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Research Article

Regulation of *Ligustrum robustum* (Roxb.) Blume on intestinal flora in C57BL/6 mice fed with western high-sugar and high-fat diet

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Abstract: Long-term consumption of a high-salt and high-fat western diet (WD) is closely associated with the development of metabolic diseases. Literature suggests that *Ligustrum robustum* (Roxb.) Blume (LR) may regulate intestinal flora to improve adverse reactions and even metabolic diseases caused by WD. Therefore, this study investigated the effects of LR on serum biochemical indexes and intestinal flora structure in mice fed WD. The experimental groups were divided into regular feed group (SD), WD feed group (WD), regular feed+ (18 g/kg) high-dose LR group (SRH), WD feed+(6 g/kg) low-dose LR group (WRL), WD feed + (12 g/kg) medium-dose LR group (WRM) and WD feed + (18 g/kg) high-dose LR group (WRH). Body weight was measured weekly throughout the experiment. After 10 weeks, the serum and colon contents were collected for analysis. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) in mouse serum were determined using an automatic biochemical analyzer. Additionally, microbial diversity in colon contents was assessed through 16S rDNA amplicon sequencing. There were no significant changes in body weight, serum Glu, ALT, AST, TG, TC, and LDL-C of SRH mice. The abundance of *Alloprovettella* in the gut microbiota was increased, and the abundance of *Lachnospiraceae* was decreased. The body weight, Glu, ALT, AST, TG, TC, LDL-C of WD mice were increased significantly. The abundance of *Erysipelotrichia*, *Proteobacteria*, and *Gammaproteobacteria* in the gut microbiota were increased, and the abundance of *Rikenellaceae*, *Alisipes*, and *Ruminococcaceae* were decreased. Compared with WD mice, the body weight and serum AST content in WRL group (WD feed +6 g/kg LR i.g.), WRM group (WD feed +12 g/kg LR i.g.), and WRH group (WD feed+18 g/kg LR i.g.). were significantly decreased, ALT and TG in WRL group were significantly decreased, TC and LDL-C in WRM and WRH groups were significantly increased. The abundance of *Ruminococcaceae* was increased in WRL group, while the abundance of *Bacilli* and *Lactobacillaceae* were decreased. The abundance of *Akkermansia*, *Deltaproteobacteria*, *Desulfovibrionaceae* and *Lachnoclostridlum* in the intestinal flora of WRH group were increased with decreasing of *Erysipelotrichia*. In conclusion, drinking low concentrations of LR (less than 6 g/kg) can improve WD-induced intestinal microflora disorder, thus regulating triglycerides and transaminases. However, high concentrations of LR can aggravate WD-induced hypercholesterolemia, which may be related to increased abundance of cholesterol-related flora such as *Desulfovibrionaceae*. This experiment provides a scientific basis for different dietary groups to drink different concentrations of LR in a healthy way, and provides a new experimental idea for clinical research on the prevention of metabolic diseases caused by poor diet, such as hypercholesterolemia.

Keywords: western high-salt and high-fat diet; gut microbiota; *Ligustrum robustum* (Roxb.) Blume; metabolic diseases

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1 Introduction

In recent years, there has been an increasing trend in the consumption of unhealthy foods high in sugar, fat, and cholesterol. These include sweet drinks, fried foods, and various cream cakes, which are characteristic of the high-salt and high-fat western diet (WD)[1,2]. These unhealthy foods are typically high in fat, sugar, and cholesterol while being low in dietary fiber and lacking essential vitamins and minerals. Some may also contain carcinogenic chemicals such as heterocyclic amines and polycyclic aromatic hydrocarbons[3]. The long-term effects of a WD can lead to nutritional deficiencies, cognitive impairment[4,5], increased levels of hypersensitive C-reactive protein leading to inflammation[1], sepsis[6], obesity, non-alcoholic fatty liver disease (NAFLD)[7-11], as well as an increased risk of type 2 diabetes due to pressure on the pancreas[12-14]. Recent reports have also indicated that a high-fat and high-sugar diet is one of the reasons for the rapid increase in the prevalence of diabetes[15] and can exacerbate diabetes complications[16]. Furthermore, long-term WD can increase the risk of cancer occurrence[3], vascular dysfunction[17], and chronic lymphocytic leukemia development[18]. These damages are not entirely caused by the direct effects of poor diet but indirectly caused by long-term disturbances of the gut microbiota[19]. For example, fat inflammation and metabolic syndrome induced by WD are related to the increase in relative abundance of *Lachnospiraceae*[20]. There is also a positive correlation between hypercholesterolemia and the abundance of *Desulfovibrionaceae* and *Lachnospiraceae*[21]. Modern studies have shown that *Roseburia*, *Blautia*, *Allobaculum*, *Alistipes,* and other microbiota are closely related to glucose metabolism and inflammation processes. The reduced abundance of these microbiota can lead to glucose (Glu) metabolism disorders leading to diabetes and inflammation[22]. A 7-month WD can even cause microbiota, leading to increased arterial stiffness and endothelial dysfunction[17]. WD can also increase the recurrence rate of tumors after colorectal tumor resection by inducing gut microbiota disorders [23]. The imbalance of intestinal flora is closely related to many diseases, especially metabolic diseases. It is worth noting that basic research shows that genetics and improper eating habits are the main causes of many diseases. Interestingly, studies have shown that the gut microbiota disorder caused by diet is hereditary[24]. This means that the effects of WD do not end with one generation of mice but are extremely profound. With the development of medicine, people pay more and more attention to the influence of unhealthy diet on human health and begin to look for solutions. Exercise [2], nutritious diet[18,25] and pharmacological interventions are the main ways to improve the body damage caused by poor diet.

According to the plant source of Ligustrum robustum (Roxb.) Blume (LR, also known as kuding tea), it can be divided into the kuding tea of big-leaf and the kuding tea of small-leaf. *Chinese Materia Medica* records that the plant sources of the kuding tea of big leaf are leaves of Ilex cornuta Lindl.ex Paxt and Ilex latifolia Thunb. It has the effects of dispelling wind, clearing heat, improving eyesight and promoting body fluids. The kuding tea of small leaf is the leaves of Ligustrum pricei Hayata, LR and Ligustrum henryi Hemsl. It has the functions of dissipating wind heat, eliminating polydipsia, and clearing the head. The kuding tea used in this study was mainly distributed in Sichuan, Yunnan, Guizhou, southern Anhui, western Hunan, eastern Hubei, India, Bangladesh, Myanmar, Vietnam, Cambodia and other regions of China. In Junlian County, Yibin City, Sichuan Province, the altitude and climate are very suitable for the growth of LR, so it is also the producing area of the best quality LR varieties. The LR used in this study was picked from Yibin City, Sichuan Province, and the processing process is shown in Fig. 1. The modern research shows that LR can lose weight and control obesity[26,27], treat fatty liver[28], improve liver damage[29], has antitumor effects[30,31], it also has an antioxidant effect, and it is sometimes better than vitamin C[32]. And these functions may be related to the improvement of related gut microbiota disorders[26,27].

Currently, researchers have primarily focused on studying the effects of LR. However, there are limited studies on the impact of LR on the intestinal flora of poor diet models. Literature suggests that LR may improve adverse reactions and metabolic diseases caused by WD by regulating the intestinal flora[26-28]. Therefore,



Figure 1 Preparation of LR into potable tea. The experimental LR is processed in Junlian County, Yibin City, Sichuan Province.

this study utilized C57 mice to investigate the impact of LR on serum biochemical indexes and gut microbiota composition following administration of WD, providing a new solution for preventing metabolic diseases caused by poor diet.

