

# Probiotic *Lactiplantibacillus Plantarum* JS5 Lowers Cholesterol by Modulating the Gut Microbiota in the High-cholesterol Diet-fed Mice

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Received May 19, 2023; Revised June 20, 2023; Accepted June 29, 2023

**Abstract** Hypercholesterolemia is the major cause of cardiovascular ailments worldwide. Jiang-shui contains plenty of lactic acid bacteria (LAB), although nothing is known about its *in vitro* probiotic characteristics and cholesterol-lowering effect. Thirty-eight strains were isolated, identified, and classified into *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, and *Lactobacillus delbrueckii subsp.* In this study, the top five strains with the highest cholesterol-lowering rates were selected, and strain *L. plantarum* JS5 demonstrated satisfactory *in vitro* probiotic characteristics. *L. plantarum* JS5 ingestion increased serum HDL-C levels and suppressed the increase in body weight and serum cholesterol induced by a high-cholesterol diet. After *L. plantarum* JS5 treatment, the relative abundance of the genera *Eubacterium coprostanoligenes group*, *Faecalibaculum*, and *Blautia* increased significantly, whereas the genus *Desulfovibrio* decreased significantly, improving the intestinal flora composition. In addition, functional analysis revealed that *L. plantarum* JS5 consumption influenced intestinal flora metabolism in high-cholesterol diet-fed mice in defense, signal transduction, and cellular activity. Hence, *L. plantarum* JS5 isolated from Jiang-shui might be ideal for preventing and treating hypercholesterolemia.

**Keywords:** *Lactiplantibacillus plantarum*, cholesterol-lowering, probiotics, intestinal flora, High-cholesterol Diet

**Cite This Article:** Yazhou Mao, Rong Ren, Shuanghong Song, Yuanyuan Hui, Linqiang Li, Shan Wu, Aiqing Zhao, and Bini Wang, "Probiotic *Lactiplantibacillus Plantarum* JS5 Lowers Cholesterol by Modulating the Gut Microbiota in the High-cholesterol Diet-fed Mice." *Journal of Food and Nutrition Research*, vol. 11, no. 6 (2023): 441-453. doi: 10.12691/jfmr-11-6-6.

## 1. Introduction

Based on the statistics shared by the World Health Organization (WHO), noncommunicable diseases (NCDs) account for approximately 68% of global mortality each year. The four top-ranked NCDs were cancer, cardiovascular diseases (CVDs), chronic respiratory diseases, and diabetes. Most premature deaths occur in countries with high incomes due to cancer. However, CVDs are responsible for a higher proportion of deaths in low- and middle-income countries [1]. It is well acknowledged that high serum cholesterol levels are a risk factor for hypercholesterolemia that sets the stage for CVDs [2]. Thus, decreasing cholesterol levels in the serum and diet is essential to prevent these diseases. Presently, clinical treatment of hypercholesterolemia relies mainly on diet management and pharmacological administration. Dietary management necessitates long-term rigorous restriction,

whereas pharmacological therapy has adverse side effects; for instance, regularly used statins can cause skeletal muscle toxicity or elevate serum hepatic transaminases levels [3]. As a result, a new, safe, and effective strategy for reducing serum cholesterol levels to prevent CVDs is required.

LAB are commonly obtained from fermented foods and recognized as safe and ideal non-pharmacologic candidates. Because of their potential health advantages, LAB, particularly in the genus *Lactobacillus*, have received increased attention as probiotics. Numerous studies have shown that *Lactobacillus* have immunomodulatory and health-promoting effects by modulating the components of the gut microbiota, attenuating inflammation, and improving the integrity of the intestinal barrier [4,5,6,7].

A meta-analysis also demonstrated that probiotics intake improves hypertension, hyperglycemia, and hypercholesterolemia in type 2 diabetes patients [8]. The investigations concerning the hypocholesterolemia effect of probiotics found that *Lactobacillus* exhibited

cholesterol-lowering efficacy *in vitro* and via altering lipid metabolism in rats with hyperlipidemia [9,10]. *Lactobacillus* supplementation dramatically lowered total triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and total cholesterol (TC) levels while increasing high-density lipoprotein cholesterol (HDL-C) levels in hypercholesterolemic rats [11].

Jiang-shui is a traditional fermented vegetable cuisine that has been popular in Northwest China for thousands of years. It has also been identified as a rich source of LAB strains, whose main genus is *Lactobacillus* [12]. However, the microbial community in fermented vegetable food diversified due to the different vegetable materials, production processes, and natural environments [13,14]. For example, *L. fermentum*, *L. amylolyticus*, and *L. pontis* were the major *Lactobacillus* in Jiang-shui samples from Shaanxi and Gansu Provinces in Northwest China, while *L. plantarum* and *L. brevis* dominated in samples from Shaanxi Province. Although the probiotic characteristics of *Lactobacillus* have been reported in several recent studies [15], there are still inadequate investigations for their beneficial potential especially in cholesterol-lowering function *in vivo*.

As a result, this study aims to discover and select potential *Lactobacillus* strains with high-cholesterol-lowering capacity from Jiang-shui samples and then investigate their probiotic characteristics and cholesterol-lowering function using a high-cholesterol diet-fed mouse model. The findings related to the cholesterol-lowering role of the *Lactobacillus* isolates will enhance our understanding of the health benefits of *Lactobacillus* strains from Jiang-shui and used to develop probiotics-based functional foods.

## 2. Materials and Methods

### 2.1. Jiang-shui Samples

Samples of Jiang-shui were collected in local households in Shaanxi Province, China, and kept at 4°C.

### 2.2. Isolation of LAB Strains

Jiang-shui soup (1 mL) was added to Man Rogosa Sharpe (MRS) agar with incubation for 48 h at 37°C. MRS medium was added with 5% CaCO<sub>3</sub> (w/v) and adjusted pH to 6.2. Acid-producing bacteria strains were isolated from these plates by selecting colonies with soluble calcium zones. These isolates were further purified by repeated plate streaking and tested by catalase test and gram staining. Further studies were limited to catalase-negative and gram-positive bacteria and they were incubated with 20% (v/v) glycerol at -40°C.

### 2.3. Genomic DNA Extraction, 16 S rDNA Sequencing and PCR Amplification

Bacteria DNA was extracted by using the DNA Extraction Kit (Personalbio Technology Co. Ltd., Shanghai, China). Then NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to examine the purity

and concentration of DNA and its quality was assessed by gel electrophoresis with 0.8% agarose at 100 V for 40 min.

The 16S rRNA genes (about 1500 bp) were amplified by PCR using the universal primer 27F and 1492R. In Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, Massachusetts, USA), template DNA (10 ng) was mixed with primers (0.2 µL) for each PCR reaction. PCR thermal cycling was done with an initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 90 s, final extension at 72°C for 7 min, and ending at 4°C. PCR amplification products were detected, purified by QIAGEN Gel Extraction Kit (QIAGEN, Hilden, Germany), and then entrusted to a commercial biotech company (Personalbio Technology Co. Ltd., Shanghai, China) for sequencing. The sequences were compared to those reported in the Gene Bank using the BLAST algorithm to study these isolates by similarity to standard strains in the gene bank.

### 2.4. The Cholesterol-lowering Capacity of Isolates

All identified isolates were activated and their cell concentration was adjusted to 10<sup>8</sup> CFU/mL with the McFarland turbidimetry method. The freshly prepared bacterial suspension was cultured in the MRS-CHOL broth supplemented with water-soluble cholesterol (0.1 g/L) and 0.3% ox bile (Sigma) at 37°C for 24 h and centrifuged at 3500 rpm for 10 min. Using a colorimetric method to determine residual cholesterol in the supernatant [16] and the uninoculated medium as a control. The cholesterol-lowering capacity of each isolate was calculated as the cholesterol removal rate after 24 h. The top five strains were restored for subsequent assays.

