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Research article

Limosilactobacillus fermentum TY-S11 ameliorates hypercholesterolemia via promoting cholesterol excretion and regulating gut microbiota in high-cholesterol diet-fed apolipoprotein E-deficient mice

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ABSTRACT

Hypercholesterolemia is a metabolic disease characterized by elevated cholesterol level in the blood, which is a risk factor for many diseases. Probiotic intervention may be one of the ways to improve hypercholesterolemia. In this study, three strains with better cholesterol removal ability were selected from 60 strains of lactic acid bacteria, and were orally administered to apolipoprotein E-deficient mice on a high-cholesterol diet. Among the three strains, only *Limosilactobacillus fermentum* TY-S11, which was isolated from the intestine of a longevity person, significantly improved serum and liver lipid levels in hypercholesterolemic mice. Further study found that *L. fermentum* TY-S11 promoted the excretion of cholesterol in the feces and inhibited the absorption of cholesterol in the small intestine. As for gut microbiota, the results showed that *L. fermentum* TY-S11 not only prevented the reduction of diversity caused by high-cholesterol diet, but also increased the contents of short-chain fatty acids in feces. These results confirmed the ameliorative effect of *L. fermentum* TY-S11 on hypercholesterolemia.

1. Introduction

Hypercholesterolemia is a metabolic disease caused by abnormal cholesterol metabolism characterized by cholesterol level in the blood exceeding the normal range. If patients with hypercholesterolemia do not receive treatment for a long time, the high cholesterol level over time may cause atherosclerosis, fatty liver and other diseases over time, and eventually lead to cirrhosis, renal function

Abbreviations: ABCG8, ATP-binding cassette transporters G8; ACAT2, acyl-coenzyme A: cholesterol acyltransferase 2; ApoE$^{-/-}$, apolipoprotein E-deficient; CON, control group; HCD, high-cholesterol diet; HDL-c, high-density lipoprotein cholesterol; LAB, lactic acid bacteria; LDL-c, low-density lipoprotein cholesterl; LEfSe, linear discriminant analysis effect size; LXR, liver X receptor; NPC1L1, Niemann-Pick C1 Like 1; PCoA, pricipal coordinates analysis; RT-qPCR, real-time quantitative polymerase chain reaction; SCFAs, short-chain fatty acids; TC, total cholesterol; TG, triglyceride.

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damage and cardiovascular diseases [1]. In addition to changing lifestyle habits, the existing therapeutic methods are mainly drugging therapy. Traditional drugs mainly relagulate the serum cholesterol level through three ways: inhibiting the endogenous biosynthesis of cholesterol [2], inhibiting intestinal absorption of cholesterol [3] and increasing the excretion of bile acid [4]. Some traditional drugs have side effects, such as muscle soreness caused by statins [5], and indigestion caused by bile acid chelating agents [6]. Therefore, hypercholesterolemia has always been the focus of research in order to develop new treatments that are more effective and have fewer side effects.

Cholesterol in the body is mainly divided into endogenous and exogenous: synthesized in the liver and peripheral tissues, or reabsorbed in the intestine [7]. Apolipoprotein E (ApoE) is responsible for transporting lipoproteins, fat-soluble vitamins and cholesterol. ApoE-deficient ($ApoE^{-/-}$) leads to impaired clearance of plasma chylous droplet, very low-density lipoprotein and low-density lipoprotein, resulting in elevated cholesterol level [8,9]. Therefore, $ApoE^{-/-}$ mice are often used as experimental animal model for endogenous hypercholesterolemia [10,11]. The intestinal cholesterol absorption rate of human is 29%–80 %. Although there is great individual difference, controlling the absorption of cholesterol in the intestine is still an important way to maintain systemic cholesterol homeostasis [12]. Niemann-Pick C1 Like 1 (NPC1L1) is a transmembrane protein that is highly expressed in the small intestinal mucosal epithelium in most species [13]. In 2004, Altmann et al. clarified that NPC1L1 is a key transporter of intestinal cholesterol absorption [14]. This was subsequently confirmed by numerous cell and animal experiments [15,16]. After being transported to cells by NPC1L1, cholesterol is transported to the endoplasmic reticulum and esterified by acyl-coenzyme A: cholesterol acyltransferase 2 (ACAT2) into cholesterol esters, which eventually enter the blood circulation via lymph [17]. Unesterified cholesterol is secreted into the intestinal cavity through ATP-binding cassette transporters G8 (ABCG8) and excreted in the feces [18]. Liver X receptor (LXR) is a ligand-activated transcription factor, and the down-regulation effect on NPC1L1 expression and up-regulation effect on ABCG8 expression of LXRα/β have been confirmed [19,20].

Probiotics are defined as a class of living microorganisms that are beneficial to human health when consumed in sufficient quantities [21]. In recent years, the ameliorative effect of probiotics on alleviating metabolic diseases has been gradually discovered. The regulatory effect of probiotics on cholesterol level has been reported in vitro [22], in animal [23] and in human [24]. Previous studies have found that some lactic acid bacteria (LAB) could reduce cholesterol content in the medium during the cultivation process [22,25,26]. This ability may be achieved through assimilating cholesterol during growth, binding cholesterol to cellular surface, disrupting cholesterol micelle, deconjugating bile salt and bile salt hydrolase activity [22,27]. In animal experiments, Jiang et al. reported that high-cholesterol diet (HCD) induced hypercholesterolemia and reduced gut microbiota diversity in rats, while *Lactobacillus* isolated from longevity populations improved the serum lipid levels and gut microbiota diversity [28]. The ameliorative effect of LAB on hypercholesterolemia in human has also been reported [24,29]. In conclusion, exploring probiotics with lipid-lowering effects may be a research direction for the treatment of hypercholesterolemia.

In this study, LAB with strong ability to remove cholesterol in vitro was applied to mice with hypercholesterolemia caused by increased self-synthesis and excessive external intake. The purpose of this study was to 1) screen one probiotic strain with the potential to ameliorate hypercholesterolemia caused by internal and external factors; 2) preliminary explore how this strain ameliorated hypercholesterolemia; 3) explore the regulation effect of this strain on gut microbiota in hypercholesterolemic mice.

2. Materials and methods

2.1. Materials

The strains involved in this study were provided by Tianyou Dairy (Chongqing, China). *Limosilactobacillus fermentum* TY-S11 was isolated from the intestine of a longevity person living in a longevity village (Chongqing, China), which was preserved in the China General Microbiological Culture Collection Center (Beijing, China) with the accession No. 25738.