2 Materials and methods

2.1 Materials

The stems and leaves of LR, which are grown in Junlian County, Yibin City, Sichuan Province, were identified by the School of Pharmacy at Chengdu University of Traditional Chinese Medicine. The method of preparation of liquid hydrogel is condensation reflux extraction method, 1 kg of LR was put into reflux extraction device, 10 times the amount of pure water was added into it, heated to keep a small boiling state reflux extraction for 2 h. After the solution was poured out, it was filtered through 8 layers of gauze. Then, the tea residue was subjected to reflux extraction with 10 times the amount of pure water for 2 h. Following the extraction, the solution was poured out and filtered through eight layers of gauze. The two medicines were then combined and placed into a vacuum concentration device for concentration, followed by transfer to a freeze-drying device for vacuum freeze-drying into a freeze-dried powder. The water extract of Kuding tea, with an extraction rate of 25.6% and the resulting freeze-dried powder were stored at −80 °C[33]. The concentrations were set at 153.6 mg/mL (low dose), 307.2 mg/mL (medium dose), and 460.8 mg/mL (high dose) for standby before use[28].

2.2 Animals

The 72 male C57BL/6 mice (7 weeks old) were purchased from the Experimental Animal Center of Sichuan University, with license number SCXK (Sichuan) 2018-026. The high-sugar, high-fat, high-cholesterol feed used in this study mimicked the characteristics of a Western high-salt and high-fat diet (WD). The main ingredients include sucrose (30%), cream (20%), lard (10%), cholesterol (2%), and corn oil (1%) from Beijing Hua Fukang Biological Technology Co., Ltd., batch number 1910250716. The mice were provided with free access to food and water, maintained at a temperature of 23–25 °C, humidity of 55% ± 5%, subjected to a 12 h light/dark cycle each day, and allowed one week for adaptive feeding in separate cages. They were then randomly divided into two groups: a WD feed group consisting of 48 mice and a conventional feed group consisting of 24 mice. After 6 weeks of feeding, these 24 mice were randomized for a second grouping: regular feed feeding components as the SD group (continuing regular feed feeding), SRH group (regular feed feeding +18 g/kg LR i.g.) according to the previous pre-experiment. The WD fed group was further divided into WD group (WD feed), WRL group (WD feed +6 g/kg LR i.g.), WRM group (WD feed +12 g/kg LR i.g.), WRH group (WD feed+18 g/kg LR i.g.) according to the previous pre-experiment. The experiment continued for an additional four weeks, as shown in Fig. 2. All experimental scheme was executed based on the Use of Laboratory Animals and Institutional Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine (ethical approval number: 2019-03).

2.3 Blood samples collection

Blood samples were collected from the mice's eyeballs. The serum samples were obtained by centrifugation at 1800 × g for 15 min. The serum was then separated and stored at −80 °C.

2.4 Biochemical indexes

In this experiment, the levels of mice serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were measured using an automated biochemical analyzer (TG determination kit, lot number 141719006; TC determination kit, lot number 141619006; ALT determination kit, Lot number 140119006; AST assay kit, lot number 140219005; HDLC assay kit, lot number 142119009; LDL-C assay kit, lot number 142019006; Glu-HK assay kit, lot number 141519006, Shenzhen Mindray Biomedical Electronics Co., Ltd.)

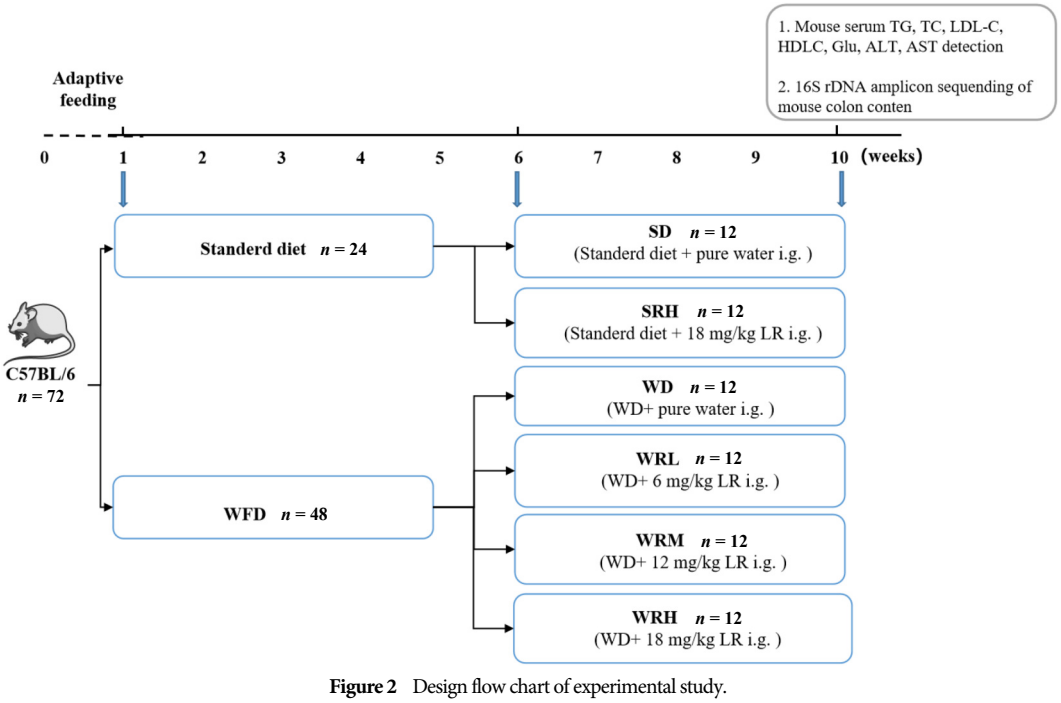


Figure 2 Design flow chart of experimental study.

2.5 Colon content collection

Mouse colons were removed by direct laparotomy on a sterile operating table. The contents of the colon were then placed in a cryotube and stored at −80 °C.

2.6 16S rDNA Amplicon Sequencing

Total genome DNA from samples was extracted using magnetic bead extraction kit. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water. The diluted genomic DNA was used as a template, 16SV3V4 was used as the amplified region, and specific primers (Sangong Bioengineering Co., Ltd.) (Table 1) were used to perform PCR (Phusion® High-Fidelity PCR Master Mix with GC Buffer, New England Biolabs). Mix same volume of 1X loading buffer (contained SYBR Green) with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400 and 450bp were chosen for further experiments (Thermo Scientific Company). PCR products were mixed in equidensity ratios. Then, the mixture of PCR products was purified with GeneJET Gel Extraction Kit (Thermo Scientific). The raw data obtained by sequencing (Raw Data) has a certain proportion of interference data (Dirty Data). Firstly, the raw data is spliced and filtered to obtain effective data (Clean Data). Perform a series of analyses based on valid data.

Table 1 Primer

Area	Primer name	Primer sequence (5'→3')
V3+V4	341F	CCTAYGGGRBGCASCAG
	806R	GGACTACNNGGGTATCTAAT

2.7 Statistical analysis

Data are expressed as mean ± SD and were analyzed statistically using SPSS 20.0 software (SPSS Chicago, IL, USA). The levels of Glu, TG, TC, HDL-C, LDL-C, ALT, and AST were performed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3 Result

3.1 Establishment of WD model mice

In the first 4 weeks, the weight of mice fed with conventional feed increased faster than that of mice fed with WD feed. After four weeks, the weight of mice fed with conventional feed gradually stabilized, while the weight of mice fed with WD feed continued to increase more than that of SD mice. On the 6th week of WD feed, the weight of the WD feed group was 29.12 ± 1.42 g, the weight of the conventional feed group was 27.58 ± 1.17 g (Table 2).

3.2 Changes in the body weight of mice at 10 weeks

The body weight of WD mice was significantly higher than that of SD mice at the 10th week ($P < 0.01$). The body weight of SRH mice decreased significantly in the first week after gavage (the 7th week of the experiment), and the body weight of the group was stabilized without significant changes in the second week after gavage (the 8th week of the experiment). At 10 weeks, the body weight was stable. It was lower than that of SD mice but no significant difference ($P > 0.05$). The body weight of WRL, WRM and WRH mice decreased significantly in the first week after gavage (the 7th week of the experiment) compared with the WD mice. After the second week of intragastric administration (week 8 of the experiment), the trend of weekly weight loss became slower. In the 10th week, the body weight of WRL mice was lower than that of WD mice ($P < 0.01$), and the body weight of WRM and WRH mice was also lower than that of WD mice ($P < 0.01$) (Table 2).

