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Original Article

Anti-hyperlipidemia efficacy of *Lactobacillus delbrueckii* on blood lipids and gut microbiota in high-fat diet-fed mice

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Abstract: *Objective:* Our aim was to explore the effects of *Lactobacillus delbrueckii* on blood lipids and gut microbiota in high-fat diet-fed mice. *Methods:* A total of 60 male C57BL/6 mice were divided into four groups (n=15 each): normal control group (CON group), *Lactobacillus* group (LAC group), high-fat group (MOD group), and high-fat group with *Lactobacillus* (MOD+LAC group). Intestinal microbiota were analyzed using traditional culturing methods. After 12 weeks, serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were measured using enzymatic methods. *Results:* Mice in the MOD group were significantly obese, compared with mice in the CON, MOD+LAC, and LAC groups (all P<0.05). TG, TC, and LDL-C levels were significantly higher in the MOD group than those in CON group (P<0.01), while HDL-C was decreased (P<0.01). TG, TC, and LDL-C levels were significantly lower and HDL-C was higher in MOD+LAC group than in the MOD group (P<0.05). Mice in the MOD group had significantly less *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* but an increase of *Enterobacter,* compared with the other three groups (P<0.05). *Conclusion:* Our results suggest that *Lactobacillus* could reduce blood lipid levels in mice, effectively improve their gut microbiota, and inhibit occurrence of obesity.

Keywords: *Lactobacillus delbrueckii*, high-fat diet, gut microbiota, obesity, hyperlipidemia

Introduction

Hyperlipidemia means that there is too much cholesterol in the blood and it is known to induce atherosclerosis. Atherosclerosis is the main cause and pathological basis of cardiovascular and cerebrovascular diseases such as cerebral and myocardial infarctions and coronary heart disease [1]. Most drugs used to reduce lipid levels in the blood are chemically synthesized. These drugs are usually associated with a series of side effects that increase the burden of lipid metabolism in the liver. Therefore, it would be beneficial to find new effective and safe drugs to reduce blood lipid levels [2, 3].

The majority of patients suffering from diseases related to lipid metabolic disorders are faced with symptoms of indigestion, such as abdominal distension and diarrhea. Meanwhile, patients that have been diagnosed with functional indigestion also tend to have diseases related to lipid metabolism, including hyperlipidemia and fatty liver. Association of bacterial composition with many diseases has been observed. Several gut bacteria such as *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, and *Enterococcus* have been shown to be closely related to lipid metabolism. Abnormal lipid metabolism can directly affect the quantity and distribution of gut microbiota [4-6].

An important probiotic microorganism in the human body, *Lactobacillus* plays a fundamental role in innate immunity. It plays a beneficial role in improving the ecological balance of host gut microflora. Moreover, *Lactobacillus* is closely associated with gut microbiota disorder, obesity, and metabolic syndromes. Therefore, it may influence the weight of the host and improve lipid metabolism by regulating gut microbiota [7-11].

In this study, we investigated the effects of *Lactobacillus* on blood lipids and gut microbio-

ta of mice fed with a high-fat diet by measuring serum lipid levels and analyzing the distribution of gut microbiota. In addition, we determined how *Lactobacillus delbrueckii* affects the development of obesity in mice.

Materials and methods

Animals and reagents

The current study was approved by the Medical Ethics Committee of Beihua University and was conducted in accordance with the Declaration of Helsinki, with the Guide for Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health [12]. Sixty male C57BL/6 mice were obtained from Vital River Laboratory Animal Technology Co. Ltd., Beijing. These mice were in a specific pathogen-free state with weights of 10-20 g. They were bred in separate cages with a 12:12-hour light-dark circle. A sufficient diet was supplied and water intake was not limited during this period. Their ordinary diet contained 50% corn, 20% bran, 15% soy beans, 10% flour, 5% fish meal, and a right amount of salt. The high-fat diet comprised the ordinary diet plus 10% lard, 5% sugar, 3% cholesterol, and 0.5% cholic acid sodium salt.

Lactobacillus selection (LBS) agar, *Bifidobacterium* culture medium, and bile esculin agar were purchased from Qingdao Hope BioTechno. Co., Ltd. Serum triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were sourced from BioSino Bio-Technology & Science Inc (Qingdao, China).

