Combined Lowering Effects of Rosuvastatin and *L. acidophilus* on Cholesterol Levels in Rat

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Introduction

The causal link between hypercholesterolemia and cardiovascular diseases (CVD) is well established [1]. Achieving lower blood lipids and blood cholesterol levels proved to be an efficient strategy in order to reduce atherosclerotic cardio vascular risk. In accordance with this strategy, statins, a group of lipid-lowering drugs, have been used routinely to treat hypercholesterolemic patients in the prevention of cardio vascular diseases [3]. Rosuvastatin is one of the most potent statins available and is effective even for patients presenting a high cardiovascular risk [4].

However, statin therapy presents many limitations. For example, only a fraction of the patients (approximately 20%) achieve the therapeutic lipid-lowering target [5–7]. In addition, high doses of statin are associated with higher risk of hepatic and muscular side effects. Additionally, patients of Asian ethnicity are more susceptible to experience side effects due to genetic variations in hepatic metabolism and drug catabolism [8, 9], especially at higher statin doses. Accordingly, those patients are usually prescribed lower doses [10]. Finally, adherence to statin therapy is generally poor [11]. Suboptimal adherence to statin therapy is a major barrier to achieving LDL-C targets [12] and statin down-titration or discontinuation occurs more frequently among patients with statin intolerance. These limitations, that cause a discrepancy between patients’ requirements and clinical practice, have led to an increased interest in non-drug therapies to improve blood cholesterol levels.

Therapeutic intervention is not the only way to normalize cholesterol levels. Similar results can be obtained through simple lifestyle changes [13], such as regular physical activity [14–17], as well as adopting a specific diet [18–22]. The use of probiotics has also been shown to improve outcomes in patients with coronary heart disease with very low risk of side effects. Probiotics are microorganisms that...
bear various health benefits for the host upon consumption such as elimination of pathogens, alleviation of lactose intolerance, decreased serum cholesterol levels, reduced risk of cancer and antihypertensive effects [23]. Many probiotic strains such as Lactobacillus (L.) acidophilus, L. plantarum, L. casei, Bifidobacterium longum, Enterococcus faecium, and Streptococcus thermophiles have been found to improve blood lipid profiles, especially in reducing total cholesterol and LDL-C levels [24]. These observations advocate for the possibility of establishing probiotics as non-drug supplements for hypercholesterolemia.

To the best of our knowledge, to date, no study has explored the efficacy of a treatment of hypercholesterolemia combining the use of rosuvastatin and probiotics. The aim of this work is to fill this gap by determining whether supplementing rosuvastatin (sp) therapy with the administration of L. acidophilus can result in improved lipid-lowering effects.

**Material and Methods**

**Bacterial Cultures**

L. acidophilus ATCC 4356 strain was chosen based on its demonstrated hypocholesterolemic effect both in vitro [25] and in vivo [26, 27]. The strain was obtained from ATCC (American Type Culture Collection, USA). The organisms were activated successively three times in sterile de Man, Rogosa, and Sharpe (MRS) broth (Hi-Media, India) before experimental use. L. acidophilus was cultured anaerobically for 24 to 36 h at 37°C until the OD value reached 3–4. L. acidophilus cells were subsequently separated from the medium through centrifugation (600 g, 10 min). The supernatant was discarded and PBS was used to carefully resuspend the cells.

**Animals, Diets, and Sample Collection**

Forty specific-pathogen-free (SPF) Sprague Dawley (SD) male rats (200 ± 20 g) were purchased from Dalian Medical University Laboratory Animal Center (application license No.: SYXK (Liao)-2013-0006). The rats were housed in stainless steel cages, in a temperature controlled room having 12 hourly light and dark cycles, at temperatures between 24–26°C and a relative humidity of 55–60%. The rats were offered food and water *ad libitum*. All animal experiments were approved by, and performed in accordance with the Institutional Animal Care and Use Committee of Dalian Medical University (Dalian, Liaoning, China).