3.3 Serum biochemical index levels in mice

Compared with SD, the serum Glu of WD, WRL, WRM and WRH mice was significantly higher than that of SD mice ($P < 0.05$). Low, medium and high concentration LR had no significant effect on blood Glu of WD diet mice ($P > 0.05$) (Fig. 3A).

The AST test showed that the serum AST content of WD mice was significantly higher than that of SD mice ($P < 0.01$). In addition, the effect of LR on WD diet mice decreased as the concentration increased, suggesting that low concentration of LR can reduce the serum AST content ($P < 0.01$) (Fig. 3B). The ALT test showed that the serum ALT content of WD mice was significantly higher than that of SD mice ($P < 0.01$). A low concentration of LR can significantly reduce AST content in serum of WD diet mice ($P < 0.01$), while there was no significant difference in AST content between WD group and medium and high concentration group ($P > 0.05$) (Fig. 3C).

In addition to affecting serum glucose and transaminase content, LR also significantly improved serum lipid concentration

Table 2 The changes of body weight in C57 mice within 10 weeks of intervention

	SD	SRH	WD	WRL	WRM	WRH
0 w	22.99 ± 0.95		22.37 ± 1.73			
1 w	25.08 ± 0.96		24.68 ± 1.75			
2 w	26.15 ± 1.07		25.10 ± 1.95			
3 w	26.63 ± 0.93		26.32 ± 1.64			
4 w	27.27 ± 1.21		27.31 ± 1.45			
5 w	27.24 ± 1.55		28.16 ± 1.26			
6 w	27.58 ± 1.17		29.12 ± 1.42			
7 w	27.43 ± 1.00	25.72 ± 1.08	30.16 ± 1.11	27.61 ± 1.73	27.10 ± 2.66	27.38 ± 1.23
8 w	26.82 ± 2.26	25.95 ± 1.24	30.32 ± 1.20	27.41 ± 1.29	27.38 ± 2.83	27.18 ± 1.54
9 w	27.61 ± 1.58	26.72 ± 1.19	30.67 ± 1.20	27.21 ± 1.47	27.31 ± 2.20	27.46 ± 1.23
10 w	27.49 ± 1.66$^{\#\#}$	27.67 ± 1.21$^{\#\#}$	30.68 ± 1.55**	27.56 ± 1.32$^{\#\#}$	27.71 ± 2.28	27.72 ± 1.55$^{\#\#}$

$^{*}P < 0.05$, $^{**}P < 0.01$, significant difference compared to SD; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significant difference compared to WD.

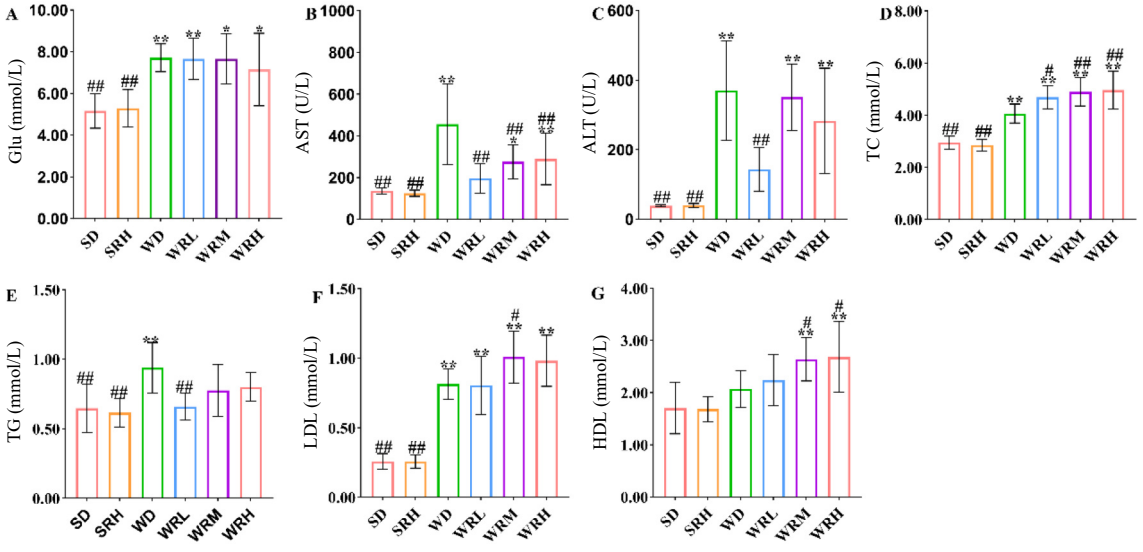


Figure 3 LR regulates serum biochemical parameters of western C57 mice on a high sugar and fat diet. The levels of Glu (A), AST (B), ALT (C), TC (D), TG (E), LDL (F) and HDL (G). One-way ANOVA analysis fllowed by a Tukey test was employed to estimate the statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$, significant difference compared to SD; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significant difference compared to WD.

in WD diet mice (Figs. 3D-3G). Serum TG, TC and serum LDL-cholesterol (LDL-C) results showed that WD diet could significantly improve the serum levels of TG, TC and LDL of mice ($P < 0.01$), but did not significantly increase the HDL-C concentration ($P > 0.05$). After LR was given at different concentrations, it was found that LR could increase the contents of TC, LDL and HDL in serum of mice after WD diet and reduce the content of TG ($P < 0.05$). The concentration of TC in serum increased significantly with the increase of LR concentration ($P < 0.05$) (Fig. 3D). The increase trend of HDL content and TC was the same ($P < 0.05$) (Fig. 3G). The medium concentration of LR had the most obvious effect on the increase of LDL content in WD diet mice ($P < 0.05$), while the other concentrations had no significant effect ($P > 0.05$) (Fig. 3F). Low concentration of LR can significantly reduce the concentration of TG in serum ($P < 0.01$), while medium and high concentration LR has no significant effect on the concentration of TG in mice after WD diet ($P > 0.05$) (Fig. 3E).

3.4 Effects of LR on the composition and diversity of intestinal microbes at the phylum level in WD diet mice

PCR-free library was constructed based on Illumina Nova sequencing platform, and Paired-End sequencing was performed. Through reads were spliced, an average of 90,012 tags was measured for each sample, and 85,193 effective data were obtained after quality control, with effective data volume of 63,673 and quality control efficiency of 70.92%. By clustering the sequences into OTUs (Operational Taxonomic Units) with 97% Identity, 2,027 OTUs were obtained. Then, by comparing with Silva132 database, species annotation was performed, and statistics of different classification levels were found: There are 2,027 OTUs in total, among which 1,759 OTUs can be annotated to the database (86.78%), 86.78% to the boundary level, 80.07% to the phylum level, 78.49% to the class level, and 74.25% to the order level. The proportion of family level was 69.36%, that of genus level was 41.64%, and that of species level was 13.86%.

The Rarefaction curve (Fig. 4B) and rank-abundance curve (Fig. 4C) indicate that the number of sequencing samples is sufficient, and the species richness and community uniformity are both high. As can be seen, the WD caused a significant decrease in the OTU richness and community Q-diversity of the intestinal flora. At the same time, the intervention of LR alleviated the decline in the OTU richness and Q-diversity caused by the long term WD fed. Low concentration of LR even made WD-fed mice have more intestinal flora OTUs than SD group. Among them, OLT showed a better alteration effect. The flower diagram of OTUs (Fig. 4E) presented 469 mutual OTUs of all groups. The peculiar OTUs in SD, SRH, WD, WRL and WRH groups were 261, 184, 66, 113 and 324 (Table 3). Non-metric multi-dimensional scaling (NMDS) analysis (Fig. 4A) showed a complete separation of gut microbiota community between the MC and NC groups. The intervention of GT and OLT reshaped the gut microbiota destroyed by the high-salt diet and brought it close to a healthy state, particularly OLT, with more effectiveness. At the phylum level (Fig. 4D), the intestinal flora structure of rats was dominated by Firmicutes, Bacteroidetes, Verrucomicrobia, Proteobacteria and unidentified_ Bacteria. Compared with the SD group, the continuous WD feeding caused a significant increase in Firmicutes and Proteobacteria, a significant decrease in Bacteroidetes, Verrucomicrobia and unidentified_ Bacteria in the intestinal microbes of the WD group. Accordingly, LR can significantly improve the diversity and structure of intestinal flora in WD-fed mice.