2.2. Measurement of cholesterol removal

The measurement of cholesterol removal was performed as previously described [22]. The filtered cholesterol (Solarbio Science & Technology, Beijing, China) solution (10 mg/ml in ethanol solution) was added to MRS broth with a final concentration of 100 μg/ml. The strains were inoculated into the broth at 3 % (v/v) and incubated at 37 °C. The broth for 0 h and 24 h was centrifuged for 10 min at 10 000×*g* and 4 °C (5804R, Eppendorf, Hamburg, Germany), and a modified colorimetric method was performed [30]. Briefly, phthalaldehyde (Solarbio) solution (1 mg/ml in ethanol solution) was added to the supernatant, after placing for 10 min, the mixed acid prepared by the 1:1 vol ratio of glacial acetic acid and concentrated sulfuric acid was added. After another 10 min, the $OD_{550\ nm}$ was determined (Synergy H1, BioTek, VT, USA) and calculated cholesterol removal according to the following formula.

$$\text{Cholesterol Removal}\ (\%) = \frac{OD_{550\ nm,0h} - OD_{550\ nm,24h}}{OD_{550\ nm,0h}}$$

2.3. Animal experiment

Eight-week-old, male, and specific-pathogen-free grade C57BL/6J and $ApoE^{-/-}$ mice (Gempharmatech, Sichuan, China) were kept in steady temperature (25 °C), humidity (55%–60 %), and aseptic conditions-controlled environment cages with a constant and standard 12/24 h-12/24 h light/dark circle. The bedding material used was corn cob. All animal procedures were performed according

to the European Community guidelines (Directive 2010/63/EU) and approved by the ethics committee of Chongqing University (CQU-IACUC-RE-202112-005).

The strains for animal experiment were cultured in MRS broth at 37 °C for 16 h. After cultured for three generations, the strains were centrifuged for 10 min at 10 000×*g* and 4 °C (5804R, Eppendorf, Hamburg, Germany) to remove the supernatant. After washed with phosphate buffered-saline three times, the strains were resuspended in physiological saline to obtain the bacterial solution of 1×10^9 colony forming units/mL.

After one week of adjustment, 10 C57BL/6J mice were assigned to the control group (CON) and fed a normal diet with energy of 3.42 kcal/g (1025, HFK Bioscience, Beijing, China). 40 $ApoE^{-/-}$ mice were randomly divided into 4 groups (one high-cholesterol diet group and three *L. fermentum* treatment groups), which were fed a high-fat diet with 1.25 % cholesterol (D12108C, Research Diets, NJ, USA). The specific dietary ingredients are shown in Table 1. The experiment period was 6 weeks, and all mice were given free access to water. The HCD + TY-S11, HCD + C4 and HCD + R48 groups were intragastricated with bacterial solution (200 μL) every day, while the CON and HCD groups were intragastricated with the same volume of saline. The weight of the mice was measured weekly during the experiment. Mice feces was collected on day 42 and frozen at −80 °C. After fasting for 12 h on day 43, all mice were put to sleep using isoflurane and sacrificed by cervical dislocation.

2.4. Biochemical analyses

Blood samples were centrifuged for 10 min at 1500×*g* and 4 °C (5804R, Eppendorf) to collect serum. Total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterl (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels in serum were measured using an automatic biochemical analyzer (BS-460, Mindray, Shenzhen, China). The atherosclerosis index was calculated according to the following formula.

$$\text{Atherosclerosis Index} = (\text{TC} - \text{HDL-C}) / (\text{HDL} - \text{C})$$

The liver and intestinal content samples pretreatment was procedured as described previously [31]. 50 mg liver and intestinal content samples refrigerated at −80 °C were taken out and homogenized (JXFSTPRP-CL, Jinxin, Shanghai, China) with a mixture of methanol and chloroform (2:1, v/v) and supplemented to 1 mL. The samples were placed in a water bath (DK-98-II, Taiste, Tianjin, China) at 45 °C for 1 h, and then centrifuged for 10 min at 8000 g and 4 °C (5804R, Eppendorf) to collect the supernatant. TC and TG levels of liver samples, TC level of intestinal content samples were measured with an automatic biochemical instrument (BS-460, Mindray, Shenzhen, China).

2.5. Histological analyses

The liver and aorta samples were soaked in 4 % paraformaldehyde (Boster Biological Technology, Hubei, China) overnight and placed in sucrose solution at 4 °C. When the tissue sank to the bottom, the samples were rinsed slightly with phosphate buffered sodium and placed in a freezable tube. After freezing at −80 °C and placing at room temperature for 2–3 h, the sections were stained with oil red O (Sigma Aldrich, MO, USA) and observed using a stereomicroscope (SZX16, Olympus Corporation, PA, USA). The ratio of aortic lesion area to vessel area was calculated.

2.6. Real-time quantitative polymerase chain reaction

100 mg small intestine samples refrigerated at −80 °C were added with 1 mL TRIzol (Invitrogen, CA, USA) to extract total RNA. The cDNA was obtained as instructed by RevertAid First Strand cDNA Synthesis Kit (Invitrogen). The mRNA expressions of *NPC1L1*, *ACAT2*, *ABCG8, LXRα* and *LXRβ* were measured using a real-time quantitative polymerase chain reaction (RT-qPCR) machine (CFX96, Bio-Rad, CA, USA). GAPDH was used as a reference gene. The primer sequences are shown in Table 2. The primers were synthesized by Maobai Technology (Chongqing, China). The relative expressions of mRNA were calculated by the comparative Ct method ($2^{-\triangle\triangle CT}$) [32].

Table 1
High-cholesterol diet ingredients.

Ingredients	g/kg	Ingredients	g/kg
Casein, 30 Mesh	222.9	DiCalcium Phosphate	14.5
L-Cystine	3.3	Calcium Carbonate	6.1
Corn Starch	236.3	Potassium Citrate, 1 H_2O	18.4
Maltodextrin 10	79.1	Vitamin Mix V10001	11.1
Sucrose 68.8	125.9	Choline Bitartrate	2.2
Cellulose, BW200	55.7	Cholesterol	12.5
Soybean Oil	27.9	FD&C Blue Dye #1	0.1
Cocoa Butter	172.7	FD&C Yellow Dye #5	0.1
Mineral Mix S10021	11.1	Energy (kcal/g)	4.5

Table 2
Primer sequences used for real-time quantitative polymerase chain reaction.

Gene		Sequence	Tm (°C)
NPC1L1	Forward	5′-CGCCCTTCTTTCTACATGGGT-3′	61.8
	Reverse	5′-GAATCTGCGCTTACGAGGGAG-3′	62.6
ACAT2	Forward	5′-CCCGTGGTCATCGTCTCAG-3′	61.8
	Reverse	5′-GGACAGGGCACCATTGAAGG-3′	62.8
ABCG8	Forward	5′-ATACCCTGGAGGTCTCATAGCA-3′	61.5
	Reverse	5′-ACGTCGAGTAGTGAGGCTCTC-3′	62.7
LXRα	Forward	5′-ACAGAGCTTCGTCCACAAAAG-3′	60.5
	Reverse	5′-GCGTGCTCCCTTGATGACA-3′	62.3
LXRβ	Forward	5′-CGTGGTCATCTTAGAGCCAGA-3′	61.0
	Reverse	5′-AGCTGAGCACGTTGTAGTGG-3′	62.1
GAPDH	Forward	5′-TGACCTCAACTACATGGTCTACA-3′	61.1
	Reverse	5′-CTTCCCATTCTCGGCCTTG-3′	61.4

2.7. Short-chain fatty acids analyses

50 mg fecal samples refrigerated at −80 °C were used for short-chain fatty acids (SCFAs) analysis as previously described [33]. Briefly, the samples were homogenized (JXFSTPRP-CL, Jinxin) with saturated sodium chloride solution. After acidified with sulfuric acid, the SCFAs were extracted with ether and centrifuged for 15 min at 10 000×*g* and 4 °C (5804R, Eppendorf). The supernatant was collected and the content of SCFAs was determined by gas chromatograph (GC-7890A, Agilent, CA, USA). The conditions of gas chromatograph were set as previously described [34].