Lactobacillus delbrueckii culture and molecular identification using 16S rDNA sequencing

Lactobacillus delbrueckii (*L. delbrueckii*) was obtained by isolation from a store-purchased yogurt on agar with the conventional culture method listed in the U.S. Food and Drug Administration Bacteriological Analytical Manual. The isolate was verified by 16S rDNA sequencing. In brief, one colony was selected for inoculation into MRS liquid culture medium and incubated in a rotary shaker at 180 rpm at 37°C for six hours. Genomic DNA was extracted from the bacteria, according to methods described previously [13]. Partial sequencing of 16S rDNA of isolates was carried out using universal primers: forward primer 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 1542R 5'-AAGGAGGTGATCCAGCCGCA-3'. PCR amplification was carried out using a GeneAmp PCR 2700 system (Applied Biosystems, Foster City, CA), with an initial denaturation step for 3 minutes at 95°C followed by 1 cycle of denaturation at 95°C for 2 minutes, 30 cycles annealing at 55°C for 1 minute, and extension at 72°C for 30 seconds. This was followed by a final extension at 72°C for 5 minutes. Five microliter PCR product was analyzed by electrophoresis (Bio-Rad) in 1% Agarose (SIGMA) gel at 100 volts for 40 minutes, followed by staining with 1% solution of ethidium bromide (50 microliter/L). Gels were visualized by UV transillumination.

DNA bands of interest were excised, purified with a QIAqick PCR purification kit, ligated to pGEM-T Easy vector (Promega), and transformed into JM109 competent cells. White clones were screened. Nucleotide sequencing was performed directly on cloned strain using ABI Prism 377 DNA sequencer. Sequence similarity search was carried out with the BLAST program available at the website of the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov).

Finally, the *L. delbrueckii* strain was maintained in MRS broth containing 15% glycerol and stored at -80°C centigrade for further analysis. When preparing for oral solution, the strain was grown overnight at 37°C in MRS broth and diluted to 3×10^8 CFU/mL in normal saline.

Animal grouping

Briefly, male C57BL/6 mice were adaptively fed for one week. Then, 15 mice were randomly selected and assigned to the normal control group (CON group) and another 15 mice were assigned to pure *Lactobacillus* group (LAC group). CON and LAC groups received a normal diet. The remaining 30 mice were fed a high-fat diet for four weeks to develop a hyperlipidemic mouse model. Successful establishment of the hyperlipidemic mouse model was verified by measuring TG and TC levels. Hyperlipidemic mice were randomly divided into the following two groups (n=15 per group): high-fat group (MOD group) and high-fat group with *Lactobacillus* (MOD+LAC group). Mice in the LAC and MOD+LAC groups received 1 mL of *Lactobacillus* solution (3×10^8 CFU/mL) by gavage, once

Table 1. Changes in serum lipid levels in mice fed with different diets (*n*=30)

Groups	Weeks	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)
Normal diet	0	2.68 ± 0.12	0.80 ± 0.15	1.67 ± 0.28
	2	2.71 ± 0.26	0.79 ± 0.77	1.74 ± 0.45
	4	2.70 ± 0.56	0.90 ± 0.57	1.71 ± 0.75
High-fat diet	0	2.88 ± 0.18	0.90 ± 0.30	1.78 ± 0.16
	2	3.19 ± 0.72*	1.17 ± 0.14	1.57 ± 0.48
	4	4.19 ± 0.63**	1.43 ± 0.56**	1.47 ± 0.19

Data are shown as mean ± SD. *P<0.05 indicates statistical significance. **P<0.01 indicates statistical significance.

Table 2. Effect of *Lactobacillus* on serum lipid levels (*n*=15)

Groups	TC	TG	LDL-C	HDL-C
CON	2.88 ± 0.22	0.84 ± 0.25	0.88 ± 0.35	1.67 ± 0.18
LAC	2.79 ± 0.56	0.82 ± 0.37	0.86 ± 0.77	1.61 ± 0.75
MOD	5.88 ± 0.78[a]	1.95 ± 0.31[a]	1.99 ± 0.36[a]	1.18 ± 0.56[a]
MOD + LAC	4.69 ± 0.72[b]	0.97 ± 0.34[b]	1.27 ± 0.88[b]	1.47 ± 0.48[b]

Data are shown as mean ± SD. [a]P<0.01, significant difference when compared with the CON and LAC groups; [b]P<0.05, significant difference when compared with MOD.

daily for 12 weeks. Mice in CON and MOD groups were gavaged with 1 mL of MRS culture medium. Animals were then fasted for 12 hours before the start of our experiment.

Blood samples were collected from the tails of mice and TG and TC levels were measured. Food and water were provided *ad libitum* throughout the experimental period. Mice were weighed on a weekly basis. After the mice were grouped, all mice were weighed once per week. Additionally, hair color, changes in diet, movement status, and fecal morphology were observed daily.