3.5 Effects of LR on the intestinal microflora structure at the genus level in C57 mice fed a WD

The intestinal microbes (top 25 genera in relative abundance) at the genus level in different treatment groups also had obvious distinctions (Fig. 5B). Additionally, Spearman correlation analysis based on the relative abundance showed more or less antagonistic or synergistic effects among various bacteria in mice of each group (Fig. 5A). Phylogenetic trees were constructed based on species annotation results and representative sequences of 16S data in SILVA database, and the top 100 dominant species among each group were compared at the genus level, confirming the differences of intestinal flora among each group at the genus level (Fig. 5C). The cladogram in Fig. 6B and linear discriminant analysis (LDA) histogram in Fig. 6A indicate that the biomarkers of SD group were *Alistipes*, *Rikenellaceae*, *Firmicutes_bacterium_M10_2*, *Intestinimonas*. The biomarkers of WD group were *Firmicutes*, *Erysipelotrichia*, *Erysipelotrichaceae*, *Erysipelotrichales*, *Lachnospiraceae*, *Burkholderiaceae*,

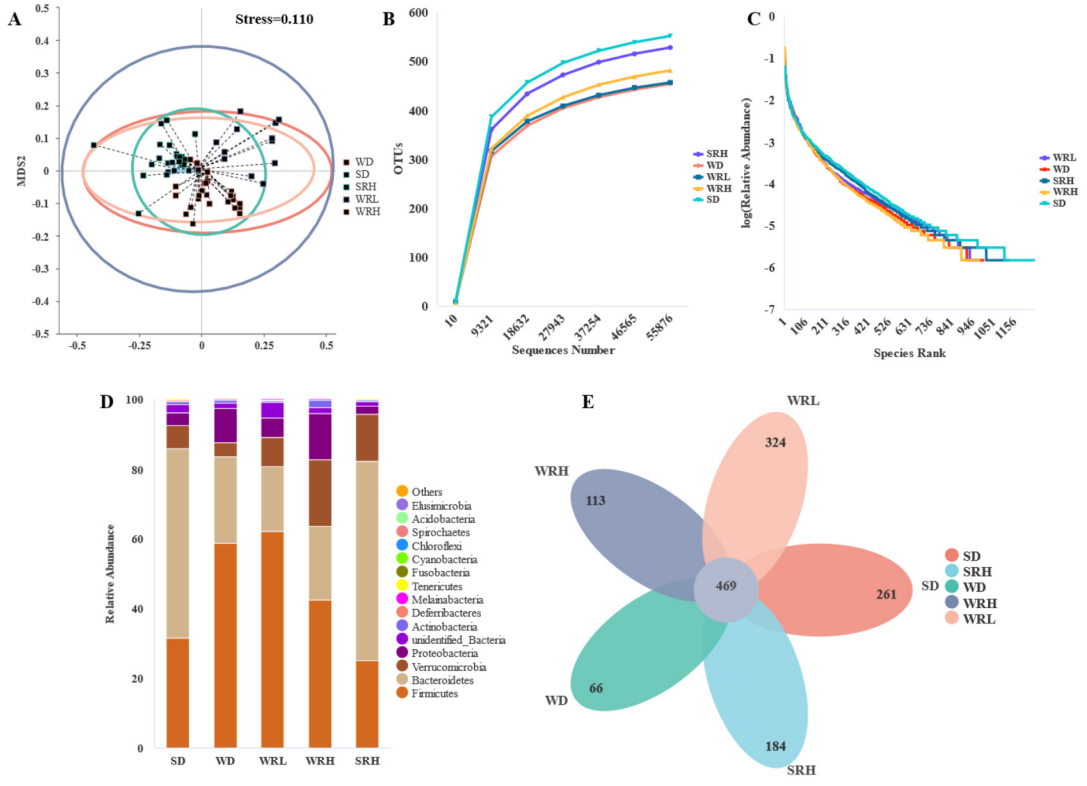


Figure 4 LR altered the composition and diversity of intestinal microbes at the phylum level in C57 mice induced by a western high-salt and high-fat diet. (A) Non-Metric Multi-Dimensional Scaling (NMDS) analysis chart depicted the differences between and within sample groups based on point distance. (B) The rarefaction curve of different sample groups reflected the adequacy of sequencing data and OTU richness. (C) Rank Abundance curve visually represented species abundance and evenness in samples. (D) Histogram illustrated the relative distribution of gut microbes at the phylum level. (E) Petal diagram displayed the number of OTUs, with each petal representing the number of OTUs in a group, and central overlap indicating shared OTUs among groups.

Table 3 The influence of different groups on gut microbiota information

Group	Number of OTUs	Dominant species (Name + Ratio%)		
		Phylum level	Genus level	Species level
SD	730	Bacteroidetes 54.4%, Firmicutes 31.36%, Verrucomicrobia 6.74%, Proteobacteria 3.59%, unidentified_Bacteria 2.33%	*Akkermansia* 6.74%, *Alistipes* 6.3%, *Dubosiella* 4.36%, *Intestinimonas* 2.35%, *Alloprevotella* 1.23%	*Firmicutes_bacterium_M10-2* 3%
SRH	653	Bacteroidetes 57.07%, Firmicutes 25.14%, Verrucomicrobia 13.52%, Proteobacteria 2.39%, unidentified_Bacteria 1.19%	*Akkermansia* 13.52%, *Alloprevotella* 5.2%, *Alistipes* 4.03%, *Dubosiella* 3.21%, *Allobaculum* 1.73%， *Parasutterella* 1.47%	\
WD	535	Firmicutes 58.63%, Bacteroidetes 24.84%, Proteobacteria 9.83%, Verrucomicrobia 4.05%, unidentified_Bacteria 1.68%	*Allobaculum* 13.92%, *Parasutterella* 5.29%, *Akkermansia* 4.05%, *Lactobacillus* 3.36%, *Ileibacterium* 1.99%, *Alloprevotella* 1.99%, *Erysipelatoclostridium* 1.52%, *Dubosiella* 1.22%, *Alistipes* 1.15%	*Burkholderiales_bacterium_YL45* 4.09%, *Ileibacterium_valens* 1.99%
WRL	793	Firmicutes 53.98%, Bacteroidetes 29.85%, Proteobacteria 7.69%, Verrucomicrobia 5.71%, unidentified_Bacteria 1.96%	*Allobaculum* 17.59%, *Akkermansia* 5.7%, *Parasutterella* 4.96%, *Alistipes* 2.77%, *Alloprevotella* 1.81%, *Dubosiella* 1.39%	*Burkholderiales_bacterium_YL45* 3.33%
WRH	582	Firmicutes 42.39%, Bacteroidetes 21.25%, Verrucomicrobia 19.12%, Proteobacteria 13.07%, Actinobacteria 2.15%, unidentified_Bacteria 1.85%	*Akkermansia* 19.12%, *Parasutterella* 4.68%, *Allobaculum* 2.87%, *Ileibacterium* 2.36%, *Lactobacillus* 2.18%, *Dubosiella* 2.11%, *Alloprevotella* 1.21%	*Burkholderiales_bacterium_YL45* 2.73%, *Ileibacterium_valens* 2.36%

The dominant species were the species with more than 1% abundance at phylum, genus and species level in the gut of mice.

Burkholderiales_bacterium_YL45, *Lactobacillales*, *Faecalibaculum*. The WRL group were *Proteobacteria*, *Desulfovibrionales*, *Desulfovibrionaceae*, *Deltaproteobacteri*, *Clostridia*, *Clostridiales*, and *Lachnoclostridium*.

