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Original Article

Long-term *lactobacillus ATCC4356* supplementation prevents formation of atherosclerosis via P65-mediated reducing of inflammation and oxidative stress in apoE~(-/-) mice

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Abstract: Lipid-reducing therapies have been widely used for the prevention and treatment on cardiovascular disease. However, common lipid-reducing medications have limited effects and are always accompanied with side effects. Recently, reducing the incidence of cardiovascular disease by the administration of *lactobacillus* has become a focus but the mechanism remains unclear. In this study, apolipoprotein E-knockout (ApoE$^{-/-}$) mice fed with different concentrations of *Lactobacillus acidophilus ATCC4356* were used to investigate the effects of *ATCC4356* on the development of atherogenesis and NF-κB p65-mediated signal pathway. The results showed that *ATCC4356* had no impact on weight and blood lipids. However, *ATCC4356* effectively relieved atherosclerotic lesions, decreased serum levels of oxidative stress factors (ox-LDL, MDA, SOD) and inflammatory cytokines (TNF-α, IL-10) in ApoE$^{-/-}$ mice after being fed with high fat diet. These results suggest that the inhibitory effect of *ATCC4356* on the atherosclerotic formation was not dependent on the reduction of cholesterol level but associated with the decreasing inflammatory reactions and oxidative stress effects via suppressing p65-mediated signaling pathway.

Keywords: Atherosclerosis, oxidative stress, hypercholesterolemia, inflammatory cytokines

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity in the modern society and largely caused by complications of clinical of atherosclerosis [1]. Chronic hyperglycemia and reactive oxygen species (ROS) are major risk factors for the development of various cardiovascular diseases, particularly atherosclerosis (AS) [2]. We know that the formation of atherosclerosis is a pathologic process including multiple inflammatory reactions. Microbial infection is a causative factor of AS and a variety of inflammatory cytokines are involved in the formation of the disease.

Concerning the side effects and limited effect of lipid-reducing medications, novel therapies are urgently needed in clinics to manage AS. One of the promising managements is to reduce cholesterol level in patients. Lacking atherogenic lipoproteins in enterocyte cells can cause atheroprotective effects for reduction of intestinal absorption of cholesterol. Relieving hypercholesterolemia is helpful to decrease clinical risk of atherosclerosis [1, 3]. *Lactobacillus acidophilus ATCC4356* is one of the important microorganisms in the intestines and virginal mucosa of healthy humans [4]. Lactic acid bacteria are considered to be beneficial microorganisms and associated with multiple potential health effects for humans. Additionally, the cholesterol-reducing effect of *Lacto acid bacillus*, such as *Lactobacillus acidophilus ATCC-4356,* has been recently confirmed the potential utility in the prevention of atherosclerosis [5]. Ying Huang et al. reported *Lactobacillus acidophilus ATCC4356* could effectively prevent atherosclerosis via inhibition of intestinal cholesterol absorption in apolipoprotein ApoE-knockout (ApoE$^{-/-}$) mice. Uchida M. et al. reported that the polysaccharides, extracted from *Lactobacillus kefiranofaciens*, could significantly inhibit aortic atherosclerotic plaque lesions

of male New Zealand white rabbits fed with high cholesterol diet. Mann et al. found that the habit of drinking yogurt fermented by *Lactobacillus acidophilus* significantly weaken the risk of hypercholesterolemia development, indicating that *Lactobacillus* had potential lipid-regulating function [5].

However, the cholesterol-reducing effect of *Lactobacillus* on atherosclerosis and its mechanism remains unclear. Only a few studies have focused on the effect of *Lactobacillus* intervention on the development of atherosclerosis in animal models. Apart from high level of cholesterol causes AS, ROS and associated oxidation products causes endothelial cells damage. Various pro-inflammatory cytokines are released during this process, which further initiate AS. In the present study we selected ApoE$^{-/-}$ mice to establish atherosclerosis model. ApoE$^{-/-}$ mice were fed with high-fat and high-cholesterol to accelerate the formation of AS model. To investigate the effects of *Lactobacillus* on arterial plaque formation and to explore the effects of *Lactobacillus* on AS and inflammatory cytokines, the intervention was taken to ApoE$^{-/-}$ mice subjected to different concentrations of *Lactobacillus acidophilus* ATCC 4356.