Rats were fed with high-fat diet (HFD), containing 87.8% ordinary feed, 10% lard, 2% cholesterol, and 0.2% propylthiouracil, for 4 weeks. Upon induction of hypercholesterolemia, where serum cholesterol levels doubled in comparison to their initial serum cholesterol levels, they were then divided randomly into four groups of ten rats each: (A) the HFD control group, 2 ml/d PBS; (B) the rosuvastatin group, 2 ml/d PBS containing rosuvastatin (AstraZeneca Pharmaceutical Co., Ltd. SFDA approval No. J20120006) (10 mg/kg); (C) the L. acidophilus group, 2 ml/d PBS containing L. acidophilus at the standard concentration of 10⁸ CFU/ml [28–30]; and (D) the rosuvastatin + L. acidophilus group, 2 ml PBS containing rosuvastatin (10 mg/kg and L. acidophilus 10⁶ cfu/ml). All four groups received their respective PBS solution intragastrically, and all groups had free access to HFD and water *ad libitum* for four weeks. Body weight and amount of feed intake were recorded weekly.

**Blood and Stool Sample Collection**

After two weeks and four weeks, the drug was withdrawn. After fasting for 12 h, the blood samples were obtained from the orbital venous plexus. Serum and red blood cells (RBC) were separated by centrifugation at 5,000 rpm for 10 min at 4°C. All serum samples were then snap-frozen in liquid nitrogen prior to storage at −80°C. The serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and C-reactive protein (CRP) were detected by using commercial kits (Ningbo Meikang Biotechnology Co., Ltd., China) according to the manufacturer’s instructions. Feces were collected from each group before treatment (week 0) and two weeks after treatment and stored at −80°C until further analysis. The fecal contents were taken after four weeks (day 29) under aseptic conditions.

**Microbial DNA Extraction from Feces**

Total bacterial DNA was extracted from 250 mg of fecal sample using QIAamp DNA Stool Mini Kit (QIAGEN, UK) with modified protocol [31]. The DNA samples were resuspended in 100 ul of TE buffer and treated with 2 ul of DNase-free RNase (10 mg/ml) at 37°C for 15 min. Proteins were removed by treatment with 15 ul of proteinase K at 70°C for 10 min. DNA was subsequently purified using QIAamp Mini Spin columns (QIAGEN) following the manufacturer’s instructions. Final DNA concentration was quantified by using NanoDrop ND-1000 (NanoDrop Technologies, DE).

**Fecal Microbiota Composition**

Feces samples were freeze-dried overnight prior to DNA extraction as previously described [32]. The V3-V4 region of the 16S rRNA gene was amplified using the primers 33F (5’-ACTCCTACGGRAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGTWTCTAAT-3’). PCR products were purified using AMPure XP magnetic purification beads (Beckman Coulter, Inc., Brea, USA) and quantified using the Quant-it PicoGreen dsDNA Assay Kit (Life Technologies Japan, Ltd, Japan). 16S rRNA sequencing (Roche Applied Science, Indianapolis, Indiana) was performed using 454 GS JUNIOR according to the manufacturer’s instructions. The resulting 16S rRNA reads were analyzed using the QIIME pipeline [33]. Taxonomic assignments and estimation of relative abundance of sequencing data were performed using...

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the analysis pipeline of the QIIME software package. Operational taxonomic units (OTUs) were taxonomically classified based on comparison with the Greengenes database using the RDP-classifier [34, 35]. Unifrac distance calculations were done using the R software package (R Development Core Team, Vienna, Austria (https://www.r-project.org).

**Statistical Analysis**

Data were subjected to one-way analysis of variance (ANOVA) using the Statistical Package for the Social Science (SPSS 19.0, SPSS, Inc., USA), and the Mann-Whitney U test was used to compare the differences among various groups. Differences were considered significant when the probability was less than 0.05. All data were expressed as the mean ± standard error of means, with n = 10 being the number of samples for each group.