### 2.5. The Probiotic Characteristics of Isolates

Tolerance of the isolates to high acidity and bile was assayed as per method reported by Andriantsoanirina, Allano [17]. Incubate the adjusted bacterial suspension of 1 mL in the modified MRS broth at 10 mL for 4 h at 37°C and adjust pH to 3.0 using 0.1 M HCL. For bile tolerance assay, add 0.3% (w/v) bile salt to the broth. The viable counts in the treated and untreated bacterial suspensions were measured, and the survival was analyzed for evaluation of acid and bile tolerance. The strain with the highest cholesterol-lowering ability and probiotic characteristics was used in the following animal experiments.

Hydrophobicity was based on the method reported by Ji, Jang [18] with some modifications. The bacterial suspension (5 mL) was mixed with xylene (1 mL), then left to stand for 1 hour to wait for stratification. Then lower aqueous phase was pipetted and measured absorbance at 600 nm. The hydrophobicity percentage was measured using equation 1:  $H\% = (A_0 - A) / A_0 \times 100$ . Where, A is the absorbance after extraction with xylene, and A<sub>0</sub> is the absorbance of bacterial suspension.

Auto-aggregation was a slight modification of the reported method [19]. The bacterial suspensions were cultured and the supernatant was carefully removed to

measure its absorbance at 600 nm. The auto-aggregation percentage was calculated using equation 2:  $Auto - A\% = (A_0 - A_t) / A_0 \times 100$ . Where  $A_0$  is time 0 h absorbance and  $A_t$  is time 4, 8, 20, and 24 h absorbance.

Co-aggregation was determined as reported by Collado, Meriluoto [20]. Briefly, equal volumes of bacterial suspension of the isolates and *E. coli* were mixed and vortexed thoroughly for 20 s. The mixture was then incubated for 5 h at 37°C under resting conditions. The supernatant was then pipetted out and determined its absorption at 600 nm. Calculate the percentage of co-aggregation with equation 3:  $Co - A\% = [(A_0 + A_{E.c} - 2A_m) / (A_0 + A_{E.c})] \times 100$ . Where,  $A_0$  is the absorbance of bacterial suspension at time 0 h,  $A_{E.c}$  is the absorbance of *E. coli* suspension, and  $A_m$  is the absorbance of mixture at time 5 h.

## 2.6. Animal Experiments

Thirty male C57BL/6 mice (6-week-old, 20-22 g, specific pathogen-free animal grade) were purchased from Chengdu Dossy Experimental Animals Co. Ltd (Chengdu, China). Feed was purchased from Research Diets Inc. agent (Biopike Biotech Co., Ltd., Chengdu, China). All mice were kept in non-toxic plastic mouse box under standard environment: humidity 50%-60%, temperature 21-25°C, and 12-h light-dark cycle. After a week of acclimatization, 30 mice were assigned into three groups of 10 mice each, with 5 per cage: contrast diet (D12102C) (CC), high-cholesterol diet (D12109C) (CM), and high-cholesterol diet plus selected strains *L. plantarum* JS5 (CI). CI group mice were intragastrically administered with 1 mL/100 g bodyweight ( $10^9$  CFU /mL) *L. plantarum* JS5 suspension each day for 8 weeks. The CC and CM groups received the same volume of the medium. In addition, the weekly weight measurements were performed at 09:00 AM. On the last weekday, the feces samples were collected using sterilized tweezers to avoid microbial contamination and stored at -80°C for analysis of the fecal microflora. After 12 h of fasting, all mice were anesthetized with 5% isoflurane and euthanized. The whole blood samples of the orbital venous plexus were collected and immediately separated by centrifugation (3500rpm, 15 min). The obtained serum was cryopreserved at -80°C until used. The kidney, liver, spleen, and epididymis fat of mice were removed, rinsed in icy PBS, dried, and then weighed. This study was authorized by the Ethics Committee of Shaanxi Normal University and all procedures were carried out in accordance with the Guiding Principles in the Care and Use of Animals approved by the American Physiological Society.

## 2.7. Analysis of the Serum Biochemical Indexes

All serum samples were analyzed by using an automatic biochemical analyzer (Beckman, AU-480, USA). Serum levels of LDL-C, HDL-C, TC, TG, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured using a commercial kit (Nanjing Jiancheng, Nanjing, China) following the instructions of manufacturer.

## 2.8. Pathological Analysis of the Liver Tissues

The liver tissue from each mouse was separated, and the residual blood in the liver aorta was rinsed with PBS. The same portions of the liver large lobe were cut off and soaked in 4% (v/v) paraformaldehyde to fix it. After successful fixation, the liver tissue was sequentially dehydrated by alcohol, cleared with xylene, embedded in paraffin, and then cut into thin slices. The sections were deparaffinized and then stained with hematoxylin and eosin (H&E). The stained sections were dripped with rhamsan gum to be sealed, observed, and photographed under an inverted fluorescence microscope (Leica, DMi8-M, Germany).

## 2.9. Bioinformatics Analysis of the Intestinal Flora

A dash of the fecal sample (200 mg) was dissolved in 0.01M sterile PBS (2 mL), and the mixture was centrifuged (4°C, 12000 rpm, 5 min). Then, the supernatant was filtered twice through a 0.45-μm filter membrane before extracting the microbial genomic DNA according to the instructions specified by the FastDNA™ Spin Kit for feces (MP Biomedicals Co., Ltd., California, USA). The PCR procedure was performed as described above, in which the primers were changed to 338F and 806R. Genomic DNA library construction and the sequencing of samples were entrusted to a commercial biotech company (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China).

Purified amplicons were sequenced using the Illumina MiSeq platform (Illumina, San Diego, USA). The raw reads were demultiplexed, quality filtered with Trimmomatic, and merged with FLASH. Operational Taxonomic Units (OTUs) were clustered at 97% similarity level using UPARSE (version 7.1, drive5.com/uparse/) and chimeric sequences were identified and eliminated. The taxonomic assignment of each 16S rRNA gene sequence was performed by the RDP Classifier (rdp.cme.msu.edu/) against the 16S rRNA database using a 0.7 confidence threshold [21]. The relative abundance of each OTU was examined at various taxonomic levels. Chao 1 and Simpson indices were calculated by QIIME to measure microbial community richness. Principal coordinate analysis (PCA) was used to investigate beta diversity in QIIME. DAVID (<https://david.ncicrf.gov/home.jsp>) was used for Gene Ontology (GO) analysis.

## 2.10. Statistical Analysis

Results were expressed as means and standard error. One-way ANOVA was employed to calculate statistical differences in data between groups using GraphPad Prism 7.0 and mean values were considered significantly different at  $p < 0.05$ .

## 3. Results

### 3.1. LAB Isolated from Jiang-shui Possesses a Potent Cholesterol-lowering Ability

A total of 38 strains with large and obvious calcium soluble rings were selected from Jiang-shui samples.



According to the findings of the gram staining and catalase tests, they were all gram-positive and catalase-negative bacteria. All isolates demonstrated typical LAB colony morphology, i.e., milky white colonies formed on MRS solid medium with a round, convex, smooth, moist surface, and neat edges (Figure 1). The isolates were taxonomically identified at the species level using 16S rDNA gene sequencing. A total of 38 strains were eventually classified as *L. delbrueckii* sp. (6 strains), *L. fermentum* (15 strains), and *L. plantarum* (17 strains), all of which belonged to the genera *Lactobacillus*. Thus, the dominating bacterial species in Jiang-shui was *L. plantarum*, and all identification results are listed in Table 1.