Materials and methods

Animals, diets and treatments

36 eight-week-old male ApoE$^{-/-}$ mice and 12 wild-type (widetype, WT) C57BL/6J mice were purchased from Xinxiang medicine school, weighted (22±2) g. High-fat and high-cholesterol diet was made by adding 15% lard and 0.25% cholesterol into normal mouse diet. ApoE$^{-/-}$ mice were randomly divided into 3 groups as the following: the model control group (Vehicle): ApoE$^{-/-}$ mice were fed daily with high-fat and high-cholesterol diet along with saline (normal saline, NS) by intragastric gavage; low-dose group of Lactobacillus acidophilus (LA. L): ApoE$^{-/-}$ mice were fed daily with high-fat and high-cholesterol diet and 10^8 CFU/mL Lactobacillus intragastrically; high-dose group of Lactobacillus acidophilus (LA. H): ApoE$^{-/-}$ mice were subjected to high-fat high-cholesterol diet daily and 10^9 CFU/mL Lactobacillus by intragastric gavage; normal control (WT) group: C57BL/6J mice were treated with daily normal diet and NS intragastrically. Each group of mice was given a daily volume of 0.5 mL via intragastric gavage for 12 weeks continuously.

Analysis of plasma lipoprotein and inflammation

Blood samples were obtained from fasting mice through retro-orbital. Blood samples were left at room temperature for 30 min, and then centrifuged to harvest serum. Total serum cholesterol (TC), triglycerides (TG), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), glucose, ALT, AST, TBIL and DBIL were analyzed using enzyme-based colorimetric assay (Hitachi 7170 automatic biochemical analyzer). The levels of interleukin (IL)-10, C-reactive protein (CRP), and tumor necrosis factor (TNF)-α were assayed using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction.

Atherosclerotic lesion analysis

Heart, liver and aortic arch were dissected after blood collection. Tissues were frozen with O.C.T. The lesions were visualized by staining with oil red O followed by a hematoxylin & esion counter-stain the cross-sections. The cross-section atherosclerotic lesions area and aortic lumens were determined by computer-assisted morphometry. Atherosclerotic lesions were normalized to aortic lumen area.

Detection of NF-κB and IκB-α protein expression of the aorta by western blotting

Total protein of frozen aorta was extracted by homogenizing in RIPA lysis buffer. Nucleoprotein and cytoplasm protein were extracted by Nc-nucleus protein and plasma protein extraction kit according to the manufacturer's instruction. Then 50 mg of total protein was loaded onto 10% SDS-PAGE, and western blot analysis was performed with antibody against Iκ-B (1:500), NF-κB P65 (1:200), histone (1:500), tubulin (1:500) or GAPDH (1:6000). Specific signals of Iκ-B, NF-κB and histone were quantified on an imaging system and normalized to internal control tubulin or GAPDH.

Statistical analysis

Statistics analysis was performed using the SPSS19.0 software. Data are presented as means ± standard deviation (SD). Differences

Table 1. Body weight, organ weight and lipid etc. al for mice after 12 weeks in each group

Variables	WT	ApoE$^{-/-}$ vehicle	ApoE$^{-/-}$ -La. L	ApoE$^{-/-}$ -La. H
Weight	135. 5±1.5%	154.4±4.7*	146.23±3.1*	151.59±6.87*
Liver index (%)	39.34±0.35	42.93±3.13*	4.43±1.15	42.04±0.89
Spleen index (%)	2.75±0.11	5.23±1.58**	4.43±1.13**	4.21±0.95**,#
HDL-C (mM)	1.68±0.20	13.42±3.36**	13.52±3.48**	15.18±3.76**
LDL-C (mM)	0.35±0.08	10.92±1.89**	11.16±2.36**	11.03±2.23**
TC (mM)	1.74±0.13	24.68±6.32**	27.32±7.73**	28.72±5.52**
TG (mM)	0.58±0.04	0.61±0.20	0.67±0.20	0.70±0.23
Glucose (mM)	7.23±3.00	9.93±3.12*	9.72±4.33	8.73±2.56
ALT (U/L)	41.09±9.47	93.92±43.21	76.81±19.23	69.12±24.12
AST (U/L)	198.34±67.20	218.52±32.89	223.78±38.23	170.49±29.28
TBIL (uM)	2.77±0.28	3.01±1.34	2.70±0.79	2.42±1.20
DBIL (uM)	1.13±0.33	1.09±0.50	0.97±0.49	0.96±0.52

*P<0.05, **P<0.01 vs. WT; #P<0.05 vs. Vehicle. N=12.

between groups were analyzed using the one-way ANOVA, and multiple comparisons were analyzed by the LSD method. *P*-value <0.05 was considered statistically significant.