2.8. Gut microbial diversity analyses

Cecum content samples were used gut microbial diversity analysis. After DNA extraction, the hypervariable region V3–V4 of bacteria were amplified with forward primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and reverse primer 806R (5′-GGACTACNNGGGTATCTAAT-3′). Followed the instruction of AxyPrep DNA Gel Extraction Kit (Axygen, CA, USA), the PCR products were recovered. After the products were quantified, the Illumina library was constructed using TruSeqTM DNA Sample Prep Kit (Illumina, CA, USA). Sequencing was performed using the Illumina NovaSeq PE250 platform (Illumina, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Table 3
24 h Cholesterol Removal in vitro of 60 Strains.

Strain Number	Genus/Species	Cholesterol Removal (%)	Strain Number	Genus/Species	Cholesterol Removal (%)
TY-S11	*L. fermentum*	55.24 ± 3.08	S26	*L. plantarum*	9.27 ± 1.66
C4	*L. fermentum*	49.69 ± 0.31	X13	*L. fermentum*	8.81 ± 5.33
R48	*L. fermentum*	43.94 ± 2.00	Q10	*L. salivarius*	8.10 ± 1.31
D49	*L. fermentum*	38.35 ± 1.92	LK30-4	*L. kefiri*	7.21 ± 3.80
X8	*L. fermentum*	31.99 ± 0.31	LC9	*L. casei*	6.33 ± 0.97
H11	*L. crispatus*	19.24 ± 3.03	LK27-4	*L. kefiri*	6.20 ± 4.44
S8	*L. fermentum*	19.13 ± 2.14	LP77-4	*L. plantarum*	5.53 ± 0.83
U46	*B. longum*	18.15 ± 1.12	A13	*L. fermentum*	5.07 ± 1.29
B6	*L. crispatus*	17.77 ± 1.33	R31	*L. salivarius*	4.97 ± 1.17
B13	*L. salivarius*	17.31 ± 2.86	LP102-4	*L. plantarum*	4.65 ± 5.07
D28	*L. gasseri*	16.91 ± 2.33	E3	*L. salivarius*	4.38 ± 1.54
M29	*L. gasseri*	16.42 ± 2.47	B13	*L. salivarius*	4.30 ± 2.65
A23	*L. plantarum*	14.77 ± 1.37	LPC3-4	*L. paracasei*	4.20 ± 2.49
R32	*L. gasseri*	14.70 ± 2.79	B6	*L. crispatus*	4.16 ± 3.59
U38	*L. fermentum*	14.52 ± 1.42	D25	*L. crispatus*	3.99 ± 0.59
X28	*B. longum*	14.48 ± 2.57	A9	*L. fermentum*	3.45 ± 1.00
W1	*B. longum*	13.58 ± 1.44	B14	*L. salivarius*	3.25 ± 2.43
U22	*L. fermentum*	13.53 ± 1.33	B15	*L. salivarius*	3.02 ± 0.89
S9	*B. longum*	12.81 ± 0.71	LP104-4	*L. plantarum*	2.79 ± 3.67
L31	*L. crispatus*	12.43 ± 1.76	X12	*L. fermentum*	2.37 ± 2.14
U6	*L. plantarum*	11.56 ± 0.68	LP81-4	*L. plantarum*	2.30 ± 0.97
U55	*L. fermentum*	11.56 ± 1.28	U21	*B. coagulans*	1.47 ± 1.37
U39	*L. plantarum*	10.93 ± 0.61	Q7	*L. salivarius*	1.30 ± 2.90
B12	*L. paracasei*	10.66 ± 1.34	LPC1-3	*L. paracasei*	1.28 ± 0.99
X6	*L. plantarum*	10.30 ± 0.71	LC2	*L. casei*	1.20 ± 1.10
B20	*L. fermentum*	10.18 ± 0.55	A17	*L. salivarius*	1.09 ± 1.80
B16	*L. salivarius*	9.75 ± 1.28	M9	*L. paracasei*	0.78 ± 1.74
B19	*L. salivarius*	9.56 ± 0.72	L15	*L. salivarius*	0.61 ± 1.69
U2	*L. fermentum*	9.31 ± 1.40	R27	*L. fermentum*	0.51 ± 1.03
W17	*L. plantarum*	9.31 ± 1.47	LP18-4	*L. plantarum*	0.49 ± 1.78

2.9. Bioinformatics analyses

The bioinformatics analysis of gut microbiota was analyzed on the online platform of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/). Alpha diversity was characterized by ACE index and Shannon index. Beta diversity was characterized by pricipal coordinates analysis (PCoA) based on Brayvd-Curtis distance. Linear discriminant analysis effect size (LEfSe) was used to identify biomakers by all-against-all methodology. Correlation diversity was characterized by heatmap based on Spearman. The difference between groups was tested by one-way ANOVA and Tukey-Kramar. $p < 0.05$ was considered to indicate statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

2.10. Statistical analyses

Major statistical analysis was performed using GraphPad Prism (version 8, GraphPad, CA, USA). The data are expressed as mean ± standard deviation. Anderson-Darling test was used to detect homogeneity of variance. If the variance was homogeneity, one-way ANOVA method was applied and Dunnett's test was used for multiple comparison. If the variance was not homogeneity, Kruskal-