All 38 LAB isolates were evaluated for their *in vitro* cholesterol-lowering activity. As shown in Table 2, there is a significant variation in cholesterol elimination present in the medium after 24 h between all the strains, ranging from 4.51% (*L. fermentum* JS38) to 40.98% (*L. plantarum* JS6). However, only eight strains with over a 30% cholesterol-lowering potential were found. Among the eight identified species, *L. plantarum* accounted for the largest proportion of the species (75.0%), and *L. plantarum* JS6 strain had the strongest cholesterol

lowering potential (40.98%). Thus, the top five strains with the highest lowering rates were subsequently evaluated for probiotic characteristics to identify potential probiotic isolates.

### 3.2. *L. plantarum* JS5 Represents Good Probiotic Characteristics

Probiotics should be able to resist stressful conditions in the host gastrointestinal tract to colonize the gastrointestinal tract more effectively. Therefore, to determine their viability in an artificial gastrointestinal tract, the five strains isolated from Jiang-shui were evaluated for acid and bile tolerance (Figure 2A). The results of acid tolerance *in vitro* digestion showed that all strains screened in this study could grow in a pH 3.0 environment, with *L. plantarum* JS5 showing high tolerance to acid stress with a survival rate of 74.58%, while others had only 40%–60%. All five strains demonstrated strong tolerance in the bile salt concentration of a 3g/L culture environment, with survival rates greater than 80%. In general, *L. plantarum* JS5 exhibited the highest viability in the artificial gastrointestinal tract.

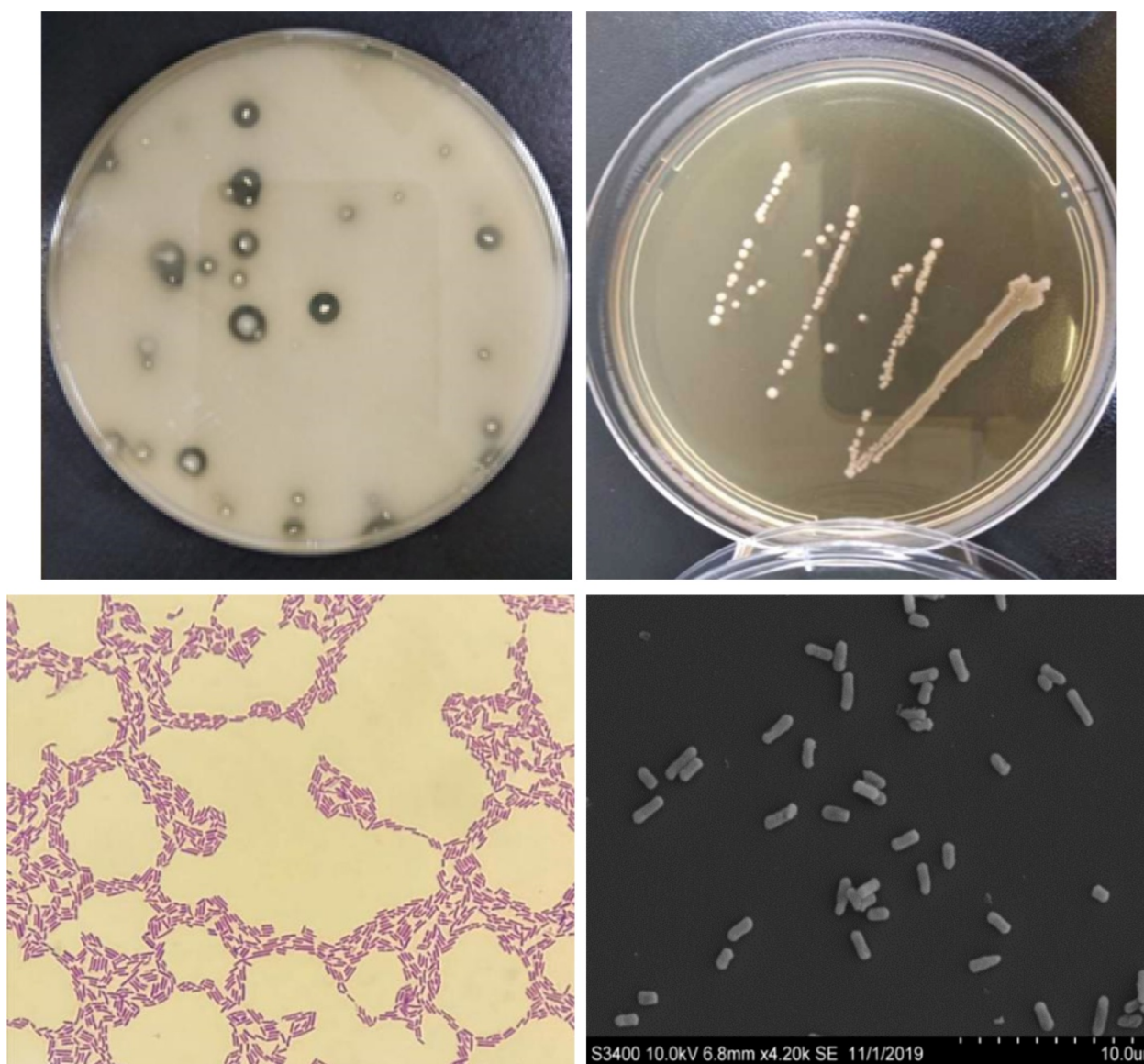


Figure 1. Morphological photos of isolated colonies

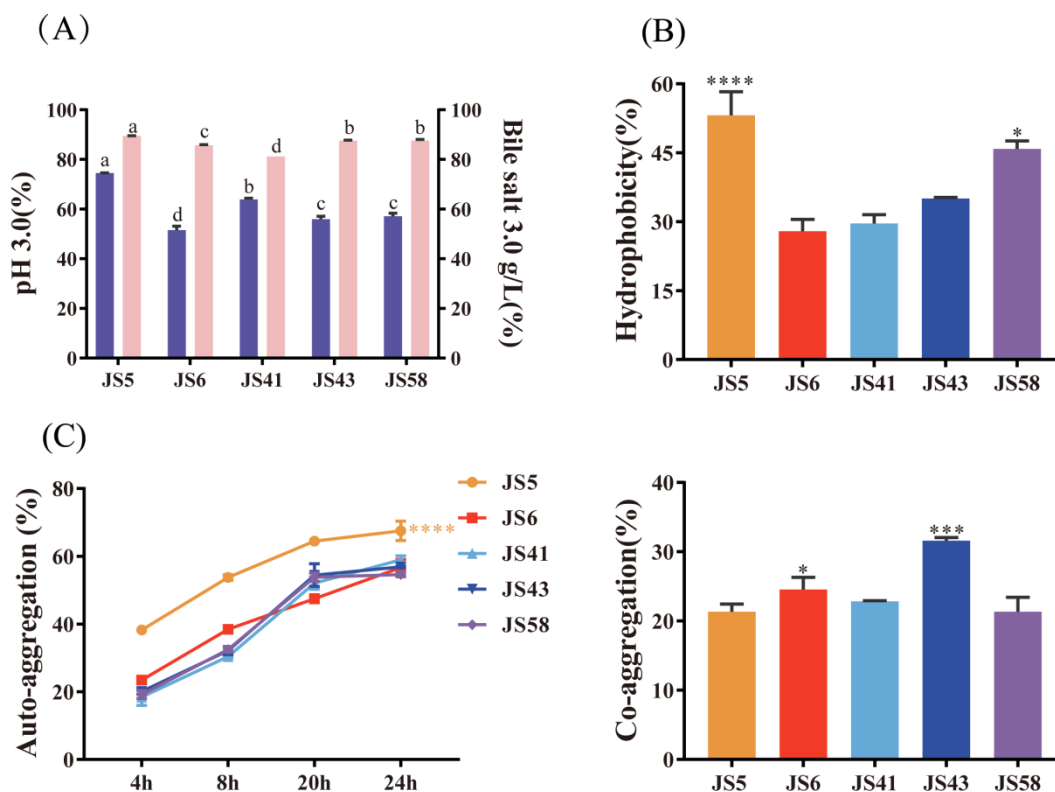
**Table 1. Isolated strains identification results**