3.6 Effects of LR on OTUs abundance and dominant species in WD-fed C57 mice

According to the species annotation and abundance information of each group at the genus level, the maximum ranking method was adopted to select the top 35 genera in abundance and draw a heat map. It was found that LR had an impact on the intestinal flora of WD-fed mice at both the genus (Fig. 7A) and species (Fig. 7B) level. The terrane shows the differences between SD + WD + WRL (Fig. 8A) and SD + WD + WRH (Fig. 8B) among the top 35 dominant species at the species level. The dominant species in SD group are *Firmicutes_*

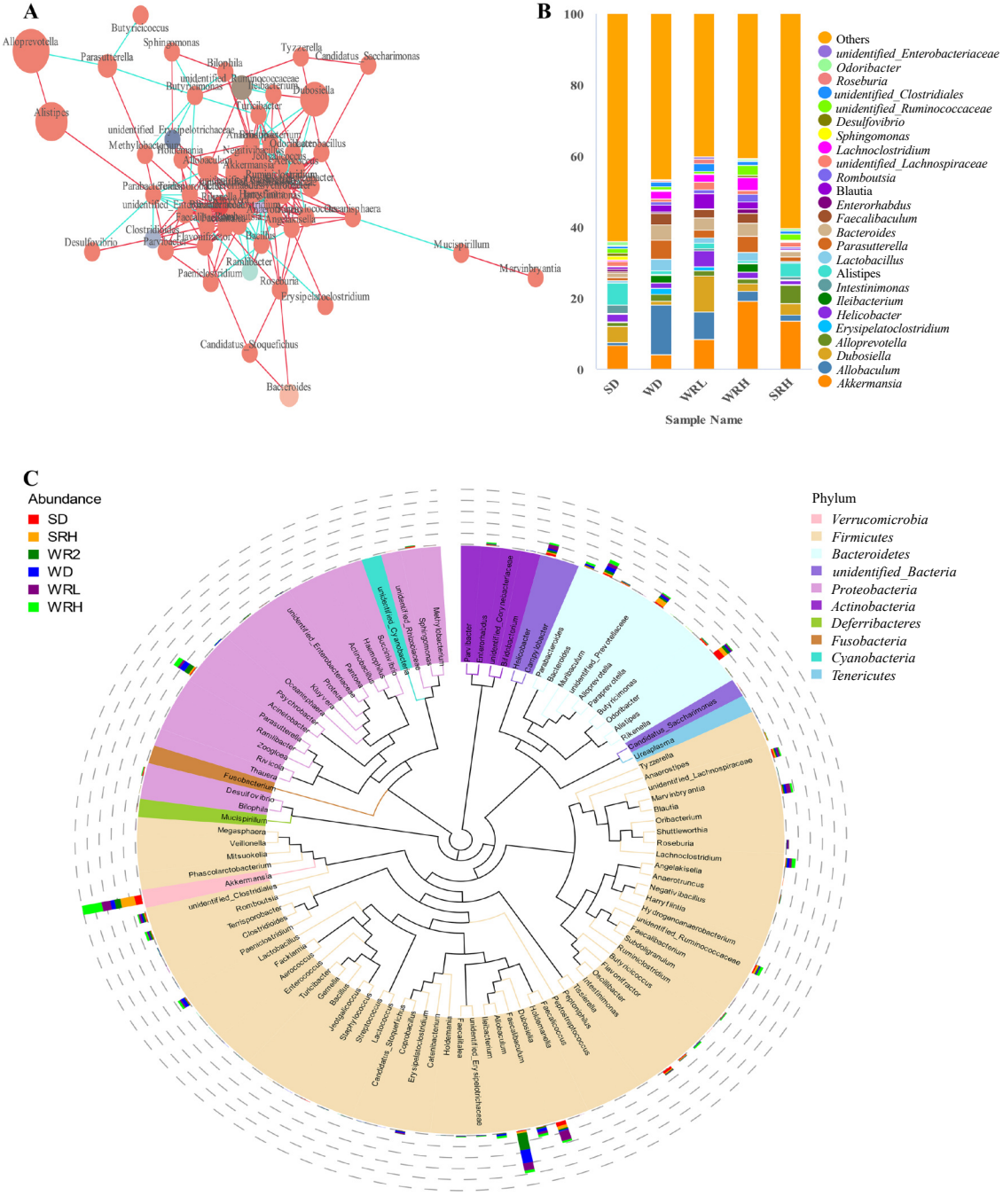


Figure 5 LR changed gut microbiota structure at the genus level in C57 mice induced by western high-salt and high-fat diet. (A) Microbial interaction network diagram at the genus level based on Spearman correlation analysis. The circle represents a genus, the size represents its relative abundance, different colors represent different phylum classifications, the line between the dircles represents a significant correlation between the two bacteria ($P < 0.05$). The red line represents a positive correlation, and the blue line represents a negative correlation. The thicker the line, the greater the absolute value of the correlation coefficient. (B) Histogram of the relative distribution of gut microbes at the genus level. (C) Phylogenetic tree at genus level, which can obtain representative sequences of top100 genera through multiple sequences alignment.

bacterium_M10-2, *Burkholderiales_bacterium_YL45*, *Sphingomonas_Panni* and *B Blautia_sp_YLS8*. The dominant species in WD group were *Bifidobacterium bifidum*, *[Ruminococcus]_gnavus* and *Parabacteroides_merdae*. The dominant species in WRL group were *Eubacterium_sp_14-2*, *Burkholderiales_ bacterium_YL45* and *Escherichia_ coli*. And the dominant species in WRH group was *Desulfovibrio_sp_ UNSW3caefatS*. In this study, the unweighted Unifrac distance was used to calculate the sample distance matrix, and UPGMA clustering was used to generate clustering tree. It was found that LR could increase WD feeding *Firmicutes* and *Proteobacteria* in the intestinal tract of mice. Decreased *Bacteroidetes*, *Verrucomicrobia* and unidentified_Bacteria (Fig. 8C and Table 3).

3.7 The effect of LR on species with significant differences between groups

The representative biomarkers was separately proposed (Figs. 9A-9D), and the T test analysis was performed according to its abundance. Compared with SD mice, the abundance of

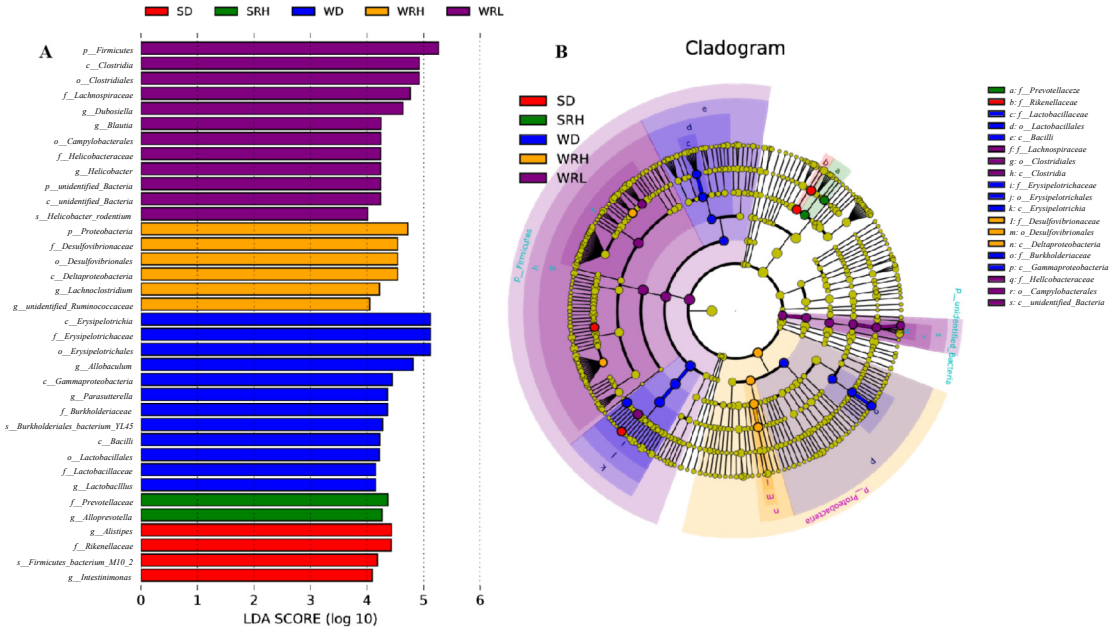


Figure 6 LR changed the composition and distribution of intestinal microflora OTUs in C57 mice induced by western high-salt and high-fat diets at the genus level. (A) LDA histogram based on LEtSe analysis. Each horizontal column represents a kind of bacterium, and the length of the column corresponds to the LDA value, The higher the LDA value, the greater the diference. The color of the column corresponds to the characteristic. (B) Cladogram based on LEtSe analysis. The cladogram corresponds to different levels of intestinal microbial from the inside to the outside, and the connection between the levels represents the belonging relationship. Each circled node represents a classification of bacteria, yellow nodes represent insignificant differences between groups, and non-yellow nodes represent that the bacterium is characteristic microorganisms of the corresponding group (significantly higher abundance in this group), The colored fan-shaped area marks the subordinate classification intervals of the characteristic microorganisms.