Detection of serological indexes

Mice were strictly fasted for 12 hours before samples were obtained. Eyeballs of the mice were then extracted for blood sampling. Briefly, 0.8-1.0 mL of blood was extracted from each mouse and obtained samples were centrifuged at 3,500 rpm for 20 minutes at 4°C, to separate serums. Serum was transferred to EP tubes and stored at -80°C until analysis. In addition, 0.1 g of mouse fecal samples were obtained, aseptically transferred to EP tubes, and stored at -80°C until analysis. TG, TC, LDL-C, and HDL-C were all detected using enzymatic methods, according to manufacturer instructions.

Detection of changes in mouse gut microbiota with pour plate method

Fresh feces (0.1 g per mouse) were collected by gently pressing the rectum. These samples were transferred into pre-sterilized EP tubes. Aseptic glass beads were added to the EP tubes. Aseptic normal saline (10 mL) was poured into each tube and the samples were thoroughly homogenized on an oscillator. Then, the samples were serially diluted from 10^{-1} to 10^{-8}. In order to determine changes in gut microflora, mouse feces were cultured and 0.5 mL of an appropriately diluted sample was analyzed using the pour plate method. *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Enterobacter* were cultured in LBS, *Bifidobacterium* BS, bile esculin agar, and eosin methylene blue (EMB) agar, respectively. All species of strains were determined, according to 16S rDNA sequencing. Three replicates were performed for each dilution. After culturing, bacterial colonies were counted. Results were presented as logarithm values of the number of bacteria per gram of feces.

Statistical analysis

SPSS software package version 16.0 (SPSS Inc, IL, Chicago, USA) was used for statistical analysis. Effects of *Lactobacillus* on weights of mice in the different groups was analyzed using analysis of variance (ANOVA). Student Newman Keuls (SNK) method was employed for multiple comparisons. Differences in blood lipids among the groups and impact of *Lactobacillus* on blood lipid levels in mice were analyzed by ANOVA. The influence of *Lactobacillus* on intestinal lactic acid bacteria of mice including *Bifidobacterium*, *Enterococcus*, and *Enterobacter* was analyzed using general non-linear model.

Results

Bacteriological identification of Lactobacillus delbrueckii

When cultured on MRS plates, *L. delbrueckii* colonies appeared milky white, glossy, flat, and

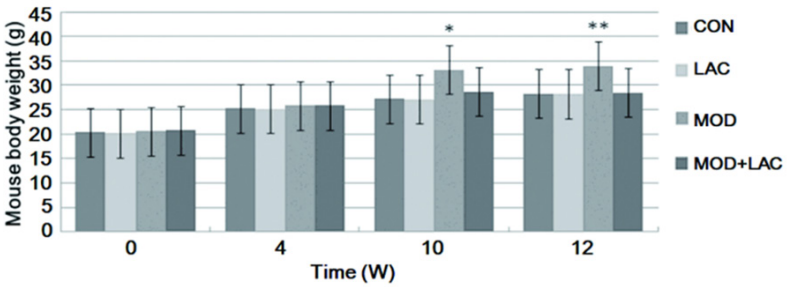

Figure 1. Effect of *Lactobacillus* on body weight. **P*-value <0.05 indicates a statistical significance. ***P*-value <0.01 indicates a statistical significance.

opaque with neat edges and notable calcium-dissolving zones. *L. delbrueckii* cells were thick long single gram-positive rods with slightly round edges. Cells were arranged in parallel or appeared as short chains. Bacteria could grow at a pH of 4.5. Furthermore, the bacteria generated a turbid and acid production when cultured in liquid MSR, demonstrated to be facultative anaerobes. *L. delbrueckii* tested negative for catalase, nitrate reduction, gelatin liquefaction, indole, and H_2S. Glucose fermentation by *L. delbrueckii* produced acid, but no gas, while gluconate fermentation could generate both. Sequencing of 16S rDNA, produced by polymerase chain reaction of bacterial DNA using universal primers, revealed that superior gram positive isolates were closely related to *Lactobacillus delbrueckii* (data not shown).

Establishing an experimental hyperlipidemia mouse model

TC and TG levels in serum were used to verify whether our hyperlipidemic animal model was established. Serum TC, TG, and HDL-C concentrations in mice in CON group remained stable throughout the study ($P>0.05$). After 2 weeks of feeding with a high-fat diet, serum TC concentrations of mice in MOD groups significantly increased relative to the control group ($P<0.05$) while the difference of TG was not statistically significant. After four weeks, TG was found to increase significantly in MOD groups ($P<0.05$) but HDL-C had an increase without a statistical difference (**Table 1**). These results suggest that we successfully established our hyperlipidemia mouse model.