| strain | NCBI accession No. | Closest relative                       |
|--------|--------------------|----------------------------------------|
| JS3    | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS5    | KJ779096.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS6    | AB830324.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS9    | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS10   | MT641196.1         | <i>Limosilactobacillus fermentum</i>   |
| JS14   | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS16   | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS19   | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS20   | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS21   | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS24   | MT538474.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS26   | MT641196.1         | <i>Limosilactobacillus fermentum</i>   |
| JS28   | MT641196.1         | <i>Limosilactobacillus fermentum</i>   |
| JS29   | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS34   | MT613525.1         | <i>Limosilactobacillus fermentum</i>   |
| JS35   | MT613525.1         | <i>Limosilactobacillus fermentum</i>   |
| JS36   | MT613617.1         | <i>Limosilactobacillus fermentum</i>   |
| JS38   | MT613617.1         | <i>Limosilactobacillus fermentum</i>   |
| JS39   | MG551083.1         | <i>Lactobacillus delbrueckii subsp</i> |
| JS40   | MT538464.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS41   | MT538458.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS42   | MT613617.1         | <i>Limosilactobacillus fermentum</i>   |
| JS43   | MG551080.1         | <i>Lactobacillus delbrueckii subsp</i> |
| JS44   | MG551080.1         | <i>Lactobacillus delbrueckii subsp</i> |
| JS45   | MT538464.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS46   | MF357331.1         | <i>Lactobacillus delbrueckii subsp</i> |
| JS47   | MG551080.1         | <i>Lactobacillus delbrueckii subsp</i> |
| JS48   | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS49   | MG551083.1         | <i>Lactobacillus delbrueckii subsp</i> |
| JS50   | MT641196.1         | <i>Limosilactobacillus fermentum</i>   |
| JS51   | MT641196.1         | <i>Limosilactobacillus fermentum</i>   |
| JS52   | MT641196.1         | <i>Limosilactobacillus fermentum</i>   |
| JS53   | MT641196.1         | <i>Limosilactobacillus fermentum</i>   |
| JS54   | MT641196.1         | <i>Limosilactobacillus fermentum</i>   |
| JS55   | MT613525.1         | <i>Limosilactobacillus fermentum</i>   |
| JS56   | MT613617.1         | <i>Limosilactobacillus fermentum</i>   |
| JS58   | MT645503.1         | <i>Lactiplantibacillus plantarum</i>   |
| JS59   | MG646766.1         | <i>Lactiplantibacillus plantarum</i>   |

**Table 2. Cholesterol lowering ability of 38 isolates (%)**

| Strains | Removal                      | Strains | Removal                      |
|---------|------------------------------|---------|------------------------------|
| JS3     | 24.97±1.72 <sup>ghijk</sup>  | JS40    | 18.57±2.35 <sup>mno</sup>    |
| JS5     | 31.19±0.49 <sup>bcd</sup>    | JS41    | 31.97±0.38 <sup>bc</sup>     |
| JS6     | 40.98±0.27 <sup>a</sup>      | JS42    | 16.99±2.02 <sup>op</sup>     |
| JS9     | 26.98±2.36 <sup>efghi</sup>  | JS43    | 31.12±1.11 <sup>bcd</sup>    |
| JS10    | 26.00±1.26 <sup>ghij</sup>   | JS44    | 28.03±0.25 <sup>cdefgh</sup> |
| JS14    | 25.52±0.35 <sup>ghij</sup>   | JS45    | 18.74±4.21 <sup>mno</sup>    |
| JS16    | 26.40±1.78 <sup>efghi</sup>  | JS46    | 26.73±0.87 <sup>efghi</sup>  |
| JS19    | 27.61±0.36 <sup>defghi</sup> | JS47    | 23.54±0.46 <sup>ijkl</sup>   |
| JS20    | 28.11±0.81 <sup>cdefg</sup>  | JS48    | 30.19±0.47 <sup>bcdef</sup>  |
| JS21    | 23.89±0.87 <sup>hijkl</sup>  | JS49    | 22.46±4.81 <sup>ijklm</sup>  |
| JS24    | 28.43±1.81 <sup>cdefg</sup>  | JS50    | 26.02±2.04 <sup>ghij</sup>   |
| JS26    | 21.03±2.02 <sup>klmn</sup>   | JS51    | 23.94±1.01 <sup>hijkl</sup>  |
| JS28    | 13.82±1.38 <sup>p</sup>      | JS52    | 23.59±0.33 <sup>ijkl</sup>   |
| JS29    | 25.82±3.33 <sup>ghij</sup>   | JS53    | 13.77±1.31 <sup>p</sup>      |
| JS34    | 16.99±0.20 <sup>op</sup>     | JS54    | 20.90±1.97 <sup>lmno</sup>   |
| JS35    | 18.77±1.09 <sup>mno</sup>    | JS55    | 24.97±2.31 <sup>ghijk</sup>  |
| JS36    | 18.29±1.62 <sup>no</sup>     | JS56    | 30.39±0.96 <sup>bcdef</sup>  |
| JS38    | 4.51±1.05 <sup>q</sup>       | JS58    | 32.63±0.99 <sup>b</sup>      |
| JS39    | 20.17±1.48 <sup>lmno</sup>   | JS59    | 30.82±0.33 <sup>bcde</sup>   |

All data were expressed as mean ± standard deviation (n=3), and different superscript letters indicated significant differences in the row (P < 0.05).



**Figure 2.** Probiotic characteristics of 5 selected strains (A: acid and bile tolerance; B: hydrophobicity; C: auto-aggregation and co-aggregation). (All data were expressed as mean values and SD (n=3). Different superscript letters on the same color column or \* in one subgraph indicated significant differences (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.0001$ ; \*\*\*\*,  $P < 0.00001$ )

The adhesion ability of isolated strains was further investigated and determined in terms of both cell surface hydrophobicity (Figure 2B) and aggregation properties (Figure 2C). The hydrophobicity of LAB strains varied. The highest hydrophobicity was observed in *L. plantarum* JS5 (53.15%), followed by *L. plantarum* JS58 (45.85%) among these isolates. In terms of auto-aggregation, the percentages of all strains steadily rose with culture time. *L. plantarum* JS5 had the highest proportion throughout the culture time, reaching 67% after 24 h. As compared to co-aggregation, *L. delbrueckii* subsp. JS43 had the highest co-aggregation value of 31.61%, while the rest were only 20%. Taken together, *L. plantarum* JS5 could be the potential probiotic strain with both high-cholesterol-lowering capabilities and satisfactory probiotic characteristics.