**Results and Discussion**

**Generation of Hypercholesterolemic Rats**

All rats appeared healthy throughout the study; the body weight gain, feed intake, and feed efficiency were stable during the normal diet period. After being fed the HFD for four weeks, the animals exhibited slightly higher body weights and significantly higher TC, LDL-C and HDL-C levels in serum (p < 0.001) showing that the HFD indeed induced hypercholesterolemia (Fig. 1).

**Lipid Profiles**

After four weeks of HFD, the rats were subjected to four weeks of therapy trial while maintaining a high fat diet. The levels of cholesterol, including TC, HDL-C, and LDL-C in the serum of all rats were measured after two weeks and four weeks of therapy (Figs. 2A and 2B). The concentration of serum TC for rats in the HFD control group (no treatment, group A) was higher than for those in all other treatment groups (p < 0.05). Rosuvastatin at a dose of 10 mg/kg (group B) decreased TC concentrations by 15.6% (after two weeks) and 12.8% (after four weeks), when compared to the HFD-control (group A) (p < 0.05). *L. acidophilus* at doses of $10^9$ CFU/ml, 2 ml/day (group C) decreased TC concentrations by 20.1% (after two weeks) and 24.8% (after four weeks), when compared to the HFD-control (group A) (p < 0.05). The combination of those 2 treatments together (group D) further decreased the TC concentration by 25.6% (after two weeks) and 31.8% (after four-weeks). The HDL-C concentrations for rats in all groups remained at similar levels, except for the group with combined treatments showing higher HDL-C levels compared to the control and rosuvastatin groups, although this difference does not

![Fig. 1](image1.png)

**Fig. 1.** Serum TC, HDL-C, and LDL-C concentrations of hypercholesterolemia-induced rats before and after four-week HFD feeding. Error bars represent standard error of means; n = 40, p < 0.05.

![Fig. 2](image2.png)

**Fig. 2.** Serum TC, HDL-C, and LDL-C concentrations of hypercholesterolemia-induced rats after two weeks (A) and four weeks (B) of treatment. Error bars represent standard error of means; n = 10 for each group, p < 0.05.
appear to be statistically significant. After two weeks of treatment, LDL-C levels of hypercholesterolemic rats administered with *L. acidophilus* at doses of $10^9$ CFU/ml, 2 ml/day (group C) and combined treatments (group D) were, however, significantly lower than that of the HFD control group by 39.7% and 46.1%, respectively ($p < 0.05$). Surprisingly, the hypocholesterolemic effect of *L. acidophilus* was found to be better than that of rosuvastatin, at least under this experimental setup.

C-Reactive Proteins (CRP) and Arterial Stiffness Index (ASI) Profiles

To assess the risk of coronary heart disease (CHD) of these hypercholesterolemic rats, we also monitored CRP and ASI levels (Fig. 3). The groups with treatments compared to the control group experienced a decrease of CRP levels ($p < 0.05$). The changes after 4 weeks of treatment in CRP levels were similar between the combination (D), *L. acidophilus* (C) and rosuvastatin (B) groups. The ASI, which is an indicator of arterial stiffness, is significantly lower for all the groups with drug and/or probiotic treatments when compared to the control group ($p < 0.01$). Furthermore the ASI for the *L. acidophilus* group was lower than for the rosuvastatin group and the ASI for the combination treatment group was even lower than for the *L. acidophilus* alone group ($p < 0.01$).

Intestinal Microbiota Composition

Fecal microbiota composition of 3 randomly selected animals in each group was analyzed by 16S rRNA sequencing of fecal samples. A stacked bar chart showing the microbiota composition for the rosuvastatin, the *L. acidophilus* alone, the combination and the control groups after the four-week therapy trial is shown in Fig. 4A. Data showed that there was an increase in *Lactobacillus*...

