deconjugation and increased fecal and urine BA excretion. Clinical studies have evaluated the safety and tolerability of *L. rhamnosus GG* in liver cirrhosis patients, have shown minimal hepatic encephalopathy and investigated the mechanism of gut microbial transformation (Bajaj et al., 2014). Endotoxin and TNF-α levels were reported to be reduced, and the gut microbiota was changed to reduced *Enterobacteriaceae* abundance and increased *Clostridiales Incertae Sedis XIV* and *Lachnospiraceae* relative abundance. Many studies have suggested that *L. rhamnosus GG* is effective in lowering AST and ALT levels in alcoholic liver disease models (Marotta et al., 2005; Segawa et al., 2008).

In animal models of liver fibrosis induced by DDC diet or CCl_4 injection, dysbiosis was confirmed, similar to the microbiome analysis in the human cirrhosis patient group. Although it was not significant, a decrease in bacterial diversity occurred, and a change in the composition of the taxa was observed. No dramatic change in abundance or composition occurred due to *Lactobacillus* administration, but as confirmed by beta diversity analysis, strain migration and compensation of some taxa induced by *Lactobacillus* were observed. Compensation of lactic acid bacteria taxa was confirmed, and among the lactic acid bacteria taxa, changes in *L. gasseri* (Carroll et al., 2007), *L. murinus* (Pan et al., 2018), and *L. reuteri* (Wang et al., 2020), which have been confirmed to have an anti-inflammatory effect, were prominent. Thus, *Lactobacillus* administration can be expected to relieve intestinal inflammation and inhibit harmful bacteria in the body in the presence of liver fibrosis, but further research is needed.

Probiotics have been verified in many studies to prevent various diseases. Although the underlying mechanism has not been well elucidated, there are certain probiotics that act specifically on specific diseases. In our study, liver fibrosis was induced via two methods, and in each model, *Lactobacillus* showed different protective effects against liver fibrosis. These results suggest that certain microbial species may act specifically to protect against liver fibrosis, suggesting their potential as customized treatments. The *Lactobacillus* strain we used showed a protective effect against liver fibrosis in an induced liver fibrosis model. Although more research is needed, we propose that the *Lactobacillus* strain might be a preventive and therapeutic agent against liver fibrosis.

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Data availability Data available within the article or its supplementary materials.

Declarations

Conflict of interest The authors declare that there is no conflict of interest, including relevant financial interests, activities, relationships, affiliations, and any other conflict of interest as explicitly and implicitly expressed in the Editorial Policies for Authors.

Ethical approval This study was controlled in accordance with ethical guidelines from the 1975 Helsinki Declaration as reflected by prior approval by the institutional review notice for human research in the hospital participating in the trial (2016-134). Basic informa-

tion has been registered on ClinicalTrials.gov for registration in the public trial registry (NCT04339725). Informed consent for enrollment was received from each participant. This experiment was designed by selecting a candidate for the treatment of liver disease among the probiotics that approved by Ministry of Food and Drug Safety, Korean. All the procedures were licensed by the Institutional Animal Care and Use Committee of the College of Medicine, Hallym University R1(2019-04).

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