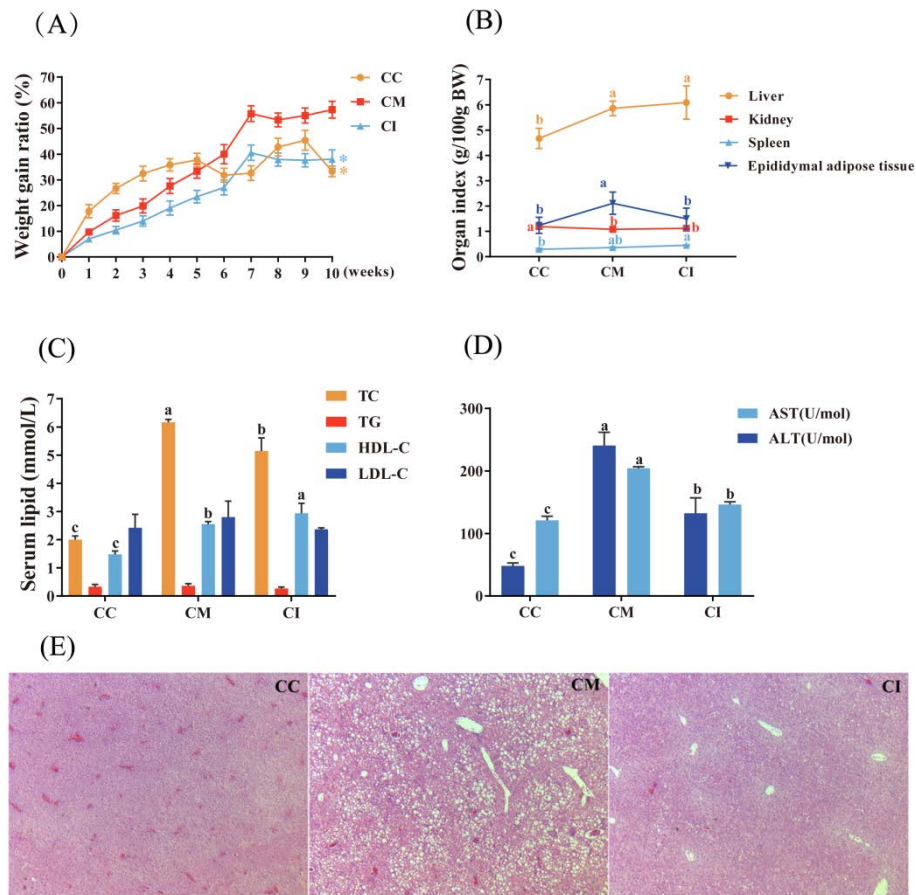
### 3.3. *L. plantarum* JS5 Ameliorates High-cholesterol-induced Weight Gain

Figure 3A depicts the rates of body weight growth in mice over ten weeks. After six weeks of feeding, CM group mice showed the highest rate ( $P < 0.05$ ), while *L. plantarum* JS5 administration remarkably reduced the values induced by high-cholesterol feeding ( $P < 0.05$ ). Figure 3B shows a significant increase in liver and spleen weights in mice of CM and CI groups ( $P < 0.05$ ), but the kidney weights were the opposite ( $P < 0.05$ ). It was also shown that high-cholesterol feeding led to a significant rise in epididymal fat weight ( $P < 0.05$ ). As predicted, the epididymal fat weight of mice in CI group was lower than CM group ( $P < 0.05$ ).

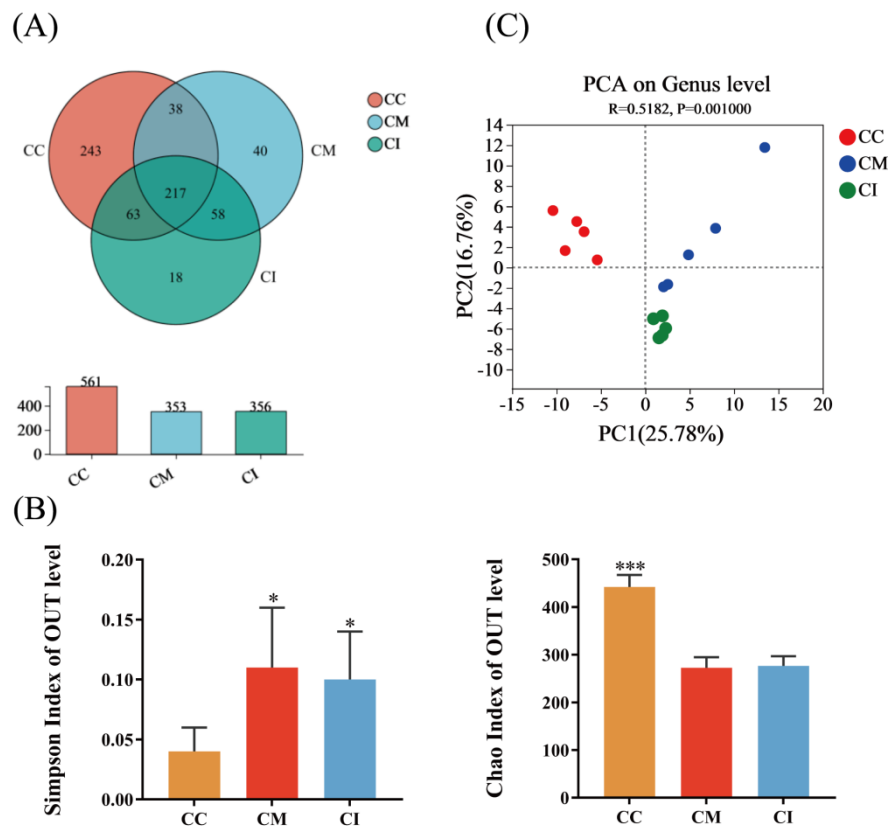
### 3.4. *L. plantarum* JS5 Alleviates High-cholesterol-induced Dyslipidemia and Hepatic Damage

As shown in Figure 3C, HDL-C and TC levels were markedly increased in CM group mice compared with CC group, indicating that high-cholesterol feeding may cause lipid disorders in mice ( $P < 0.05$ ). In comparison with CM group mice, CI group mice had significantly lower TC and higher HDL-C levels ( $P < 0.05$ ). However, the mice showed no significant differences in TG and LDL-C levels. Thus, *L. plantarum* JS5 intervention might influence the serum lipid profile by significantly lowering TC and increasing HDL-C, thereby lowering CVDs morbidity.

In clinical studies, the extent of liver damage can be determined by ALT and AST levels [22]. As depicted in Figure 3D, serum ALT and AST levels in CM group mice were remarkably higher, demonstrating that elevated cholesterol caused hepatic damage ( $P < 0.05$ ). According to our expectation, *L. plantarum* JS5 treatment decreased serum ALT and AST levels in high-cholesterol patients ( $P < 0.05$ ). A liver histology examination with H&E staining was undertaken, as shown in Figure 3E. H&E staining photomicrographs revealed that the mice liver tissues in CC group were nearly arranged with obvious nuclei, while the liver mice tissues in CM group displayed obvious lipid droplets with the nucleus pushed to the side compared to CC group. As hypothesized, *L. plantarum* JS5 intervention might improve hepatohistological alterations by decreasing lipid droplet vacuoles. This analysis suggested that *L. plantarum* JS5 might prevent high-cholesterol-induced liver injury.