![Fig. 3. CRP (A) and ASI (B) levels of hypercholesterolemia-induced rats after four weeks of treatment. Error bars represent standard error of means; $n = 10$ for each group, $p < 0.05$.](image-url)

![Fig. 4. Intestinal microbiota composition of all the groups. (A) Fecal microbiome structure variation among groups A, B, C, and D. Each of the stacked bar plots illustrates the relative abundance of the fecal microbiota at the genus levels for each of the individuals. (B) Analysis of the level of genus difference between group AB and CD shown by the anova one-way method at four weeks of treatment.](image-url)
population for both the *L. acidophilus* and the combination groups. The bray method was used to calculate the difference in abundance of the genus population between the samples. Groups A and B were clustered as there was no significant difference in the species structure between these samples. The groups C and D were clustered as well as they also display similar structures.

The one-way analysis of variance (ANOVA) analysis showed that *Lactobacillus* in cluster CD is higher than that in cluster AB revealing the differences generated by the administration of *L. acidophilus* (Fig. 4B).

**Dietary Influence on Hypercholesterolemic Rats**

Previous studies have suggested that the fat and cholesterol levels in diet are highly correlated with the cholesterol concentrations in blood [36, 37]. Indeed, blood cholesterol has both an endogenous and a dietary origin through the reabsorption of biliary cholesterol in the small intestine [38]. Since we are interested in exploring the non-drug therapies complementary to rovustatins, we also conducted a feeding trial to analyze the effects of a change in diet on hypercholesterolemic rats. After 2 weeks on a normal diet, we observed that the blood levels of TC, LDL-C, and HDL-C were significantly lower. After 4 weeks, the risk of hypercholesterolemia continued to decrease as shown by a composite score reflecting the variations in TC, LDL-C, and HDL-C ($p < 0.01$) (Fig. 5). Thus, daily diet has a concurrent and clinically significant effect on the blood cholesterol levels of hypercholesterolemic rats.

A number of studies have shown that beneficial bacterial additives, by changing the composition of the intestinal microflora and increasing the intestinal population of *Lactobacillus, Bifidobacterium* can help to lower cholesterol blood level [39–41]. Xie et al. fed rats with a high cholesterol diet, *Lactobacillus acidophilus* ATCC 4356 was intragastrically administered as the lipid-lowering properties of this strain have been shown both in vitro [25] and in vivo [26, 27]. According to the volume of the fecal contents, the number of bacteria was calculated according to the rat (10 g * 10^9/d) for 4 weeks. Compared to the control group, hypercholesterolemic SD rats given *L. acidophilus* experienced a decrease in TC and LDL-C after 2 weeks of 20.1% and 39.7% respectively, indicating that the blood cholesterol was significantly reduced. Those observations are consistent with the previous reports showing that *Lactobacillus* exerts a significant role in cholesterol metabolism.

In this work, a high-cholesterol rat model was established, and *Lactobacillus acidophilus* ATCC 4356 was intragastrically administered as the lipid-lowering properties of this strain have been shown both in vitro [25] and in vivo [26, 27]. According to the volume of the fecal contents, the number of bacteria was calculated according to the rat (10 g * 10^9/d) for 4 weeks. Compared to the control group, hypercholesterolemic SD rats given *L. acidophilus* experienced a decrease in TC and LDL-C after 2 weeks of 20.1% and 39.7% respectively, indicating that the blood cholesterol was significantly reduced. Those observations are consistent with the previous reports showing that *Lactobacillus* exerts a cholesterol-lowering effect [44–46].

Illuminate Miseq PE300 16S rDNA sequencing technology (6S V3-V4) high-throughput sequencing analysis was applied to the DNA of intestinal microflora of 3 randomly selected animals in each group at the 4th week of treatment to establish a taxonomic comparison and determine the species difference. The results showed that the *Lactobacillus* in groups C and D at the genus level was significantly higher than that for the A and B groups. Plasma TC and LDL-C levels in the C and D groups were also significantly lower than those in the A and B groups, and negatively correlated with the levels of *Lactobacillus* in the intestinal tract, similar to previously reported results [47]. Other genera also showed significant differences between the two groups. The abundance of *Lactobacillus, Enterorhabdus, Bacillus, Lactococcus, Halomonas, Pseudomonas, Massilia, and...
Pelagibacterium was significantly increased in the CD group compared to the AB group. Meanwhile, the abundance of Anaerotruncus, Bacteroides, Alistipes, Papillibacter, Sutterella significantly decreased ($p \leq 0.05$). Interestingly, in addition to Lactobacillus, Bacillus also has a demonstrated effect on blood lipid differences [48].