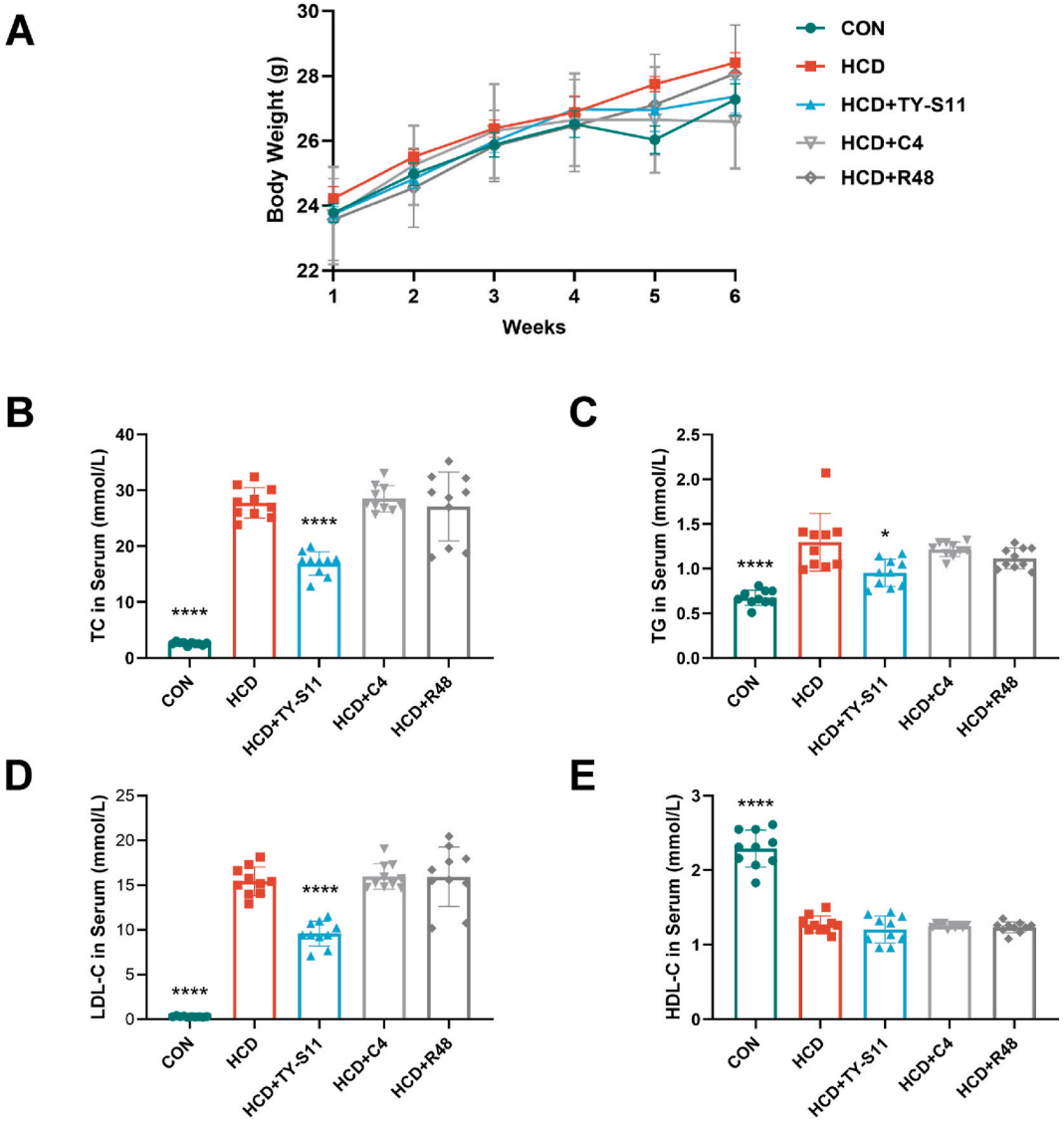

Fig. 1. Effect of L. *fermentum* TY-S11 on body weight and serum lipid in hypercholesterolemic mice. (A) Body weight. (B) TC, (C) TG, (D) LDL-c and (E) HDL-c in serum. Data are shown as mean ± SD (n = 10/group). *$p < 0.05$ and ****$p < 0.0001$ as compared with the HCD group. CON, control group; HCD, high-cholesterol diet group; HF + TY-S11, high-cholesterol diet group treated with *L. fermentum* TY-S11; HF + C4, high-cholesterol diet group treated with *L. fermentum* C4; HF + R48, high-cholesterol diet group treated with *L. fermentum* R48. TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

Wallis test was applied. The comparison was all compared with the HCD group. $p < 0.05$ was considered to indicate statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

3. Results

3.1. L. fermentum TY-S11 had Capacity of cholesterol removal in vitro

24 h cholesterol removal in vitro of 60 strains were determined, including 16 strains of *L. fermentum*, 12 strains of *Ligilactobacillus salivarius*, 11 strains of *Lactiplantibacillus plantarum*, 5 strains of *Lactobacillus crispatus*, 4 strains of *Lacticaseibacillus paracasei*, 4 strains of *Bifidobacterium longum*, 3 strains of *Lactobacillus gasseri*, 2 strains of *Lacticaseibacillus casei*, 2 strains of *Lactobacillus kefiri* and 1 strain of *Bacillus coagulans*. The cholesterol removal in vitro of the strains varied greatly, even among strains of the same genus (**Table 3**), which is consistent with literature reports [22,27]. Among 60 strains, the cholesterol removal rate of *L. fermentum* TY-S11 was the highest (55.24 % ± 3.08), followed by *L. fermentum* C4 (49.69 % ± 0.31) and R48 (43.94 ± 2.00), while the removal rates of other strains were all below 40 %. The results suggested that the 3 strains might have the potential to improve blood lipid.

3.2. L. fermentum TY-S11 improved serum lipid levels in hypercholesterolemic mice

After 6 weeks of different feed interventions, the weight of ApoE$^{-/-}$ mice feeding with HCD was slightly higher than that of C57BL/6J mice feeding with a control diet (Fig. 1A), and this is consistent with literature reports [35,36]. At the end of the experiment, the levels of serum TC, TG and LDL-C of mice in the HCD group were significantly higher than mice in the CON group, and only TY-S11 of the 3 strains of *L. fermentum* significantly suppressed this increase (Fig. 1B–D). The level of serum HDL-C of mice in the HCD group was significantly lower than mice in the CON group, but no strain suppressed this decrease (Fig. 1E). The strains in previous literature also failed to suppress decrease of HDL-C caused by HCD [28,37]. The results showed that *L. fermentum* TY-S11 had the potential to relieve hypercholesterolemia.

3.3. L. fermentum TY-S11 improved liver lipid levels in hypercholesterolemic mice

After 6 weeks of HCD feeding, the levels of liver TC and TG of mice in the HCD group were significantly higher than mice in the CON group, and *L. fermentum* TY-S11 suppressed this increase (Fig. 2A and B). Oil red O strained section of liver showed that hepatocytes in the CON group were normal in shape and tightly packed, and there was no obvious fat vacuole in the cytoplasm, while lipid accumulation, swelling and vacuole were observed in hepatocytes of the HCD group. *L. fermentum* TY-S11 obviously improved the accumulation of lipid in hepatocytes, and improved hepatocytes swelling and vacuole to a certain extent (Fig. 2C). The results showed that *L. fermentum* TY-S11 improved liver lipid levels in hypercholesterolemic Mice.