Lactobacillus delbrueckii could reverse effects of high-fat diet on serum lipid levels

We used the established hyperlipidemia mouse model to investigate effects of *Lactobacillus delbrueckii* on MOD mice. TC, TG, and LDL-C levels were significantly higher in the MOD group compared with CON group, while HDL-C was significantly lower than that in the CON group. All differences were statistically significant ($P<0.01$) (**Table 2**). Furthermore, serum TC, TG, and LDL-C levels in the MOD+LAC group were significantly lower than corresponding levels in the MOD group, while HDL-C was significantly higher ($P<0.05$). *Lactobacillus* did not influence lipid levels in mice in the LAC group, compared with the CON group ($P>0.05$, **Table 2**). Therefore, these findings suggest that *Lactobacillus delbrueckii* could reverse effects of a high-fat diet on serum lipid levels.

Effects of Lactobacillus delbrueckii on weight of mice

Next, we investigated *Lactobacillus delbrueckii* effects on weight of the mice. At the start of the experiment, the average weight of mice was 20.46 ± 0.30 g. After four weeks of feeding mice either with a high-fat diet or an ordinary diet, there were no significant differences in body weight between the CON and LAC groups ($P>0.05$). However, after 10 weeks, mice in the MOD group became significantly heavier than mice in the other three groups (CON, LAC, and MOD+LAC) ($P<0.05$). Nevertheless, differences between the MOD+LAC group and the CON or LAC groups were not statistically significant ($P>0.05$, **Figure 1**). These results demonstrate that increased body weight induced by a high-fat diet could be attenuated with *Lactobacillus delbrueckii*.

Effects of Lactobacillus delbrueckii on bacteria in mice intestines

Intestinal microorganisms are connected to blood lipids and diet-induced obesity. Thus, we asked whether *Lactobacillus delbrueckii* could affect microorganisms in mice intestines. *Lactic acid bacteria*, *bifidobacterium*, *enterococcus*, and *enterobacter* in mouse intestines were relatively constant in the CON group. As shown in **Figure 2**, *enterobacter* tended to slightly increase with age, while the other three bacteria exhibited a slight decrease. Furthermore,

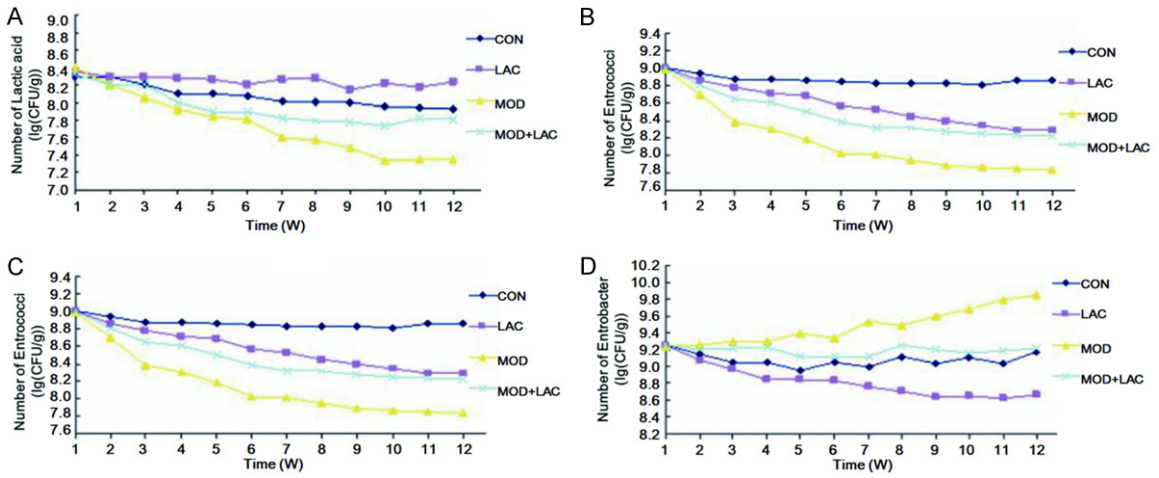

Figure 2. Effect of *Lactobacillus* on gut microbiota in various diet groups. A: Intestinal lactic acid bacteria counts; B: Intestinal *Bifidobacterium* counts; C: *Enterococcus* counts; D: *Enterobacter* counts.