Lactobacillus exerts a cholesterol-lowering effect through various possible mechanisms [49–54]: (a) through the reduction of the synthesis of cholesterol due to the inhibition of the enzyme HMGCR; (b) it interferes in the reduction of the synthesis of cholesterol due to the various possible mechanisms [49–54]; (c) it increases liver LDL-R expression: LDL-R can remove blood cholesterol contained in LDL particles to reduce total cholesterol levels. (d) Cell absorption and assimilation: Lactobacillus can absorb cholesterol into its own bacterium and is then discharged with the feces. (e) Organic acid salt production: is involved in the lowering of plasma TC and LDL-C levels.

The combined effects of rosuvastatin and another factor have already been observed, as Fei et al. reported a synergic protective effect on Acute Myocardial Infarction (AMI) involving rosuvastatin together with the Vascular Endothelial Growth Factor A (VEGF-A) [55]. However, to the best of our knowledge, there has been no study on the combined cholesterol-lowering efficacy of Lactobacillus and rosuvastatin. Rosuvastatin is mainly transported by the anion transporter polypeptide (OATP1B1) into hepatocytes [56]. Therefore, its lipid-regulating effect mainly relies on the presence of the transporter OATP1B1 on the cell membrane. The expression and activity of OATPs are regulated by various factors such as endocrine hormones, inflammatory factors and drugs [57]. Additionally, the modulation of OATP by pro-inflammatory cytokines is pervasive. A number of studies have shown that alterations in Bifidobacteria and Lactobacillus can cause changes in TNF-α and IL-6 levels [58–61]. High lipid levels can have severs effects on the intestinal flora, and decrease significantly the proportion of beneficial bacteria such as Lactobacillus. The decrease of Lactobacillus leads to an overexpression of the inflammatory cytokines TNF-α and IL-6, which in turn inhibits the expression of OATP1B1. The down-regulation of OATP1B1 in hepatocytes prevents the uptake of rosuvastatin which in turn enhances its lipid-lowering properties. The results of our study showed the combined cholesterol-lowering effect of the combined administration of Lactobacillus acidophilus and rosuvastatin. Indeed, the combined effect of Lactobacillus and rosuvastatin was better than that of statins and Lactobacillus alone. We assume that the combined effect is due to the lowering effect of increased Lactobacillus population on the level of inflammatory cytokines such as IL-6, therefore up-regulating the expression of OATP1B1 and promoting the uptake of statins by hepatocytes.

The cholesterol in the body is mainly derived from food and biosynthesis. Cholesterol in food is converted into cholesterol monomers in the intestine, absorbed into the lymphatic system after absorption by the small intestine, and then entered into the bloodstream from the lymphatic system in the form of chylomicrons with various apolipoproteins. In this work, TC and LDL-C levels increased significantly in SD rats by high-cholesterol intake for one month. After adjusting to dietary structure, cholesterol levels decreased significantly after 2 weeks of eating regular diets, among which TC decreased by 76.3%, $p < 0.0001$; LDL-C decreased by 80%, $p < 0.001$. It is suggested that the dietary structure has a great influence on serum cholesterol levels in SD rats. Martínez et al. studied the interactions between diet, gut microbial ecology and cholesterol in model hamsters with hypercholesterolemia and found that the grain sorghum lipid extract (GSL) and the intestinal microbiota changes are closely related, suggesting that the effect of diet on cholesterol metabolism is, to some extent, achieved through the influence of intestinal flora [62].

The results of this work revealed that L. acidophilus showed good lipid-regulating effects on hypercholesterolemic rats, either on its own or used together with rosuvastatin. Our study also raises important questions such as the role of L. acidophilus in improving arteriosclerosis, its specific mechanism of lipid-lowering effect and its long-term efficacy, tolerability and safety.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References