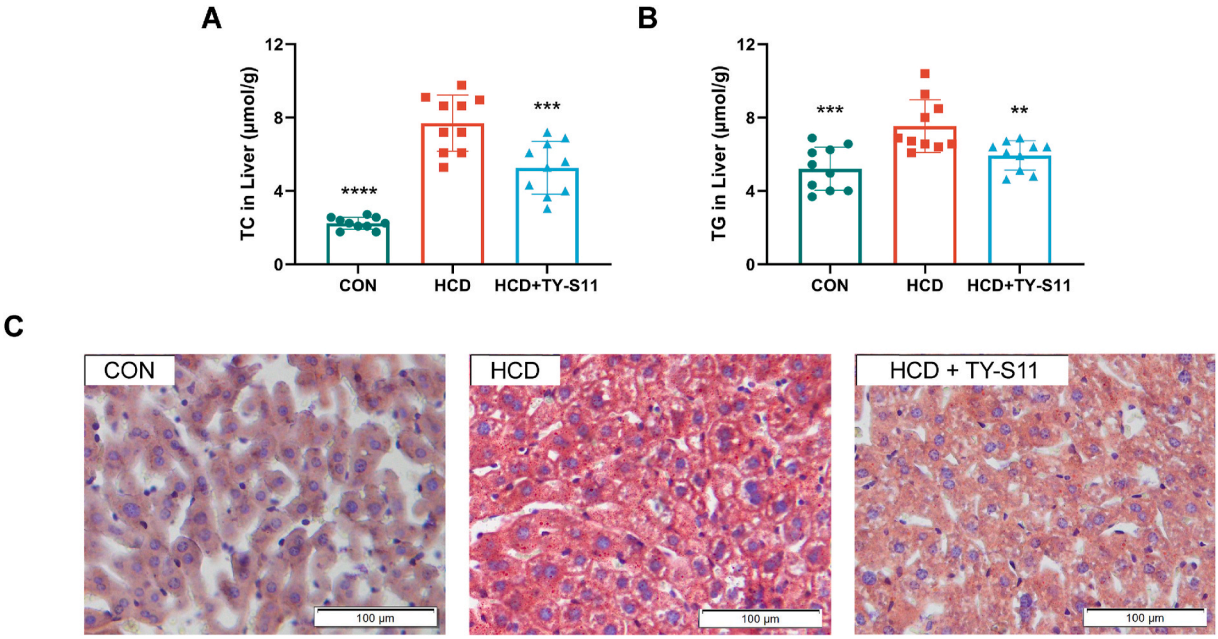

Fig. 2. Effect of L. *fermentum* TY-S11 on liver lipid in hypercholesterolemic mice. (A) TC and (B) TG in liver. (C) Oil red O staining of the liver. Data are shown as mean ± SD (n = 10/group). **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ as compared with the HCD group. CON, control group; HCD, high-cholesterol diet group; HF + TY-S11, high-cholesterol diet group treated with *L. fermentum* TY-S11. TC, total cholesterol; TG, triglyceride.

3.4. *L. fermentum* TY-S11 did not improve atherosclerotic lesion formation in hypercholesterolemic mice

The atherosclerosis index showed that the risk of atherosclerosis in mice of the HCD group was higher than that in mice of the CON group, while the risk was reduced after *L. fermentum* TY-S11 intervention (Fig. 3A). Oil red O strained section of aorta showed that the aorta of the CON group was normal, while lipid accumulation was observed in aorta intima both of mice in the HCD group and the HCD + TY-S11 group (Fig. 3B). The ratio of aortic lesion area to vessel area of every group was further calculated, and the results showed that there was no significant difference between the HCD group and the HCD + TY-S11 group (Fig. 3C), suggesting that *L. fermentum* TY-S11 did not prevente atherosclerotic lesion formation.

3.5. *L. fermentum* TY-S11 inhibited cholesterol absorption in small intestine in hypercholesterolemic mice

The detection of TC level of intestinal content showed that mice in the HCD group could not metabolize excessive TC caused by HCD and excreted it through feces. On this basis, *L. fermentum* TY-S11 further promoted TC emission through feces (Fig. 4A). The effect of *L. fermentum* TY-S11 on cholesterol absorption in the small intestine was assessed by RT-qPCR. Compared with the CON group, the mRNA expression of *NPC1L1* and *ACAT2* in small intestine of the HCD group were increased, while the mRNA expression of *ABCG8*, *LXRα* and *LXRβ* were decreased. *L. fermentum* TY-S11 suppressed this trend (Fig. 4B–F). The results showed that *L. fermentum* TY-S11 inhibited cholesterol absorption in the small intestine in hypercholesterolemic mice.

3.6. *L. fermentum* TY-S11 regulated the gut microbiota and short-chain fatty acids metabolism in hypercholesterolemic mice

In order to explore the effect of *L. fermentum* TY-S11 on gut microbiota, diversity analysis was conducted. ACE and Shannon index on genus level showed that the richness and evenness on genus level in hypercholesterolemic mice were both decreased, while *L. fermentum* TY-S11 inhibited this decrease (Fig. 5A and B). PCoA on genus level showed that significant difference between CON and HCD groups, and on this basis, *L. fermentum* TY-S11 further changed gut microbial diversity (Fig. 5C).

To reveal changes in metabolism of gut microbiota, fecal SCFAs levels were measured. The levels of acetic, propionic and butyric acid in feces were significantly decreased in hypercholesterolemic mice, while *L. fermentum* TY-S11 promoted propionic and butyric acid secretion (Fig. 5D–F). These results indicated that *L. fermentum* TY-S11 had a regulatory effect on gut microbial diversity and SCFAs metabolism in hypercholesterolemic mice.

In order to further explore the effect of *L. fermentum* TY-S11 on gut microbiota, a series of analyses were performed. On phylum level, the abundance of Firmicutes and Actinobacteriota was the highest in the CON group, while Firmicutes greatly increased and Actinobacteriota greatly decreased in the HCD group. *L. fermentum* TY-S11 suppressed this trend (Fig. 6A). Community barplot analysis