Results

Effects of ATCC 4356 on hematology indexes in ApoE$^{-/-}$ mice

Firstly, we defined the basic hemodynamic measurement on all mice. The weight of ApoE$^{-/-}$ mice is higher (P<0.05) compared to WT group (**Table 1**). ApoE$^{-/-}$ mice in vehicle group exhibited a higher liver (P<0.05) and spleen index (P<0.01). However, feeding with *Lactobacillus acidophilus ATCC4356*, the spleen index decreased significantly in La. H group compared to vehicle group (P<0.05) (**Table 1**). We also found that high-fat diet (HFD) significantly increased serum TC, HDL-C and LDL-C levels (P<0.01) while TG, TBIL, DBIL and ALT, AST remained the same (**Table 1**). In La. L group and La. H group, HDL-C level had a tendency to rise, and blood glucose, ALT, DBIL and TBIL had a trend to decrease. However, it was not statistically significant (**Table 1**).

Lactobacillus acidophilus ATCC 4356 reduces the incidence of atherosclerotic lesion in ApoE$^{-/-}$ mice

Next we evaluated the atherosclerotic lesions area. We calculated the proportion of atherosclerotic lesions area in aortic intima by imaging the section of aortic arch and thoracic aorta. After 12 weeks' treatment, atherosclerotic lesions in aortic arch and thoracic aorta were rarely observed in WT group while atherosclerotic plaques were observed clearly in vehicle group. In *lactobacillus* treated group, atherosclerotic lesions area on aortic arch intima decreased to (17.14±2.92)% in La. L group and (6.82±1.88)% in La. H group (P<0.05) (**Figure 1A**).

In the aorta thoracic segments, similar trend was observed. In vehicle group, the atherosclerotic lesions area was (12.11±3.60)%, while it was decreased to (9.28±1.34)% in La. L group and (4.40±0.27)% (P<0.05) in La. H group (**Figure 1B**). The results suggested that *lactobacillus acidophilus* ATCC 4356 treatment in ApoE$^{-/-}$ mice decreased the atherosclerotic lesions area, which indicated the treatment prevented the formation of atherosclerotic lesion.

In WT group, a morphologically intact endothelium covering the cell-free subendothelial space could be found, and there was no plague formation. In vehicle group, plagues and foam cells accumulated, and extracellular lipid pools deposited and intima atrophy were observed (**Figure 1C**). In addition, the structure of blood vessel was unclear, and part of intima irregularly thickened. Besides atheroma was developed and large amount of foam cells were presented. Compared to the vehicle group, the plague amount, the intima thickness, the foam cells number and the inflammatory cells infiltration of the group treated with *lactobacillus* was down regulated.