Alloprevotella in the flora of SRH mice increased ($P < 0.05$), and the abundance of *Lachnospiraceae* decreased ($P < 0.05$). Compared with SD mice, the abundances of *Bacilli* and *Lactobacillus* in WD mice were increased ($P < 0.01$), *Intestinimonas* ($P < 0.05$), and *Alistipes* ($P < 0.01$) were decreased. Compared with WD mice, the *Rikenellaceae abundance* in WRL mice was increased but not statistically significant, while *Rikenellaceae abundance* in WRL mice decreased. The abundance of *Ruminococcaceae* in WRL and WRH mice both increased ($P < 0.01$). In WRL, the abundance of *Lactobacillus* in WRH mice increased, but it was not statistically significant. The abundance of *Bacilli* in WRL ($P < 0.05$) mice decreased and was statistically significant, the abundance of Bacilli in WRH mice was decreased but not statistically significant, and the abundance of *Desulfovibrionaceae* in WRH mice was significantly increased ($P < 0.01$).

4 Discussion

C57BL/6 mice are particularly sensitive to metabolic syndrome and are prone to obesity and are widely used in research[34]. Therefore, C57BL/6 mice were selected as the experimental animals. The weight of WD-fed mice at the sixth week was significantly higher than that of conventionally fed mice[7]. The difference from other studies is that this study adjusted the low, medium and high concentration settings to the concentration of the toxicity experiment[28]. The specific concentration setting is based on the toxicity study of LR in our laboratory.

WD feeding can induce a significant increase in the weight of mice, which is consistent with the results of previous studies[34,35], the intervention of LR can significantly reduce the weight of mice fed with WD[30]. However, different from other studies, this experiment found that the level of concentration is not directly proportional to the weight control effect. LR below 6 g/kg has a better weight control effect than LR above 12 g/kg. Routinely fed mice with high concentration of LR in the 7th week did not significantly reduce their body weight.

WD feeding can induce a significant increase in blood glucose, blood lipids (except HDLC) and transaminase in mice. This corresponds to the results of modern research on the greater harm of WD to the lipid metabolism, sugar metabolism and liver of mice[7,8,11]. However, LR intervention did not achieve the effect of lowering blood sugar starting from the 7th week onward. Different concentrations of LR can improve the high AST induced by WD, and it is consistent with body weight measurement. The lower the concentration, the more significant the effect. Different from AST, only the intervention of LR lower than 6 g/kg can improve the high ALT induced by WD. LR higher than 12 g/kg does not significantly improve the high ALT. There are some differences in the study of LR[29]. This may be due to WD induction LR intervention of less than 6 g/kg can improve WD-induced hypertriglyceridemia, especially the effect of LR intervention of 6 g/kg is better. However, it is worth noting that any concentration of LR cannot significantly improve WD-induced hypercholesterolemia, and even LR higher than 12 g/kg can cause WD-induced hypercholesterolemia to significantly worsen. It is consistent with modern studies on the toxicity of high concentration kuding tea. This study showed that the long-term (high sugar and high fat) WD increased dose of kuding tea extract can affect the liver fat of mice. This can increase liver weight and fat accumulation in the liver. Hepatocyte toxicity was increased in a dose-dependent manner after treatment with kuding tea in HepG2 cells[28]. This result is different from other lipid-lowering studies of Daye kuding tea, and the reason may be related to the dose of the experimental group[27,36,37].

After comparing the abundance of WD mice with the differential species analysis of SR mice, it was found that WD

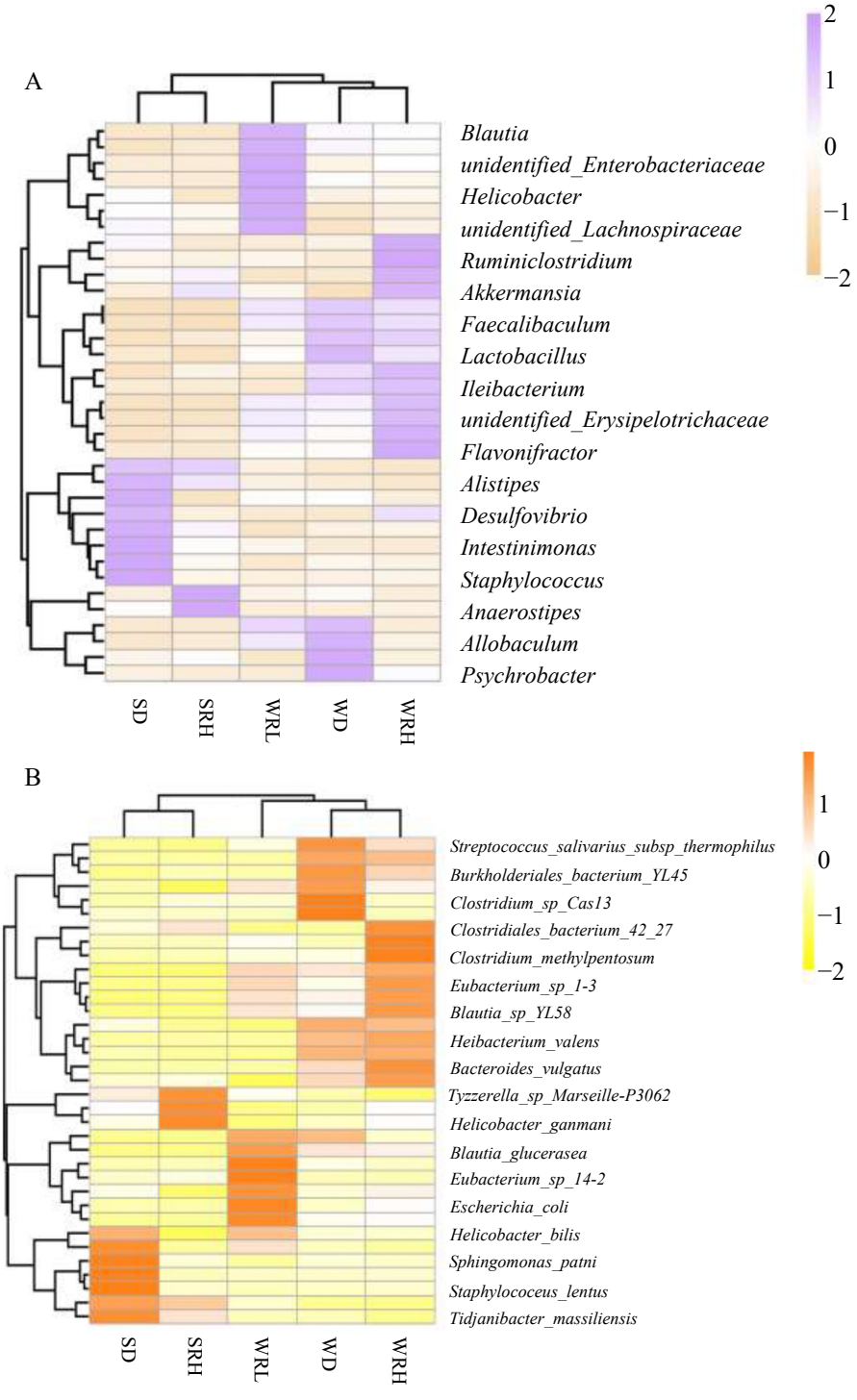


Figure 7 LR changed the abundance of intestinal flora OTUs in C57 mice induced by western high-salt and high-fat diets. The clustering heat maps of OTUs abundance at genus level (A) and species level (B) suggested the aggregation and content information of the top 35 OTUs abundance LR changed the dominant species in the intestinal flora of C57 mice induced by high-salt and high-fat diets in the west.