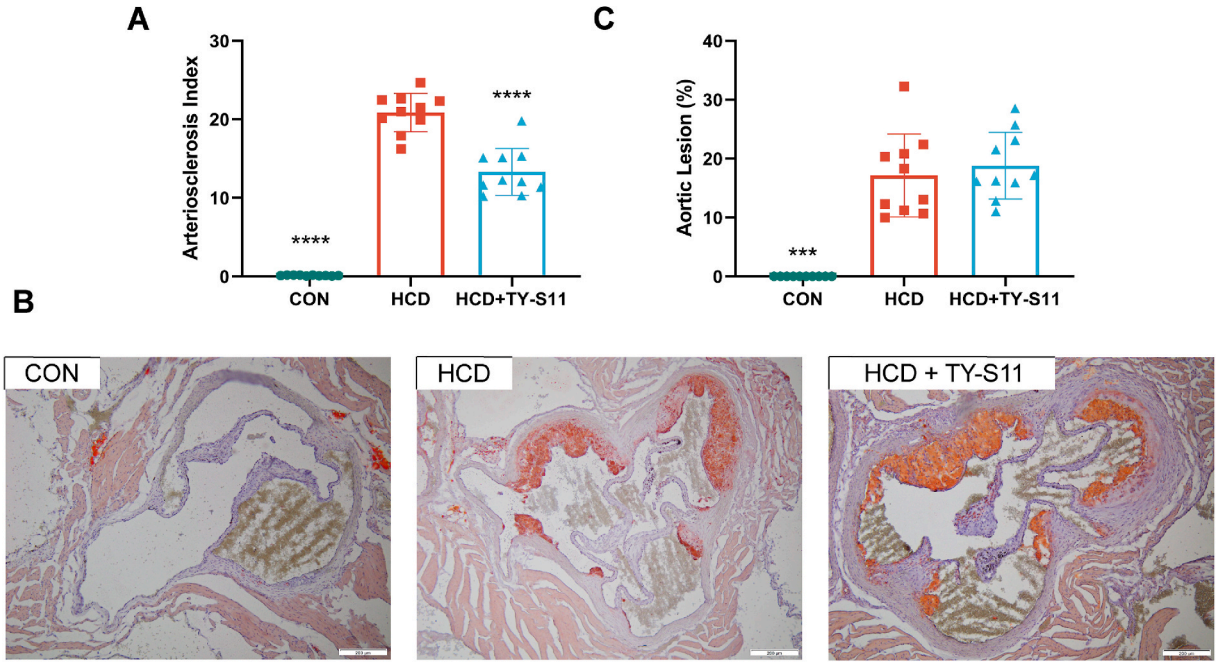

Fig. 3. Effect of L. *fermentum* TY-S11 on atherosclerotic lesion formation in hypercholesterolemic mice. (A) Atherosclerosis index. (B) Oil red O staining of the aorta. (C) The ratio of aortic lesion area to vessel area. Data are shown as mean ± SD (n = 10/group). ***$p < 0.001$ and ****$p < 0.0001$ as compared with the HCD group. CON, control group; HCD, high-cholesterol diet group; HF + TY-S11, high-cholesterol diet group treated with *L. fermentum* TY-S11.

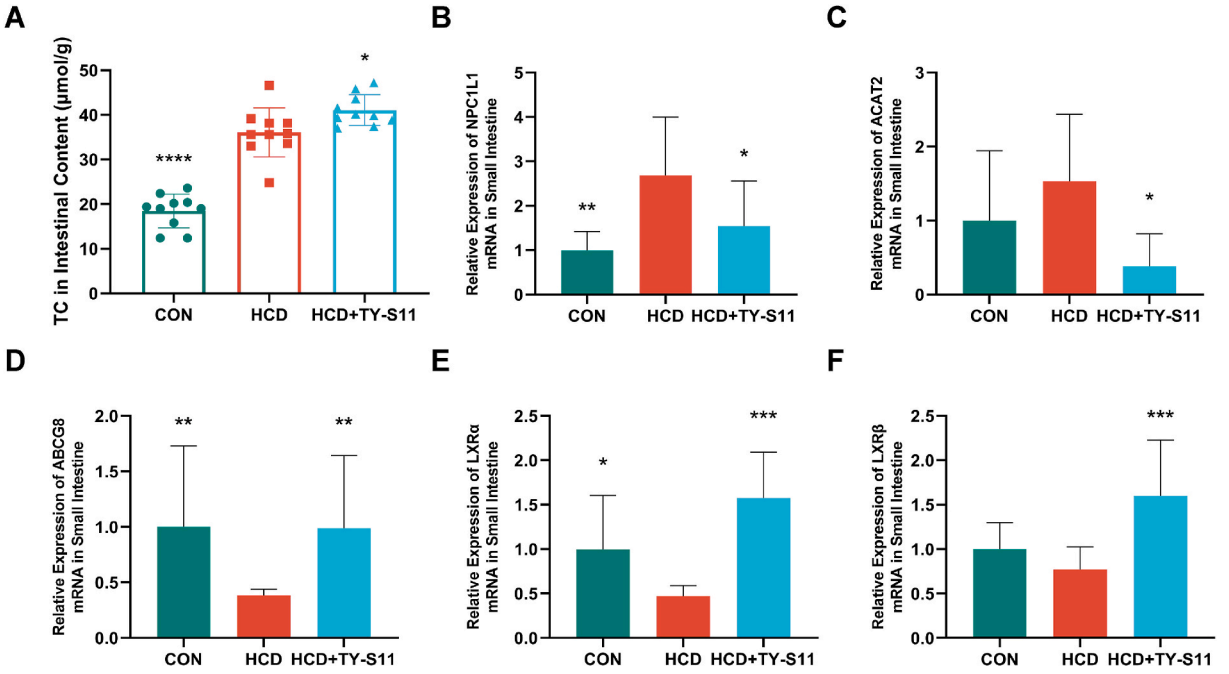

Fig. 4. Effect of L. *fermentum* TY-S11 on cholesterol absorption in small intestine in hypercholesterolemic mice. (A) TC in intestinal content. (B) *NPC1L1*, (C) *ACAT2*, (D) *ABCG8*, (E) *LXR-α* and (E) *LXR-β* mRNA relative expression in small intestine. Data are shown as mean ± SD (n = 8–10/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ as compared with the HCD group. CON, control group; HCD, high-cholesterol diet group; HF + TY-S11, high-cholesterol diet group treated with *L. fermentum* TY-S11. TC, total cholesterol.

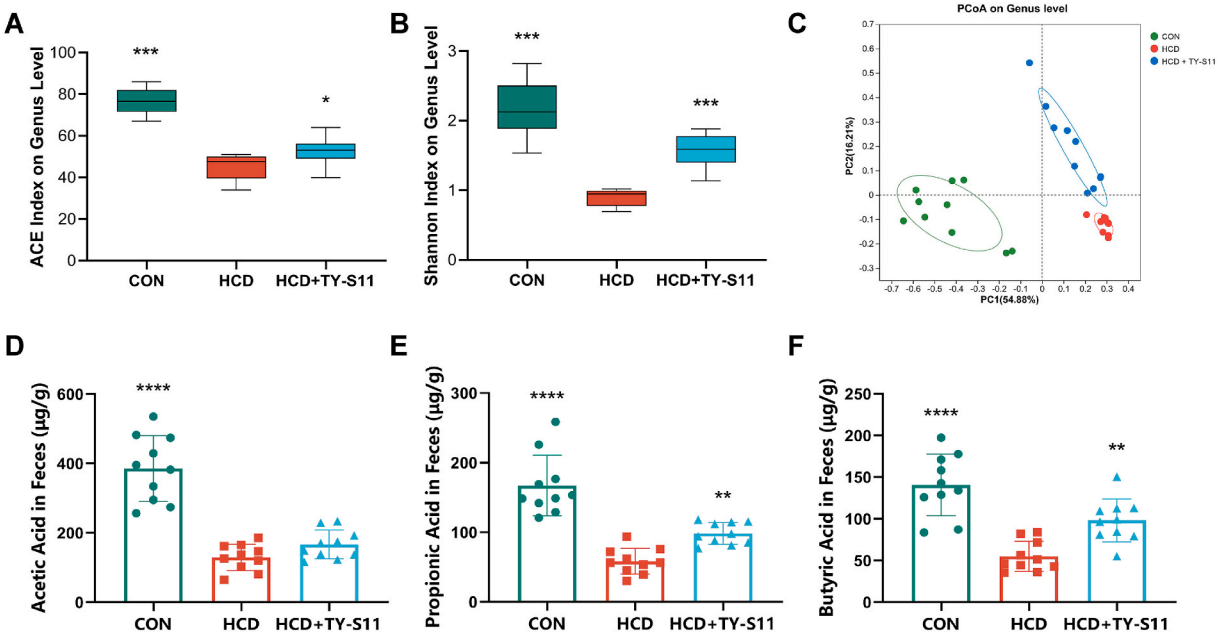

Fig. 5. Effect of L. *fermentum* TY-S11 on gut microbial diversity and SCFAs metabolism in hypercholesterolemic mice. (A) ACE and (B) Shannon index on genus level. (C) PCoA on genus level. (D) Acetic acid, (E) propionic acid and (E) butyric acid in feces. Data are shown as mean ± SD (n = 10/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ as compared with the HCD group. CON, control group; HCD, high-cholesterol diet group; HF + TY-S11, high-cholesterol diet group treated with *L. fermentum* TY-S11.