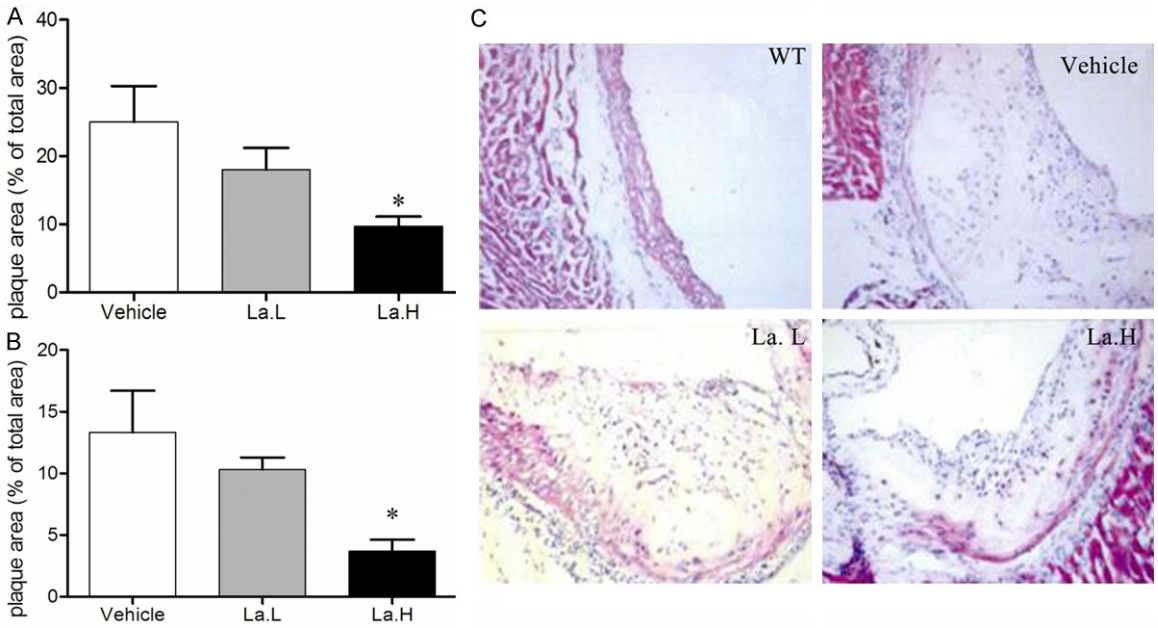

Figure 1. Lactobacillus acidophilus ATCC 4356 reduces the incidence of atherosclerotic lesion in ApoE$^{-/-}$ mice. A. Atherosclerosis lesion area of aortic arch segment and thoracic intimal surface. B. Atherosclerosis lesion area of thoracic aortic intimal surface. C. Frozen sections of aortic root in different groups stained by oil red O method. WT, wide type C57BJ/L mice treated with normal diets for 12 weeks. Vehicle group, ApoE$^{-/-}$ mice were given normal saline by gavage with high-fat diets for 12 weeks to develop atherosclerosis. La. L group, low dose (each mouse 5×10^7 CFU/d) of ATCC 4356 were given to atherosclerosis mice model by gavage. La. H group, high dose (each mouse 5×10^7 CFU/d) of ATCC 4356 were given to atherosclerosis mice model by gavage. Data are presented as mean ± standard deviation (SD), *P<0.05 vs. WT, #P<0.05 vs. vehicle, n=12/group.

Effects of lactobacillus acidophilus ATCC4356 on inflammatory cytokines and ROS level

As described above that feeding the mice with *Lactobacillus acidophilus ATCC4356* reduces the incidence of atherosclerotic lesion. Based on these findings, we then determined the effects of *Lactobacillus acidophilus ATCC4356* on the inflammatory cytokines and the ROS levels in ApoE$^{-/-}$ mice. We selected glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) to assess the oxidative stress. These molecules are involved in various steps of ROS processes [REFS]. In the WT group treated with normal diets, the ox-LDL level was 1553.98±204.87 nmol/L. But after fed with HFD, the ox-LDL level was 2964.78±577.83 nmol/L, which was significantly increased compared to the WT group (P<0.05) (**Figure 2A**). *Lactobacillus* treatment decreased ox-LDL level to 1648.07±361.50 nmol/L (P<0.05) (**Figure 2A**). No significance was observed in mouse serum GSH levels (**Figure 2B**).

Compared with the mice of WT group fed with ordinary diet, the serum MDA level in ApoE$^{-/-}$ mice of vehicle group fed with high-fat high-cholesterol diet increased significantly (P< 0.05). After the oral administration of *L. acidophilus* strain ATCC4356, the MDA level of the mice decreased, and the MDA level of La. H group was significantly different from that of the vehicle group (P<0.01), as shown in **Figure 2C**. However, the trend of serum SOD activity was the opposite. Compared with WT group, the SOD activity of ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet declined considerably by 66.03% (vehicle group, P<0.01). The SOD activity increased after oral administration of *L. acidophilus* ATCC4356, by 31.13% in La. H group as compared with the vehicle group (P<0.05) (**Figure 2D**).