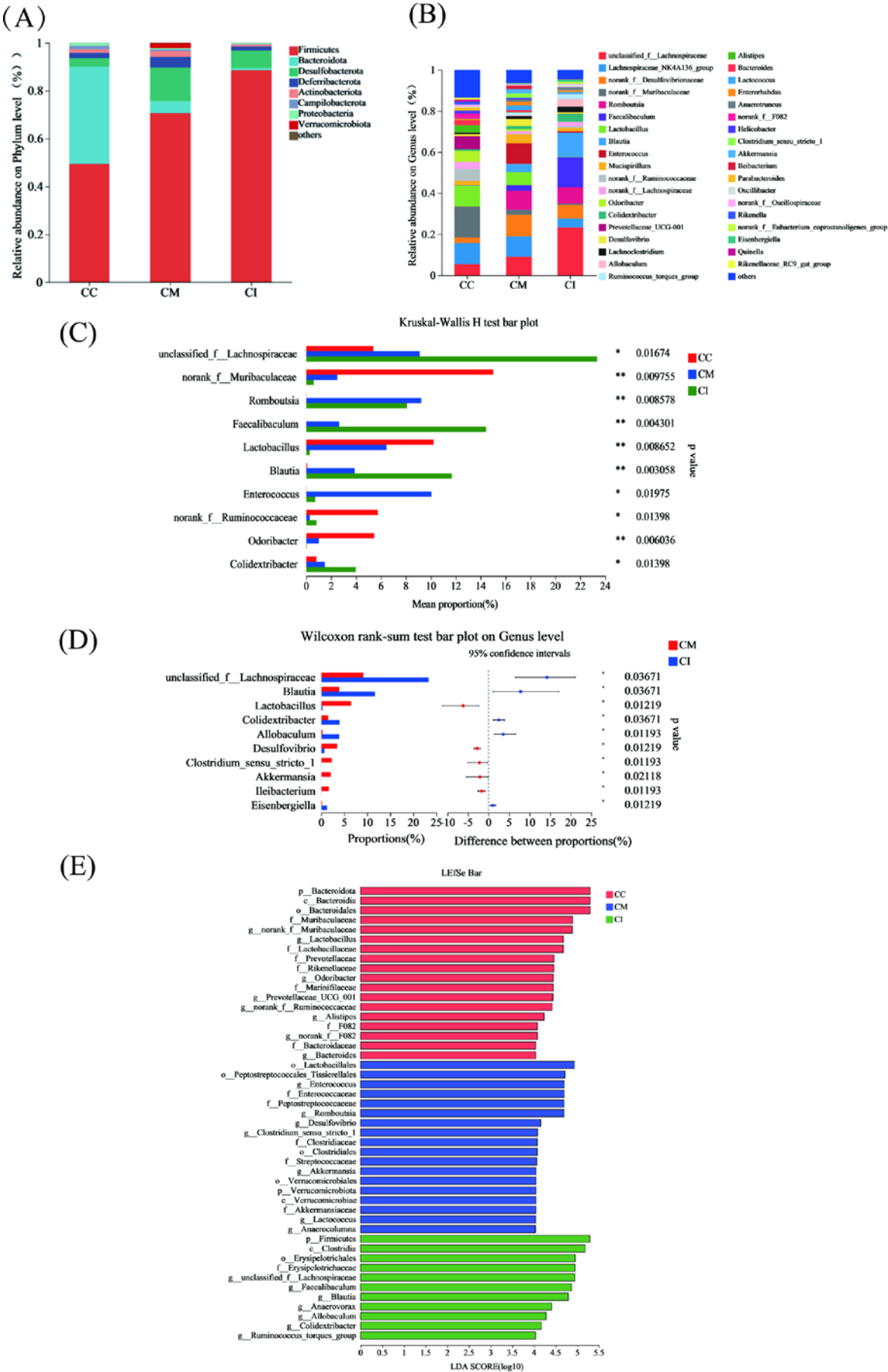


**Figure 3.** Changes in physiological properties of mice (A: weight gain ratio; B: various organs weight; C: serum lipid indices; D: levels of serum ALT and AST; E: liver H&E staining). (BW=body weight. All data were expressed as mean values and error bars (n=5). Different superscript letters on the same color column or \* in one subgraph indicated significant differences ( $P < 0.05$ ). The magnification of the staining photo was 40 $\times$ )



**Figure 4.** A: Venn diagram; Alpha diversity (B: Simpson Index and Chao Index) and Beta diversity (C: PCA graph) of intestinal flora at Genus level. (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ )





**Figure 5.** Community barplot analysis of intestinal flora at Phylum (A), Genus, (B) level; C: Kruskal-Wallis H test on the genus level; D: Wilcoxon rank-sum test bar plot at genus level; E: Biomarker taxon generated from LEfSe analysis, LDA > 4.0 (Representative bacteria in all groups at different taxonomic levels were presented (p: phylum, c: class, o: order, f: family, g: genus, and s: species))



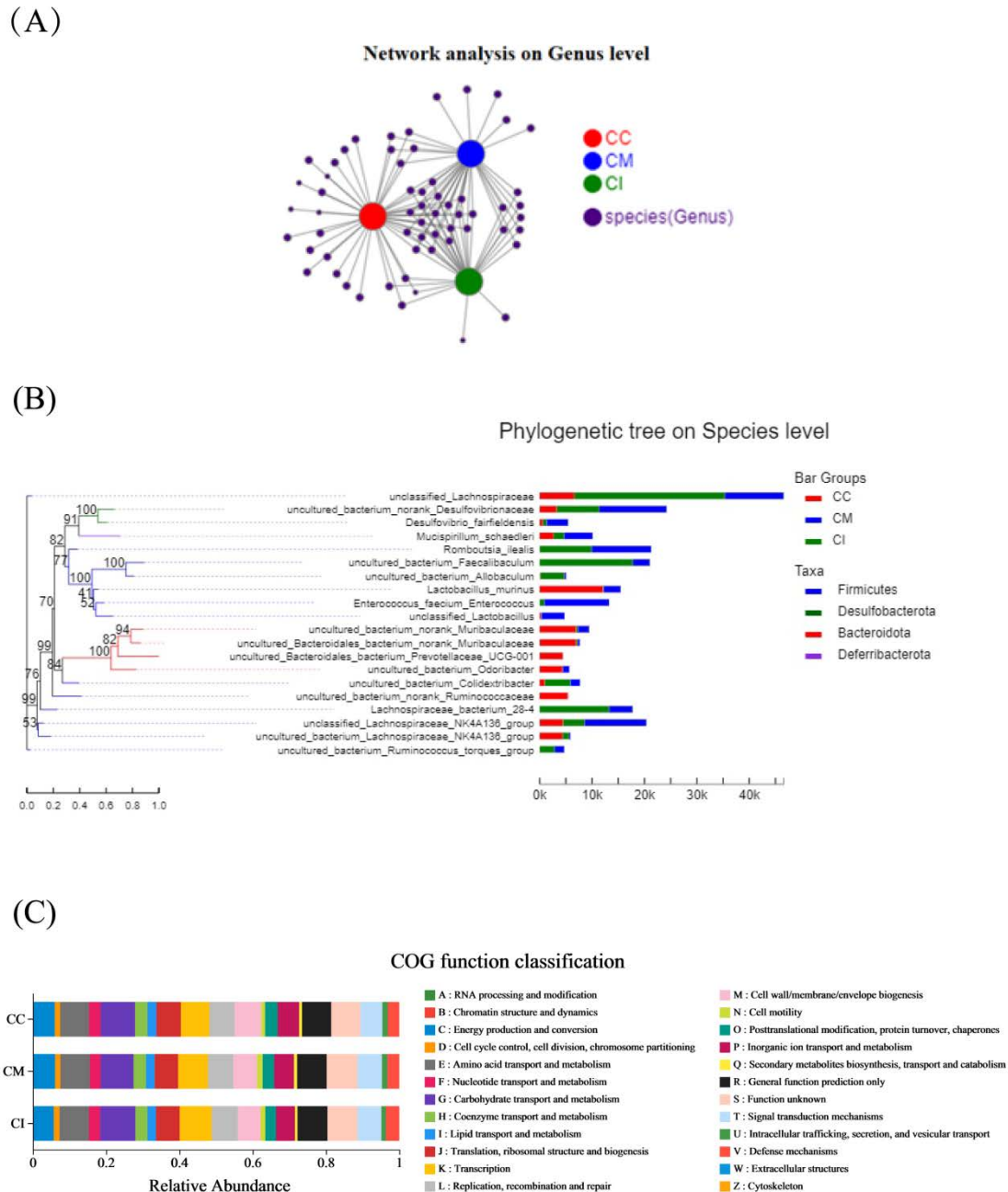


Figure 6. A: Network analysis on genus level; B: Phylogenetic tree on species level; C: COG functional classification