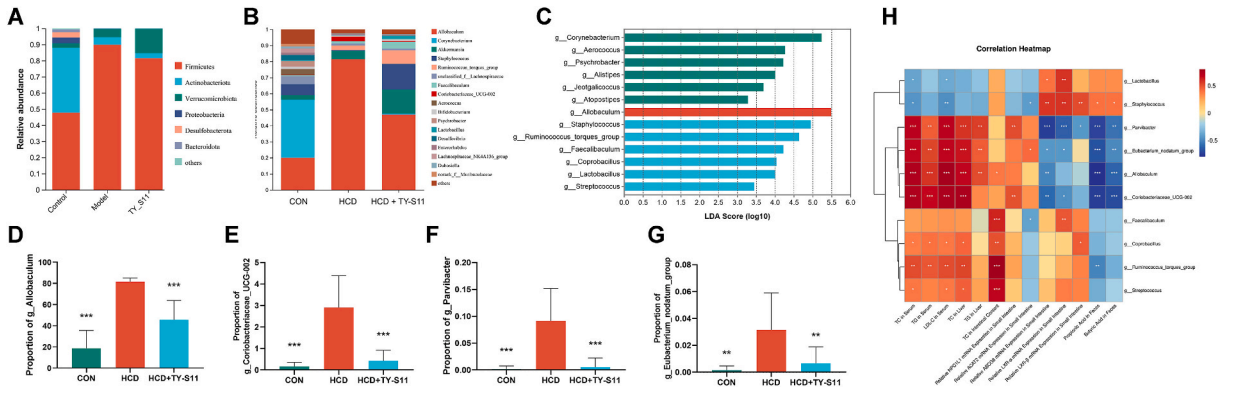

Fig. 6. Effect of L. *fermentum* TY-S11 on gut microbial composition in hypercholesterolemic mice. Community barplot analysis on (A) Phylum and (B) genus level. (C) LEfSe bar on genus level. (D) *Allobaculum*, (E) *Coriobacteriaceae_UCG-002*, (F) *Parvibacter* and (G) *Eubacterium_nodatum_group* relative abundance. Data are shown as mean ± SD. (H) Spearman correlation analysis between clinical symptoms, small intestine protein levels, SCFAs levels, and gut microbial abundance. (n = 10/group). $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the HCD group. CON, control group; HCD, high-cholesterol diet group; HF + TY-S11, high-cholesterol diet group treated with *L. fermentum* TY-S11.

on genus level showed that the composition of gut microbiota changed significantly in hypercholesterolemic mice, while *L. fermentum* TY-S11 restored that change to some extent (Fig. 6B). LEfSe analysis showed that *Allobaculum* was the biomaker in the HCD group compared to other groups, which was totally different from biomaker of the CON group, suggesting hypercholesterolemia caused changes in gut microbiota composition. After L. *fermentum* TY-S11 intervention, *Staphylococcus*, *Ruminococcus_torques_group*, *Faecalibaculum*, *Coprobacillus*, *Lactobacillus* and *Streptococcus* became the biomakers, suggesting that *L. fermentum* TY-S11 had a regulatory effect on the composition of gut microbiota (Fig. 6C). Inter-group difference analysis of gut microbiota abundance showed that the relative abundance of *Allobaculum*, *Coriobacteriaceae_UCG-002*, *Parvibacter* and *Eubacterium_nodatum_group* in the HCD group increased significantly, while *L. fermentum* TY-S11 suppressed this trend (Fig. 6D–G). These results suggested that *L. fermentum* TY-S11 could regulate and restore the dysbacteriosis caused by hypercholesterolemia.

3.7. Correlation between clinical symptoms, small intestine protein levels, short-chain fatty acids levels, and gut microbial abundance

According to the results of LEfSe analysis (Fig. 6C) and inter-group difference analysis (Fig. 6D–G), core gut microbiota was selected for correlation analysis with clinical symptoms, small intestine protein levels and SCFAs levels. Correlation heatmap (Fig. 6H) showed the biomakers *Lactobacillus* and *Staphylococcus* in the HCD + TY-S11 group were negatively correlated with TC level in serum, suggesting that *Lactobacillus* and *Staphylococcus* might be beneficial gut microbiota to ameliorate hypercholesterolemia. Other biomakers in the HCD + TY-S11 group, including *Ruminococcus_torques_group*, *Faecalibaculum*, *Coprobacillus*, and *Streptococcus,* were all positively correlated with TC level in intestinal content, suggesting the above gut microbiota might be helpful for promoting TC emission through feces. *Allobaculum*, *Coriobacteriaceae_UCG-002*, *Parvibacter* and *Eubacterium_nodatum_group* were positively correlated with serum and liver lipid levels. It was further confirmed that *L. fermentum* TY-S11 could suppress the increase of the above gut microbiota abundance caused by hypercholesterolemia, thus ameliorating the symptoms.

4. Discussion

In this study, we explored the regulatory effects of LAB on hypercholesterolemia, and found that *L. fermentum* TY-S11 effectively improved the pathological features and regulated gut microbiota composition of hypercholesterolemic mice.

In previous studies, some LAB showed consistency of efficacy in vitro and in vivo: removed cholesterol in vitro and reduced serum cholesterol level in vivo [38]. But some strains did not show consistency [39]. In this study, a total of 60 strains of different LAB were tested for cholesterol reduction in the medium before and after culture, so as to reflect the cholesterol removal ability of the strains. A total of 3 strains of *L. fermentum* had cholesterol removal rates higher than 40 % (Table 3). Further, these 3 strains of *L. fermentum* were administered to ApoE$^{-/-}$ mice with HCD by oral administration.