The serum level of IL-10 in ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet was significantly lower than that of the WT group (P<0.05). Although the oral administration of *L. acidophilus* strain ATCC4356 tend to be increased the serum level of IL-10 in ApoE$^{-/-}$ mice, the difference was not significant (**Figure 2F**). Compared with WT group, the TNF-α level in the vehicle

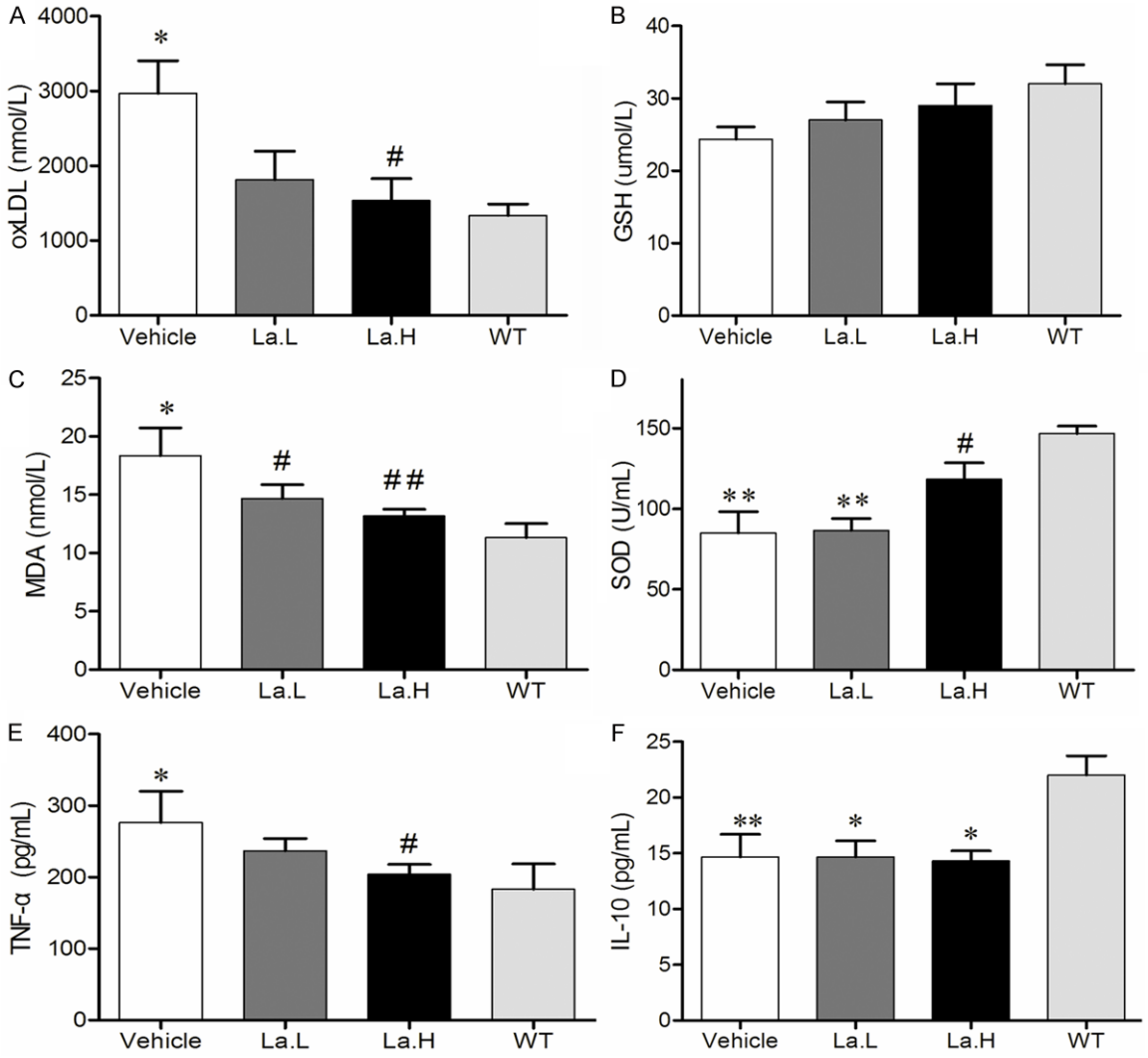

Figure 2. Effect of *ATCC4356* on the serum level of ox-LDL, GSH, MDA, SOD, IL-10 and TNF-α of ApoE$^{-/-}$ mice. A. Effect of ATCC 4356 on ox-LDL. B. Effect of ATCC 4356 on GSH. C. MDA. D. Effect of ATCC4356 on SOD. E. Effect of ATCC 4356 on TNF-α. F. Effect of ATCC4356 on IL-10. Data are presented as mean ± standard deviation (SD), *P<0.05 versus WT; **P<0.01 versus WT; #P<0.05 versus vehicle, n=12.

group increased considerably (P<0.05) (**Figure 2E**). The serum TNF-α level in ApoE$^{-/-}$ mice declined after oral administration of *L. acidophilus* strain ATCC4356, compared with the vehicle group (P<0.05) (**Figure 2E**).

Effects of L. acidophilus strain ATCC 4356 on NF-κB signaling pathway in atherosclerosis-susceptible regions of the aorta of ApoE$^{-/-}$ mice

In this study, we also determined the expression profiles of NF-κB p65 and IκB-α protein in the atherosclerosis-susceptible regions. IκB-α inhibits NF-κB by blocking nuclear localization signaling [REF]. IκB-α expression in ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet was decreased compared with the mice in WT group (**Figure 3A** and **3B**). After oral administration of *L. acidophilus* strain ATCC 4356, there was an upregulation of IκB-α in the aortacompared with vehicle group (P<0.01, **Figure 3A** and **3B**). NF-κB P65 was mainly found in the cytoplasm in WT group and very rare in the nuclei (**Figure 3C** and **3D**). The NF-κB p65 nuclear translocation was enhanced in ApoE$^{-/-}$ mice that fed with high-fat high-cholesterol diet. In the vehicle group, the NF-κB p65 expression was greatly up-regulated in the nuclei (**Figure 3E** and **3F**). These results showed that oral administration of *L. acidophilus* strain ATCC 4356 inhibited