### 3.5. *L. plantarum* JS5 Improves Intestinal Microbiota Diversity in Hypercholesterolemia Mice

As shown in Figure 4A, an OTU level-based Venn diagrams reveals the total number of species and differences between them for each group. A total of 561, 353, and 356 OTUs were identified in the CC, CM, and CI groups, respectively. There were 217 mutual OTUs, indicating that these bacteria were frequent inhabitants in mice intestines. The Simpson and Chao indices were used for analyzing the diversity and richness of the microbiota (Figure 4B). The results show that a high-cholesterol diet significantly increased the Simpson index while decreasing the Chao index, indicating that the diversity and richness of microbial were markedly reduced in CM group mice ( $P < 0.05$ ). However, *L. plantarum* JS5 intervention

was unable to effectively reverse this reduction. Beta diversity was assessed to make a comparison of the microbial community composition in all samples. Principal component analysis (PCA) (Figure 4C) suggested that the microbiota cluster in CM group was distinct from those in CC and CI groups, indicating that a high-cholesterol diet altered gut microbial community structure.

### 3.6. *L. plantarum* JS5 Improves Intestinal Microbiota Composition in Hypercholesterolemia Mice

We analyzed the intestinal flora composition of the CC, CM, and CI groups to determine which bacteria were ameliorated by *L. plantarum* JS5 intervention in the progression of hypercholesterolemia. Bacteroidetes, Firmicutes, Desulfobacterota, and Deferribacterota were found to be

the four dominant phyla in all samples (Figure 5A). When compared to CC group mice, a high-cholesterol diet led to an increase in Desulfobacterota, Deferribacterota, and Firmicutes relative abundance and a decrease of Bacteroidetes ( $P < 0.05$ ). The relative abundance of *norank\_f\_Ruminococcaceae*, *norank\_f\_Lachnospiraceae*, and *Quinella* were decreased in the CM group than CC and CI groups at the genus level, whereas intestinal normal inhibitory bacteria such as *Enterococcus* and *Mucispirillum* were highly expressed (Figure 5B). When compared to CC and CM groups, the relative abundance of *norank\_f\_Lachnospiraceae*, *Faecalibaculum* and *Blautia* rose significantly in CI group, whereas that of *Lachnospiraceae\_NK4A136\_group* and *norank\_f\_Ruminococcaceae* decreased. These results showed an alteration in the intestinal flora composition in high-cholesterol-fed mice due to the intake of *L. plantarum* JS5.

### 3.7. Compositional Comparison among Three Groups of Intestinal Flora

To further find the impact of *L. plantarum* JS5 on specific intestinal flora microbiota in high-cholesterol-fed mice, taxonomic differences in the intestinal microbiota of CC, CM, and CI groups were examined. Kruskal-Wallis H test (Figure 5C) displayed that *L. plantarum* JS5 intervention reduced the relative abundance of *Lactobacillus*, *norank\_f\_Muribaculaceae*, *Enterococcus*, and *Odoribacter* in high-cholesterol-fed mice, while significantly increasing the relative abundance of *Faecalibaculum*, *Blautia*, *Colidextribacter*, and *norank\_f\_Lachnospiraceae* by more than 53%. In addition, the Wilcoxon rank-sum test bar plot (Figure 5D) also demonstrated that the relative abundance of these genera in CI group was decreased than in CM group, including *Ileibacterium*, *Akkermansia*, *Clostridium\_sensu\_stricto\_1*, *Desulfovibrio*, and *Lactobacillus*, while *Eisenbergiella*, *Allobaculum*, *Colidextribacter*, *Blautia*, and *norank\_f\_Lachnospiraceae* were significantly increased. Furthermore, the linear discriminant analysis effect size (LEfSe) (Figure 5E) displayed that the genus *Enterococcus* markedly bloomed by high-cholesterol treatment, whereas *Faecalibaculum* and *Blautia* were enriched in the *L. plantarum* JS5 intervention group. The findings indicated that *L. plantarum* JS5 promotes the growth of beneficial intestinal flora in high-cholesterol-fed mice.

### 3.8. Network, Evolution Analysis, and Functional Prediction

The co-occurrence network analysis (Figure 6A) showed the top five genera in terms of weighted degree were *Eggerthella*, *Gordonibacter*, *norank\_f\_Bacteroidales\_BS11\_gut\_group*, *Bacillus*, and *Lachnospiraceae\_UCG-010*. Evolutionary analysis of the phylogenetic tree (Figure 6B) demonstrated that the top five species with the closest affinities were *Desulfocobrio\_fairfieldensis*, *Romboutsia\_ilealis*, *uncultured\_bacterium\_Allobaculum*, *uncultured\_bacterium\_Colidextribacter*, and *Lachnospiraceae\_bacterium\_28-4*. Furthermore, the functional prediction analysis based on Cluster of Orthologous Groups of proteins (COG) data (Figure 6C)

revealed that the main cellular functions were amino acid transport and metabolism, ribosomal structure and biogenesis, translation, carbohydrate transport and metabolism, and transcription. From these results, *L. plantarum* JS5 might enhance cellular functioning in high-cholesterol-fed mice.

## 4. Discussion

Hypercholesterolaemia is a leading risk factor for the progression of CVDs. Growing evidence indicated that probiotics or LAB have some preventive effects on hypercholesterolemia due to their better cholesterol removal ability [23,24,25]. Traditional fermented vegetable food, such as Jiang-shui are reported to be beneficial for human health due to their rich probiotic LAB [26]. However, different LAB isolates exhibited different biological activities. *Limosilactobacillus fermentum* JL-3 alleviates hyperuricemia via degradation of uric acid [27], while we recently discovered that *L. plantarum* JS19 prevents acute and chronic ulcerative colitis induced by DSS. [28]. However, the effect of Jiang-shui LAB on hypercholesterolemia has seldom been reported. In this work, 38 LAB strains were successfully isolated and compared their cholesterol-lowering capacity *in vitro*. Only eight strains exhibited a cholesterol-lowering potential of more than 30%, which was in line with the cholesterol-lowering capacity of *L. plantarum* KR105940 (45.8%) [29] and *L. plantarum* AR511 (37.14%) [30]. The results indicate that cholesterol-lowering capacity might be species- and strain-specific. The differences in bacterial growth circumstances may be ascribed to differences in ox bile concentration, pH, and growth status. However, the mechanisms for lowering cholesterol require additional investigation.

Probiotics must endure not just a strongly acidic environment but also bile salts from the upper intestine to reach and colonize the small intestine of the host. The top five strains with high cholesterol-lowering effects that we identified all had high acid and bile salt tolerance, especially *L. plantarum* JS5, with over 70% acid tolerance and over 80% bile salt tolerance. These data were remarkably higher than the previously reported acid tolerance of 15%–34% and bile salt tolerance of 60%–80% [31]. The above results revealed that *L. plantarum* JS5 has strong gastrointestinal viability. To further exert probiotic effects, probiotics must have good adherence to the gastrointestinal tract, reducing pathogenic bacteria in the gut and maintaining intestinal homeostasis [32,33]. Our results in terms of cell surface hydrophobicity and aggregation properties demonstrated that *L. plantarum* JS5 exhibits similar values to those previously reported [34,35]. Regardless of aggregation properties, the present results were all higher than the values reported by Topcu, Kaya [36]. This indicated that *L. plantarum* JS5 with good probiotic characteristics might prevent pathogenic bacteria colonization and maintain host intestinal homeostasis by remaining high viability and adhesion to the gastrointestinal tract. The specific proteins on the strain's surface must yet be studied further. *L. plantarum* JS5 was selected for additional investigations to evaluate its cholesterol-lowering function in high-cholesterol diet-fed mice due to its

substantial cholesterol-lowering ability and probiotic features.