During the 6-week animal intervention experiment, among the 3 strains with high-cholesterol removal rates in vitro, only *L. fermentum* TY-S11 effectively reduced the serum TC level of the mice (Fig. 1B). Based on this, the cholesterol-lowering effect of LAB in vitro and in vivo seemed to be not absolutely consistent, but this needs to be further verified. Meanwhile, only *L. fermentum* TY-S11 effectively reduced the serum TG and LDL-C levels (Fig. 1C–E), indicating that *L. fermentum* TY-S11 was the only effective strain to ameliorate hypercholesterolemia. However, *L. fermentum* TY-S11 failed to improve HDL-C level (Fig. 1E), and similar results have been reported in the previous literature [28,37]. As an anti-atherosclerotic lipoprotein, HDL-C has a direct protective effect on arterial wall and can promote rapid atheroma regression [40]. In this study, although *L. fermentum* TY-S11 reduced atherosclerotic index, it did not reduce HDL-C level or improve atherosclerotic lesion formation (Fig. 3).

As the central organ of cholesterol metabolism, liver directly affects the body cholesterol metabolism homeostasis, and the process

of cholesterol entering and leaving the liver is in a dynamic balance [41]. The disruption of this dynamic balance will not only cause abnormal cholesterol accumulation in the liver, but also lead to hypercholesterolemia [42]. In this study, the levels of liver TC and TG of mice in the HCD group increased significantly, which may be due to the liver ingesting of foodborne cholesterol in the form of chyme particle residues through surface receptors [43]. The morphology of liver cells in mice of the HCD group was seriously damaged, which showed a large number of lipid droplets and fat vacuoles of different sizes, while the nucleus was squeezed to one side and the steatosis was obvious (Fig. 2C). These findings suggested that hypercholesterolemia caused liver damage, which could be mitigated by *L. fermentum* TY-S11 intervention.

In addition to using acetyl-coenzyme A to synthesize cholesterol from scratch through more than 30 steps of enzymatic reaction, the direct absorption of cholesterol from food by the small intestine is another main way for the body to obtain cholesterol [7]. NPC1L1 is a transmembrane protein that mainly mediates the absorption of cholesterol in the small intestine [14] and is the primary target of the lipid-lowering drug Ezetimibe [44]. Moreover, it has been reported that NPC1L1 null mice are resistant to diet-induced hypercholesterolemia [45]. ACAT2, ABCG8 and LXRα/β also involved in the reabsorption of cholesterol in the small intestine [46,47]. In this study, *L. fermentum* TY-S11 inhibited the expressions of *NPC1L1* and *ACAT2* in small intestine, and promoted the expressions of *LXRα/β* and *ABCG8* in small intestine, at the same time, increased TC content in feces (Fig. 4). These results suggested that *L. fermentum* TY-S11 could reduce the absorption of cholesterol in the small intestine and promote the excretion of cholesterol through feces, so as to ameliorated hypercholesterolemia.

The link between gut microbiota and lipid metabolism has been established before [48,49]. Through 16S rDNA sequencing technology, Chen et al. found changes in the structure and abundance of gut microbiota in patients with hyperlipidemia compared to people without hyperlipidemia [50]. In this study, we analyzed the gut microbial diversity of colon contents in different groups of mice. Alpha diversity reflects the species richness and evenness of the sample, where richness shows how many different species are present, and evenness measures the difference in the number of different species [51]. As indicators of alpha diversity, the ACE index and Shannon index reflected the decrease in the richness and evenness of gut microbiota in mice caused by hypercholesterolemia. After L. *fermentum* TY-S11 intervention, the status improved (Fig. 5A and B). Beta diversity refers to the difference in species composition between different habitat communities along the environmental gradient [52]. In this study, beta diversity showed that the composition of gut microbiota in the CON, HCD and HCD + TY-S11 group was different, indicating that hypercholesterolemia changed the composition of gut microbiota, and *L. fermentum* TY-S11 further regulated the gut microbiota composition on this basis (Fig. 5C). This was consistent with the results of community barplot analysis (Fig. 6A and B). LEfSe analysis was used to discover the respective biomarkers of different groups, while inter-group difference analysis was used to discover the different gut microbiota among groups (Fig. 6C–G). Further, the correlation between the results of above analysis and characteristics were analyzed (Fig. 6H). A series of analyses found that the development of hypercholesterolemia led to an increase in the abundance of *Allobaculum*, while *L. fermentum* TY-S11 suppressed this trend (Fig. 6D). Although *Allobaculum* has been reported to be beneficial to health [53,54], the association between increased abundance of *Allobaculum* and abnormal lipid metabolism has also been reported [55,56]. It was also found that *L. fermentum* TY-S11 might ameliorate hypercholesterolemia by increasing the abundance of *Staphylococcus* and *Lactobacillus* in the gut of mice. Although *Staphylococcus aureus* is well known as a kind of pathogenic bacteria, some *Staphylococcus* is harmless or even beneficial [57,58]. As a type of bacteria that can metabolize carbohydrates into lactic acid, *Lactobacillus* has great benefits for health, and its ameliorative effect on hypercholesterolemia has also been reported [59,60]. SCFAs are the products formed by the fermentation of indigestible polysaccharides by gut microbiota [61]. Researches have shown that SCFAs, especially butyric acid, can activate AMPK pathway and reduce liver lipid synthesis [62]. In this study, the occurrence of hypercholesterolemia resulted in the decrease of the content of acetic, propionic and butyric acid in feces, but the content of propionic and butyric acid significantly improved after *L. fermentum* TY-S11 intervention.

However, this study only discussed the improvement effect of *L. fermentum* TY-S11 on hypercholesterolemic mice, and did not explore the specific effective substance of the strain. Meanwhile, this study only used 16s rDNA sequencing to preliminarily understand the regulatory effect of *L. fermentum* TY-S11 on the host gut microbiota. In future research, we will further investigate the material basis, and verify efficacy of *L. fermentum* TY-S11 in the clinical trial, while using metagenomes to further explore changes in gut microbiota. In addition, we will also combine *L. fermentum* TY-S11 with different diet varieties, such as high fiber diet, to explore the effective changes of combining the two treatments on hypercholesterolemia.

5. Conclusion

In summary, *L. fermentum* TY-S11 showed the potential to ameliorate hypercholesterolemia caused by increased self-synthesis and excessive external intake. It significantly improved lipid levels of serum and liver, and prevented pathological changes in the liver. The above effects seemed to be related to *L. fermentum* TY-S11's ability to prevent cholesterol absorption in the small intestine, promote cholesterol excretion from feces, and regulate gut microbiota structure. The results of this study further deepened the correlation between gut microbiota and host lipid metabolism, and provided a basis for probiotics to be a side effect free treatment for hypercholesterolemia in the future.

Ethics statement

All animal procedures were performed according to the European Communities Council (Directive 2010/63/EU) and complied with the ARRIVE guidelines. This part was approved by the Ethics Committee of Chongqing University (CQU-IACUC-RE-202112-005).

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Author declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Yadan Deng: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Data curation. **Jing Wang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Funding acquisition, Data curation. **Ran Wang:** Validation, Supervision. **Yuying Wang:** Software, Formal analysis. **Xi Shu:** Resources, Investigation. **Pengjie Wang:** Resources. **Chong Chen:** Resources. **Feng Zhang:** Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Jing Wang reports financial support was provided by Chongqing Natural Science Foundation. Yadan Deng has patent pending to Chongqing Southwest Huayu Patent Agency Co., Ltd. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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