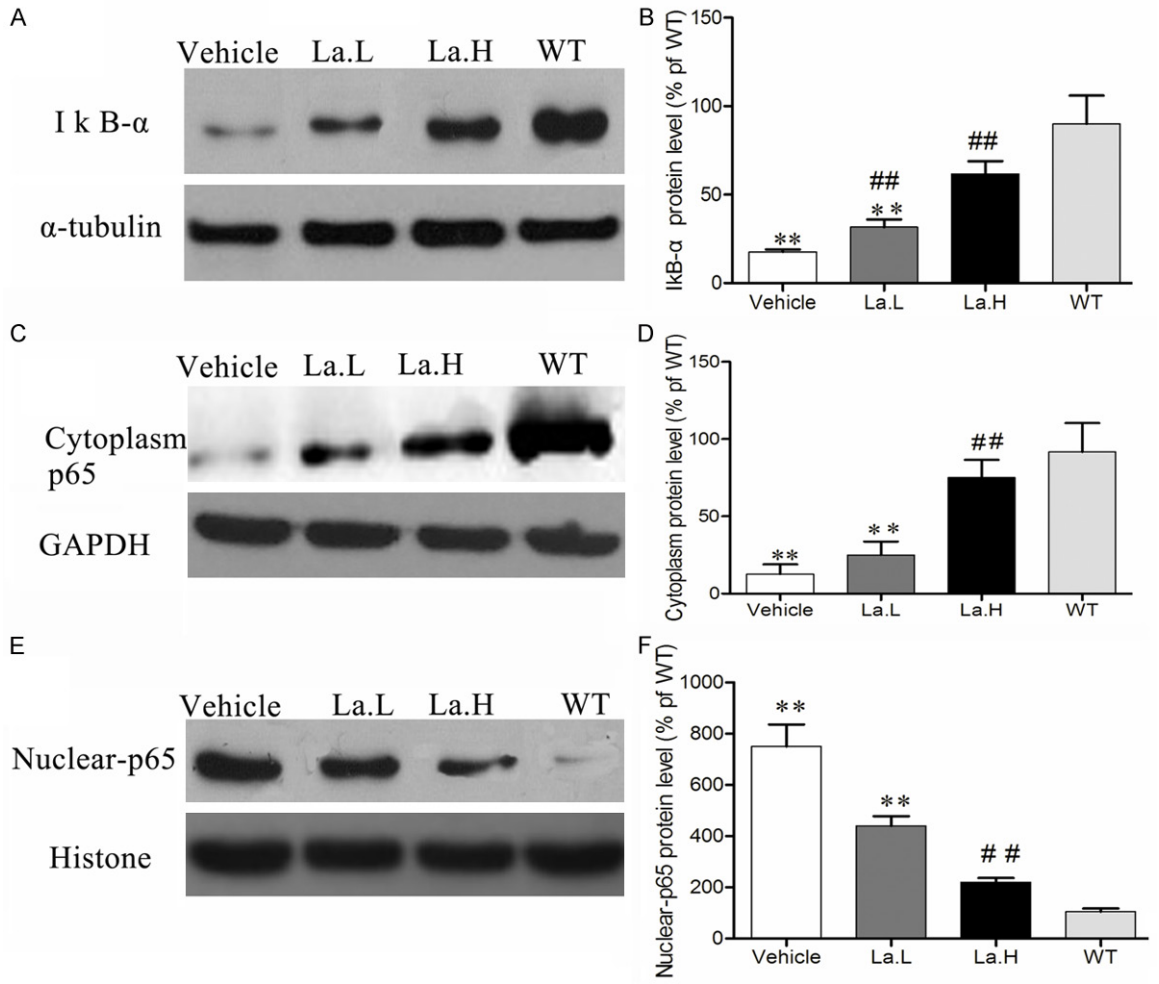

Figure 3. The expressions of IκB-α and NF-κB P65 in the aorta of each group detected by Western Blot. A-E. Western Blot images of total IκB-α, cytoplasmic NF-κB P65 and nuclear NF-κB p65, respectively; B-F. The band density of each protein, respectively. *P<0.05, **P<0.01 versus WT; #P<0.05, ##P<0.01 versus Vehicle.

NF-κB p65 nuclear translocation (**Figure 3C** and **3D**).

Discussion

The cholesterol reduction ability of Lactobacillus is now widely recognized and researched, the mechanisms involved are remained to be further investigated. We found that after gastric lavage with bacterial liquid of *L. acidophilus* strain ATCC4356 at the dose of 5×10^7 CFU/day and 5×10^8 CFU/day in ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet for 12 weeks, the serum levels of LDL-C, TC and HDL did not change significantly compared with the ApoE$^{-/-}$ mice receiving normal saline. This result indicates that *L. acidophilus ATCC4356* administration does not impact on serum cholesterol level of ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet. Moreover, the body weight and liver index of ApoE$^{-/-}$ mice receiving gastric lavage with *L. acidophilus* strain *ATCC4356* were not significantly different from that of mice receiving gastric lavage with normal saline.