increased the abundance of *Firmicutes*, *Erysipelotrichales*, *Proteobacteria*, *γ-Proteobacteria*, *Bacillus* and *Lactobacillus* in mice. And reduced the abundance of *Bacteroidetes*, *Bacteroidetes*, *Rikenaceae*, *Muribaculaceae* and *Alistipes*. The increased abundance of *Firmicutes*, *Erysipelotrichales*, *Proteobacteria*, *γ-Proteobacteria*, *Bacillus* and *Lactobacillus* were related to obesity and fatty liver caused by high-fat diet[38-40]. Studies have found that the increase of the abundance of *Alisties* and *Rikenellaceae* in intestinal flora can regulate glucose and lipid metabolism, which is negatively correlated with metabolic disorders[22, 41]. And modern studies have shown that *Alisties* can not only regulate blood sugar, but also negatively correlate with endotoxemia markers in mice caused by a high-fat and high-sugar diet[38]. It shows that it has a good anti-inflammatory effect, which may be positively correlated with the production of short-chain fatty acids and negatively correlated with disorders of glucose and lipid metabolism[22]. Studies have also shown that *Prevotella* is negatively related to diseases such as hyperlipidemia, obesity and liver inflammation

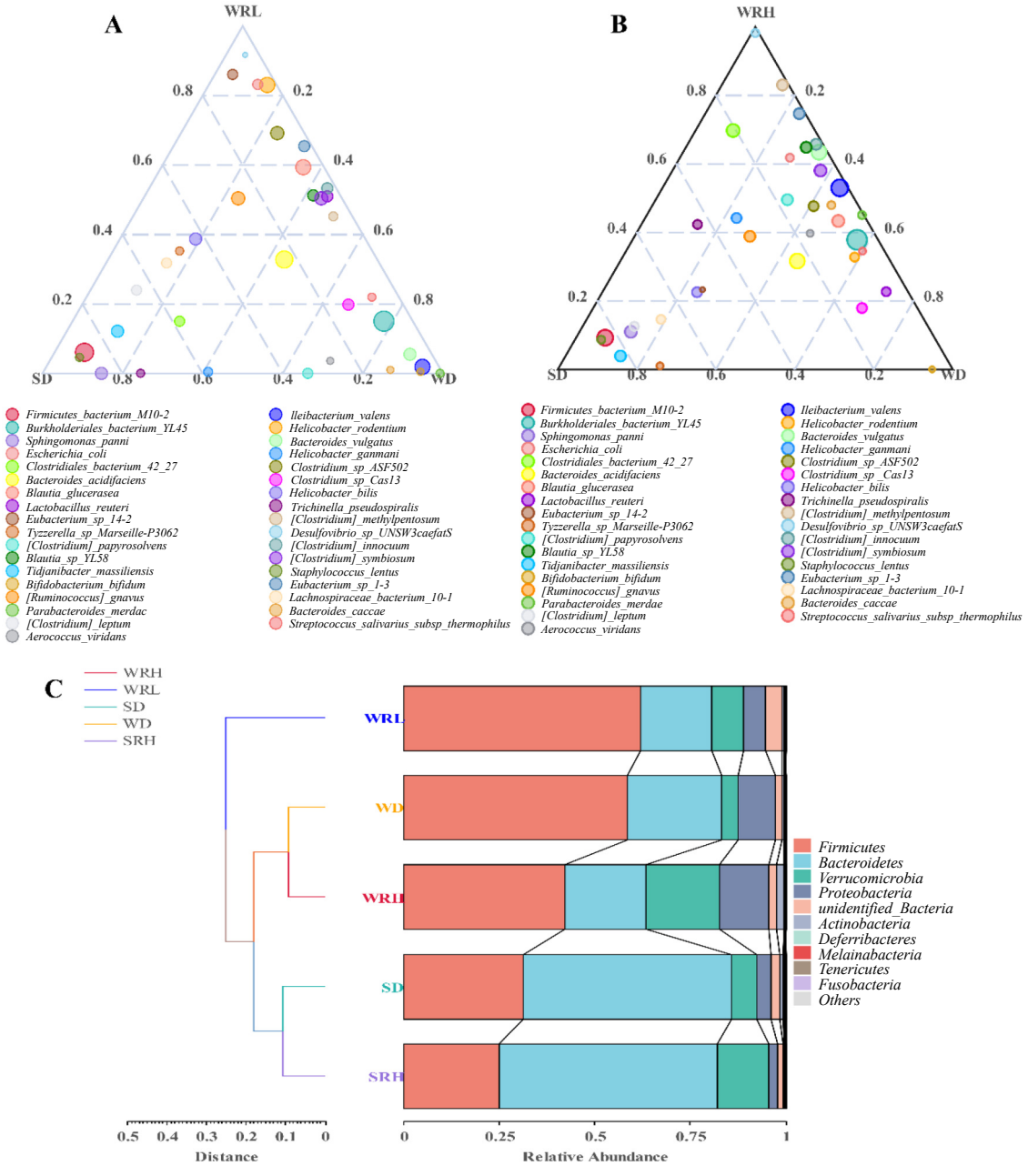


Figure 8 LR changed the dominant species of intestinal flora in C57 mice induced by high-salt and high-fat diets in the west at the species level. (A) and (B) are ternary phase diagrams, representing the differences of the top 35 dominant species at the species level in SD + WD + WRL and SD + WD + WRH groups, respectively. (C) UPGMA clustering tree, and the length of branches represents the distance between samples. If the community composition of samples is similar, they will gather into a cluster in the clustering tree.

caused by high-fat, high-protein, high-sugar and low-fiber diets[42-44]. Therefore, the changes in the abundance of these species may be related to the increase of body weight, blood lipids, blood glucose and transaminase in WD mice, and the regulation of these species abundance may help to alleviate the increase of body weight, blood lipids, Glu and transaminase caused by WD. In this study, it was found that low-dose LR could increase the abundances of *Ruminococcaceae*, *Rikenellaceae* and *Alistipes*, and decrease the abundances of *Bacillus* and *Lactobacillus* in WD mice. Modern studies have shown that the increased abundance of *Ruminococcaceae*, *Rikenellaceae* and *Alistipes* can help regulate endotoxemia indicators, participate in the production of short-chain fatty acids, and benefit glucose and lipid metabolism[22,41,45]. Other studies have also shown that *Bacillus* and *Lactobacilli* are positively correlated with obesity, and their reduction is conducive to the regulation of lipid metabolism[38]. In summary, low-dose LR significantly alleviated intestinal flora disturbances caused by WD. The increase in the abundance of *Verrucomicrobia*, *Akkermansia*, and the decrease in the abundance of *Firmicutes* and *Erysipelotrichales* are beneficial to improve non-alcoholic fatty liver. In our experiments, high concentrations of LR can significantly increase the abundance of *Prevotellaceae* and *Akkermansia* in mice on a regular diet, which is consistent with modern research. It shows that high concentrations of LR can help the health of the regular diet group by adjusting gut microbiota. It is worth noting that high concentrations of LR can also increase the abundance of *Akkermansia* in the gut microbiota of WD-fed mice, which may be related to its regulatory effect on the increase