Previous research has shown that *Lactobacillus* can help high-fat diet-fed mice lose body weight and alleviate the accumulation of tissue fat [37]. As expected, CM group mice gained body weight at a faster pace, whereas mice in the CI group treated with *L. plantarum* JS5 gained weight slowly. The high-cholesterol diet significantly increased the weight of mice's liver, spleen, and epididymal fat. However, *L. plantarum* JS5 mitigated the increasing trend caused by high-cholesterol feeding. These results suggest that LAB could have an antiobesity effect, but the detailed mechanisms are extremely complex and will need further molecular or cellular research to understand.

In general, elevated serum cholesterol levels are considered a critical risk factor for CVDs and are closely associated with LDL-C. Most probiotics lowered TC and LDL-C, and some also lowered TG. In other studies, *L. plantarum* Lp09 and *L. reuteri* LR6 significantly reduced TC, TG, and LDL-C levels, but had no significant effect on HDL-C levels in a hypercholesterolemic rat model [38,39]. However, the *L. plantarum* HT121 not only lowered serum TG, TC and LDL-C, but also increased serum HDL-C levels in hypercholesterolemic rats [40]. HDL-C has reverse cholesterol transport, which protects against CVDs and is a potential factor in the protection of health and longevity [24]. Our results indicated that *L. plantarum* JS5 gavage significantly decreased the elevated serum TC level in high-cholesterol diet-fed mice, while simultaneously increasing serum HDL-C level. These results suggest that *L. plantarum* JS5 administration could influence the serum lipid profile by significantly lowering TC and increasing HDL-C, thus reducing CVDs morbidity. Besides cardiovascular diseases, a high-cholesterol diet can also cause liver damage and fatty liver-associated liver cancer in mice [41,42]. We also detected AST and ALT levels for analysis of the damage extent of the liver in hypercholesterolemia mice. The results found that CM group had highest AST and ALT values, indicating that *L. plantarum* JS5 significantly reversed hepatic damage, which is in agreement with the liver staining results. *L. plantarum* JS5 administration attenuated the extent of lipid deposition and vacuolization in mouse liver tissue cells in histological analysis.

According to recent research, dysbiotic gut microbiota has been linked to CVDs development, while healthy microbiota has been linked to reduced cholesterol levels [43]. The gut microbiota's relative abundance in the mice was assessed to understand the preventive impact of *L. plantarum* JS5 in hypercholesterolemia. In this study, mice given a high-cholesterol diet had remarkably reduced microbial diversity and richness than CC group. So, improving gut microbiota dysbiosis may be beneficial in preventing or treating hypercholesterolemia and CVDs. Probiotic consumption by the host generally regulates the gut microbiota by increasing beneficial microorganisms and by promoting competition against pathogens via direct inhibition or competitive activity. Bacteroidetes, Firmicutes, and Desulfobacterota were the dominant phyla. A high-cholesterol diet decreased the relative abundance of Bacteroidota phylum, while increasing Firmicutes and

Desulfobacterota, but the *L. plantarum* JS5 intervention reversed the increase of Desulfobacterota. In addition, the CM group had a higher relative abundance of intestinal normal inhibitory bacteria such as *Enterococcus* and *Mucispirillum* than the CC group mice, and *L. plantarum* JS5 intake decreased their abundances at the genus level. Meanwhile, the relative abundance of beneficial intestinal microorganisms like *Faecalibaculum*, *Blautia*, *Eubacterium coprostanoligenes\_group*, and *norank\_f\_Lachnospiraceae* genera increased, while *Desulfovibrio* decreased in CI group when compared to the CC and CM groups.

There is a positive correlation between *Faecalibaculum* and short-chain fatty acid synthesis [44], and it has also been suggested that *Faecalibaculum* may benefit rodents via carbohydrate and energy metabolic pathways [45]. *Blautia* is known to be negatively associated with the visceral fat area [46] and produces acetic acid to help expel excess gas from the intestine [47]. It also reported that *Blautia* can improve glucose metabolism and inflammation correlated with obesity [48]. Our results show that *L. plantarum* JS5 intervention boosted *Faecalibaculum*, and *Blautia* in the CI group, which in turn contributed to cholesterol degradation or weight reduction. It is worth noting that the *Eubacterium coprostanoligenes\_group*, which could degrade cholesterol into non-absorbable coprosterol that expels with feces, was only found in the CI group [49]. *Desulfovibrio*, a genus of intestinal microorganisms, has been shown to modulate the genetic mechanisms of diabetes [50], and its production of H<sub>2</sub>S causes apoptosis and persistent inflammation [51]. Previous research indicated that *L. plantarum* H6 gavage reduced *Desulfovibrio* abundance and helped maintain lipid and glucose homeostasis in mice fed a high-cholesterol diet [52]. *Desulfovibrio* abundance was likewise lowered by *L. plantarum* JS5. These results demonstrated that *L. plantarum* JS5 attenuated the liver injury and the hypercholesterolemia by altering the intestinal flora composition in mice on high-cholesterol diet. The functional prediction results show that defense mechanisms, signal transduction mechanisms, and cell motility accounted for higher proportions in CI group, indicating that *L. plantarum* JS5 had a certain impact on the intestinal flora metabolism in defense, signal transduction, and cell activity.

In a nutshell, the intestinal flora directly affects intestinal function in various aspects via complex interactions or metabolites. Body stability can be maintained under the external effects of a high-cholesterol diet, such as *L. plantarum* intervention, as a result of the changes in the abundance of beneficial bacteria, normal intestinal flora, neutral bacteria, or harmful bacteria, and dynamic regulation of the signal pathways and metabolic pathways. These findings can be applied to diverse pathogenic mechanisms, drug resistance mechanisms, and pathogen-host interactions as a prospective research topic.

## 5. Conclusion

In this study, *L. plantarum* JS5 was isolated from Jiang-shui, a traditional Chinese fermented vegetable, and

shows potent *in vitro* cholesterol-lowering ability and high probiotic properties, as well as the ability to improve hypercholesterolemia in high-cholesterol diet-fed mice by modulating their intestinal flora. However, further investigations are needed into the molecular mechanisms of its regulation. This research will provide more insight into the health benefits of our traditional fermented vegetable Jiang-shui, and also provide some theoretical basis for developing novel functional probiotic-based food. However, the identification of the metabolites of the probiotic *L. plantarum* JS5 and the molecular mechanism of its regulation requires further investigation.

## Acknowledgements

This work was funded by the Key Industry Innovation Chain Project of Key R&D Program in Shaanxi Province (2019ZDLNY06-06); and the Science and Technology Program of Xi'an City (20NYF0018).

## Conflict of Interest

No potential conflict of interest was reported by the author(s).

## Authors Contribution

Ya-Zhou Mao: Data curation, Investigation, Writing – original draft; Rong Ren: Methodology, Formal Analysis; Shuang-Hong Song: Resources, Formal Analysis; Yuan-yuan Hui: Investigation, Formal Analysis; Lin-Qiang Li: Investigation, Writing – review & editing; Shan Wu: Formal Analysis, Resources; Ai-Qing Zhao: Formal Analysis; Bi-Ni Wang: Conceptualization, Funding acquisition, Writing - review & editing.

## Data Availability Statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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