Yoo Heon et al. performed gastric lavage with *L. acidophilus* strain ATCC 4356 in pigs fed with high-fat high-cholesterol diet [6]. They found neither body weight nor food intake amount was changed compared with the pigs not administered with probiotics. Hatakka K et al. performed 4-week randomized double-blinded 2-period trial for 38 healthy male subjects with normal average cholesterol levels [7, 8]. For the experimental group, 2 tablets of *P. Freudenr-*

eichii (JS) were administered daily and the blood lipid level was measured regularly. Throughout the entire course of the experiment, the levels of TC, HDL-C, LDL-C and TG did not change significantly in the experimental group. This result is consistent with our findings, but the effects of Lactobacillus on body weight, cholesterol level and the relevant mechanism should be further studied.

There are more studies about the effect of *Lactobacillus* on blood lipid rather than the effect of *Lactobacillus* on atherosclerosis. Tang et al. found that *Lactobacillus casei* produced by Yakult had an inhibitory effect on atherosclerosis. The present study found no significant effect of *L. acidophilus ATCC4356* on blood lipid and body weight of mice, but the atherosclerotic plaque formation was alleviated [9]. Typical atherosclerosis was induced in ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet for 12 weeks. It was supposed that the inhibitory effect of *L. acidophilus ATCC4356* on atherosclerotic plaque formation may be not due to the reducing cholesterol effect.