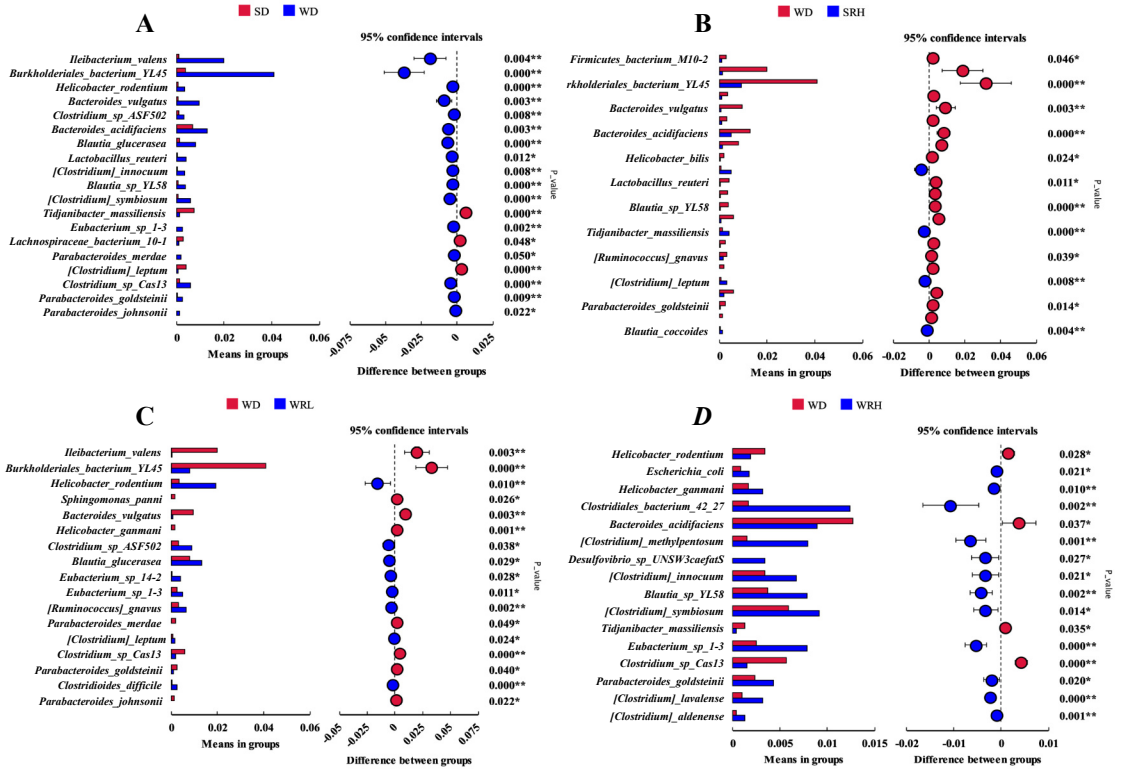


Figure 9 T test was used to analyze OTUs with significant differences between groups at the species level. (A), (B), (C) and (D) show the comparison between SD + WD, WD + SRH, WD + WRL and WD + WRH respectively, $^{*}P < 0.05$, $^{**}P < 0.01$.

in AST. According to research reports, obesity is associated with increased abundance of Firmicutes and Proteobacteria, and decreased abundance of Bacteroides[46, 47]. *Ruminococcaceae* belongs to *Firmicutes*, what is interesting is that its regulatory effect is controversial in modern research. Some studies believe that it is positively related to obesity[38]. However, some studies have shown that the increase in the abundance of *Ruminococcaceae* contributes to glucose and lipid metabolism, which is negatively correlated with non-alcoholic fatty liver and steatohepatitis induced by high-fat, low-fiber drinks[46, 47]. Our experimental results support the side that *Ruminococcaceae* is beneficial to Glu and lipid metabolism. Other studies have also shown that *Bacillus* and *Lactobacillus* are positively correlated with obesity and reducing them helps regulate lipid metabolism[45]. The increase in the abundance of *Ruminococcaceae*, *Rikenellaceae* and *Alisties* help to regulate endotoxemia indicators, participates in the production of short-chain fatty acids, and is conducive to glucose and lipid metabolism[22, 41, 48]. These studies correspond to our research results. But the most interesting result of this experiment is that high concentrations of LR not only cannot reduce WD-induced hypercholesterolemia but increase it. In recent years, there have been similar reports that long-term WD (high sugar and high fat) supplemented with large doses of kuding tea extract and γ-cyclodextrin negatively affects liver fat in mice and produces certain liver cytotoxicity[28]. It is worth noting that previous studies have shown that a high-fat diet can increase the abundance of *Desulfovibrio* and *Lachnospiraceae* in experimental animals, and are positively correlated with hypercholesterolemia[21, 23, 38]. In our experiment, LR higher than 12 g/kg caused WD to induce a significant increase in the abundance of *Desulfovibrionaceae* in the gut microbiota of mice, which indirectly proved that 12 g/kg LR in this experiment caused hypercholesterolemia in WD mice. The deterioration is closely related to the increase in the abundance of *Desulfovibrionaceae*. To sum up, low concentration (< 6 g/kg) LR can improve hypertriglyceridemia and liver dysfunction caused by WD, as well as *Ruminococcus*, *Lactobacillus*, *Bacillus* and other related intestinal flora disorders. The diagram of its action is shown in Fig. 10. However, high concentrations (> 12 g/kg) LR not only has no positive effect on hypercholesterolemia caused by WD, but also aggravates hypercholesterolemia in these diet groups, which may be related to the increased abundance of *Desulfovibrionaceae* related population.

Currently, this study still has some limitations: on the one hand, this study only uses male C57 mice as the research object, and the sample size and representativeness are slightly insufficient. On the other hand, the mechanisms of different LR doses affecting intestinal flora, liver function and lipid metabolism still needs to be further studied. In the future, the mechanism of LR affecting WD intestinal flora can be further explored by expanding the study population, increasing the detection of liver and intestinal pathology, as well as the detection of biochemical indicators such as interleukin-6, malondialdehyde and glutathione.

5 Conclusions

In conclusion, an appropriate dose of LR (< 6 g/kg) can improve AST and TG abnormalities and intestinal flora disorders caused by WD in mice, while excessive LR (> 12 g/kg) may aggravate the TC and LDL abnormalities and related flora disorders caused by WD. This experiment provides a scientific basis for different dietary groups to drink different concentrations of LR safely. It also provides some experimental insights for clinical research on dietary prevention of hypercholesterolemia.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

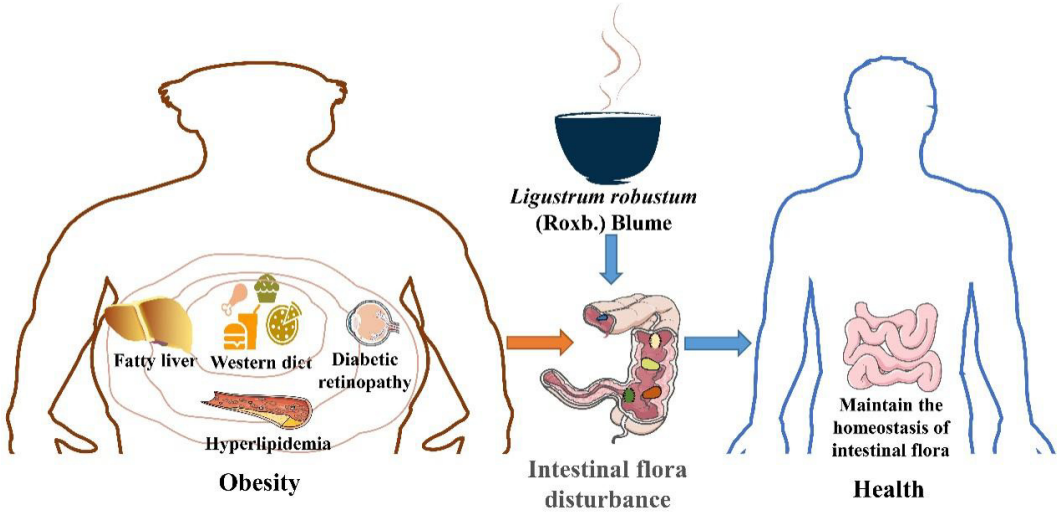


Figure 10 Schematic diagram of LR drinking effect. By changing the diversity and structure of intestinal flora, LR can improve obesity induced by high-salt and high-fat diets in western countries and increase the content of blood glucose and lipid, so as to prevent the occurrence of fatty liver and hyperlipidemia.

Acknowledgments

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