enterococci and *enterobacter* significantly decreased in the LAC group, compared with the CON group, while lactic acid bacteria and *bifidobacterium* increased. In the MOD group, lactic acid, *Bifidobacterium*, and *enterococci* significantly decreased over time, while *enterobacter* increased. Additionally, there were significant differences in the four bacteria between groups receiving *Lactobacillus* gavage (LAC and MOD+LAC) and groups that did not receive *Lactobacillus* gavage (CON *vs.* LAC and MOD *vs*. MOD+LAC, both $P<0.01$). Lactic acid bacteria and *Bifidobacterium* increased in MOD+LAC and LAC groups after 4 weeks, compared with the MOD and CON groups, while *enterobacter* decreased. For *enterococcus*, it was increased in the MOD+LAC groups, compared with the MOD group, but less in the LAC group than the CON group. These results suggest that *Lactobacillus delbrueckii* could affect other intestinal microorganisms, playing a role in disease.

Discussion

The incidence of disorders of lipid metabolism including hyperlipidemia, obesity, and fatty liver has been increasing in recent years [14]. Most patients diagnosed with diseases related to lipid-related metabolic disorders also suffer from symptoms of indigestion, such as abdominal distension and diarrhea. Additionally, the majority of patients with functional indigestion experience lipid metabolism-related diseases like hyperlipidemia and fatty liver. Microorganisms normally presented in the intestines are termed gut microbiota. They have significant differences among different individuals. It has been confirmed that gut microbiota are directly related to human health, as intestinal microorganisms can induce various diseases by affecting signal channels of their host bodies. A large body of evidence has indicated that normal intestinal microbiomes are able to induce dietled obesity [15]. The connection between intestinal microorganisms and blood lipids in a large population has been identified and decline of microorganism diversity in the gut is a new endangering factor of heart disease [16-19].

Some gut microorganisms can transform cholesterol into steroids, facilitating metabolism of serum cholesterol and triglycerides in order to reduce blood lipids. The two major probiotics in the human gut, lactic acid bacteria and bifidobacteria, positively influence intestines, and consequently, its host health. Numerous experiments and studies have proven that both lactic acid bacteria and bifidobacteria are able to metabolize and produce a series of active substances, boosting nutrition absorption as well as decreasing serum cholesterol [20-22]. This study established a hyperlipidemic mouse model by providing mice with a high-fat diet. The results of our present study revealed that serum TC, TG, and LDL-C levels in high-fat diet groups significantly increased relative to normal diet groups ($P<0.01$), while HDL-C levels notably decreased. These findings indicate the successful establishment of our hyperlipidemic mouse model. In comparison with the high-fat groups, high-fat *Lactobacillus*-gavaged groups

had significantly reduced TC, TG, and LDL-C levels ($P<0.05$) and significantly higher HDL-C levels ($P<0.05$). Thus, a high-fat diet may influence growth of intestinal probiotics. As observed in our present study, the change may reduce the rate of cholesterol conversion, leading to an increase in blood lipids.

Gut microorganisms play an important role in lipid metabolism. A high-fat diet can alter the composition of gut microbiome. Furthermore, changes in distribution of bacteria in the gut may consequently influence lipid metabolism, thereby influencing occurrence and development of hyperlipidemia. Normal intestinal microbiota, in humans and animals, have been shown to involve *Lactobacillus*, *Bifidobacterium*, *Enterococcus,* and members of the Enterobacteriaceae family [23]. Together, they preserve the ecological balance of gut microbiota. In addition, both Enterobacteriaceae and enterococci are known to induce disease in their hosts, under specific conditions [24, 25]. In this study, *Lactobacillus* by gavage was utilized to alter gut microbiota of hyperlipidemic mice to observe the effects of these bacteria on gut microbiota of hyperlipidemic mice. There was a significant reduction in levels of lactic acid bacteria, bifidobacteria, and enterococci in the high-fat groups and an increase in the population of *enterobacter.* Moreover, differences in gut microbial composition between the CON and LAC groups was statistically significant. These results indicate that abnormalities in lipid metabolism are closely tied to alterations in gut microbial composition. Lactic acid bacteria and bifidobacteria showed a gradual increase in the high-fat group supplemented with *Lactobacillus* (MOD+LAC). This increase was statistically significant compared to that in the high-fat group (MOD), indicating that *Lactobacillus* intake improved gut microbiota of mice with abnormalities in lipid metabolism.

In conclusion, we demonstrated that supplementation with *Lactobacillus* could successfully improve distribution of microorganisms in the gut and alter the imbalance of intestinal bacteria, resulting in attenuation of serum lipid levels affected by a high-fat diet. Therefore, our findings shed light on the mechanism of anti-hyperlipidemia effects of *Lactobacillus*, serving as a basis for future research.

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Disclosure of conflict of interest

None.

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