Ox-LDL is the product of LDL oxidation and one of the main reasons of endothelial cell damage. It can also induce the expression of proinflammatory cytokines by endothelial cells. Steinberg's theory of oxidation showed that the production of reactive oxygen species and ox-LDL oxidative stress played a crucial role in atherosclerosis [10]. Ox-LDL can inhibit the activity of iNOS and NO production, which leads to vasomotor dysfunction, facilitates the proliferation and migration of endothelial cells and smooth muscle cells, and promotes atherosclerosis [2, 11, 12]. Therefore, clearing and reducing ox-LDL is an important management to treat atherosclerosis. In our study, the serum level of ox-LDL increased significantly in ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet, and it decreased significantly after oral administration of *L. acidophilus* strain ATCC4356. TiiuKullisaar et al. found that compared with the 5 subjects who drank fresh goat milk, the serum oxLDL levels of 16 healthy subjects who drank fermented goat milk with *L. fermentum* ME-3, *L .buchneri* S-15 and *L. plantarum* LB-4 were lower. They also discovered that the total antioxidant activity of the subjects drank fermented goat milk was higher than the control subjects [13, 14]. These findings suggest that L. acidophilus ATCC4356 delays the formation and progress of atherosclerosis.

In this study, we found that the serum SOD activity decreased, while serum MDA level increased in ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet as compared with WT group. After oral administration of *L. acidophilus ATCC4356*, the serum SOD activity was increased, and the MDA level was declined. These results demonstrated the strong antioxidative ability of *L. acidophilus* strain ATCC-4356 which reduced the serum level of ox-LDL. Many reports have discussed about the antioxidative ability of *Lactobacillus*. T. Virtanen et al. determined the free radical clearing ability of *Lactobacillus* by fading spectrophotometry and the inhibitory effect on lipid peroxidation using liposome model by fluorescent staining [15]. They assessed the antioxidant ability of 25 *Lactobacillus* strains and found that *L. acidophilus ATCC4356* had the highest antioxidant ability.

TNF-α and IL-10 are multi-effect cytokines, which act as potential pro-inflammatory cytokines in atherosclerosis and other metabolic disorders [16, 17]. TNF-α can be found in atherosclerotic plaques and higher level of TNF-α in circulation can increase the incidence of heart attack and atherosclerotics well as triglyceride and glucose metabolism disorders [18, 19]. Caligiuri et al. reported the level of LDL and leness of plaques increased in ApoE/IL-10 double-knockout mice [20]. However, atherosclerosis was relieved after transfection with IL-10 cDNA in ApoE$^{-/-}$ mice. We found that the serum TNF-α level in ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet increased significantly while the IL-10 level decreased. Oral administration of *L. acidophilus* strain ATCC4356 reduced TNF-α level in the La. H group and Vehicle group ($P<0.05$); the serum IL-10 level showed an increasing trend. NF-κB is important in regulating the expressions of genes related to immune and inflammatory responses. The p65 subunit is responsible for the strong transcription activating potential of NF-κB, and the activation of NF-κB can be reflected by detecting p65 expression. We found that the NF-κB was activated in the aorta of ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet. In atherosclerosis model group, IκB-α was degraded significantly, and nuclear p65 expression was

upregulated, indicating p65 nuclear translocation. The administration of *L. acidophilus* strain *ATCC4356* can inhibit the degradation of IκB-α, reduce p65 nuclear translocation and achieve anti-inflammatory and antioxidant effect [21].

In conclusion, *L. acidophilus* strain ATCC4356 had no impact on the body weight and blood lipid of ApoE$^{-/-}$ mice fed with high-fat high-cholesterol diet, though it alleviated atherosclerosis. It is suggested that the inhibitory effect of *L. acidophilus* strain ATCC4356 on atherosclerosis may not be exerted by reducing cholesterol level. Instead, the oxidative stress and the expression of inflammatory cytokines are inhibited by *L. acidophilus* strain ATCC4356 through suppressing the activation of NF-κB signaling pathway.

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Disclosure of conflict of interest

None.

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