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Lactobacillus acidophilus ATCC 4356 Prevents Atherosclerosis via Inhibition of Intestinal Cholesterol Absorption in Apolipoprotein E-Knockout Mice

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The objective of this study was to investigate the effect of *Lactobacillus acidophilus* ATCC 4356 on the development of atherosclerosis in apolipoprotein E-knockout (ApoE$^{-/-}$) mice. Eight-week-old ApoE$^{-/-}$ mice were fed a Western diet with or without *L. acidophilus* ATCC 4356 daily for 16 weeks. *L. acidophilus* ATCC 4356 protected ApoE$^{-/-}$ mice from atherosclerosis by reducing their plasma cholesterol levels from 923 ± 44 to 581 ± 18 mg/dl, likely via a marked decrease in cholesterol absorption caused by modulation of Niemann-Pick C1-like 1 (NPC1L1). In addition, suppression of cholesterol absorption induced reverse cholesterol transport (RCT) in macrophages through the peroxisome proliferator-activated receptor/liver X receptor (PPAR/LXR) pathway. Fecal lactobacillus and bifidobacterium counts were significantly ($P < 0.05$) higher in the *L. acidophilus* ATCC 4356 treatment groups than in the control groups. Furthermore, *L. acidophilus* ATCC 4356 was detected in the rat small intestine, colon, and feces during the feeding trial. The bacterial levels remained high even after the administration of lactic acid bacteria had been stopped for 2 weeks. These results suggest that administration of *L. acidophilus* ATCC 4356 can protect against atherosclerosis through the inhibition of intestinal cholesterol absorption. Therefore, *L. acidophilus* ATCC 4356 may be a potential therapeutic material for preventing the progression of atherosclerosis.

Cardiovascular disease (CVD) is the leading cause of death in Western society and is caused primarily by complications of atherosclerosis (1, 2). Hypercholesterolemia is a major clinical risk factor for the development of atherosclerosis (3). Intestinal absorption of cholesterol represents an important process in cholesterol homeostasis. Studies using several animal models have demonstrated that a reduction in atherogenic lipoproteins via the inhibition of intestinal cholesterol absorption is associated with a clear atheroprotective effect (4, 5). The molecular mechanism of intestinal cholesterol absorption has recently been elucidated (6). Niemann-Pick C1-like 1 (NPC1L1) was recently found to be required for cholesterol absorption (6). Mice lacking NPC1L1 exhibit a substantial reduction in cholesterol absorption (6) and are completely resistant to diet-induced hypercholesterolemia (7, 8) and to atherosclerosis caused by apolipoprotein E (ApoE) deficiency (9).

Lactic acid bacteria (LAB) such as *Lactobacillus acidophilus* are important microorganisms in the intestines of healthy humans (10). LAB are considered to be beneficial microorganisms and have been associated with multiple potential health effects in both humans and animals (11–14). Among these effects, the cholesterol-lowering effect of LAB has recently gained interest due to its potential utility in the prevention of atherosclerosis. Only a few studies have investigated the effect of lactobacilli intervention on the development of atherosclerosis in animal models. Portugal et al. (15) investigated whether *Lactobacillus delbrueckii* reduces atherosclerosis by colonizing conventionally raised ApoE-knockout (ApoE$^{-/-}$) mice, but they observed no significant effects on atherosclerotic lesion size, which may be explained by the fact that the bacterium did not alter the blood cholesterol levels.

Previous research has found that decreased cholesterol absorption rates are associated with decreases in plasma cholesterol levels and in atherosclerosis formation (9, 16). In our previous study, we showed that the hypocholesterolemic effects of *L. acidophilus* ATCC 4356 in rats are mediated by the downregulation of NPC1L1 (17). In the present study, we investigated whether *L. acidophilus* ATCC 4356 affects atherosclerosis development in ApoE$^{-/-}$ mice by inhibiting cholesterol absorption. Our findings indicate that *L. acidophilus* ATCC 4356 reduces atherosclerosis by decreasing intestinal cholesterol absorption, improving lipoprotein composition, and stimulating macrophage reverse cholesterol transport (RCT) through the peroxisome proliferator-activated receptor/liver X receptor (PPAR/LXR) pathway.

MATERIALS AND METHODS

Source and maintenance of bacterial cultures. *Lactobacillus acidophilus* ATCC 4356 was obtained from the ATCC (Manassas, VA, USA). The stock culture was stored in 40% (vol/vol) glycerol at −80°C. The organism was subcultured three times before use in sterile de Man, Rogosa, and Sharpe broth (Difco, Detroit, MI, USA) with a 1% inoculum and was allowed to grow for 16 h at 37°C. The inoculum was stored at 4°C between transfers.

Animals, diets, and treatments. Male ApoE$^{-/-}$ mice aged 8 weeks and weighing 20 to 25 g were purchased from the Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, People's Republic of China). The animals were randomly selected and assigned to 3 groups. The 3 groups were assigned diets according to the following regimen: (i) the control group, with a Western-type diet (42% of total calories from fat; 0.15% cholesterol); (ii) the L.4356 group, with a Western-type diet plus *L. acidophilus* ATCC 4356; and (iii) the L.4962 group, with a Western-type diet plus *L.*

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TABLE 1 Mouse primer sequences for real-time PCR

Gene product	Primer sequence (5′→3′)	
	Forward	Reverse
ABCA1	CGTTTCCGGGAAGTGTCCTA	CTAGAGATGACAAGGAGGATGGA
ABCG5	CCTGCAGAGCGACGTTTTTC	GCATCGCTGTGTATCGCAAC
ABCG8	GAGCTGCCCGGGATGATA	CGGAAGTCATTGGAAATCTG
HMG-CoA-S	GCCGTGAACTGGGTCGAA	GCATATAGCAATGTCCTGCAA
LXRα	GGATAGGGTTGGAGTCAGCA	GGAGCGCCTGTTACACTGTT
PPARα	CGTACGGCAATGGCTTTATC	AACGGCTTCCTCAGGTTCTT
PPARδ	GCCTCGGGCTTCCACTAC	AGATCCGATCGCACTTCTCA
SR-BI	CACTACGCGCAGTATGTGCT	TGAATGGCCTCCTTATCCTG
NPC1L1	TTGCCTTGACCTCTGGCTTAG	AGGGCGGATGAATCTGTGC
β-Actin	GTGGGCCGGTCTAGGCACCAA	CGGTTGCCTTAGGGTTCAGG

acidophilus ATCC 4962. All mice were housed individually in metal cages under controlled temperature (21 ± 2°C) and humidity (55% ± 5%) and maintained on a 12-h light-dark cycle. Each day during the 16-week study period, the L.4356 group received 0.2 ml (10^9 CFU) of *L. acidophilus* ATCC 4356 intragastrically. The control group received an equivalent amount of normal saline. We monitored the general condition, dietary intake, body weight, and liver weight of the mice and found no significant differences in any of these parameters between the L.4356 and control groups. All animals were housed according to guidelines of the Institutional Animal Care and Use Committee of the University of Jilin. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Analysis of atherosclerotic lesions in the aorta and aortic sinus. Eight-week-old male mice were treated with *L. acidophilus* ATCC 4356 plus a Western diet (L.4356 group) ($n = 10$) or with a Western diet only as a control ($n = 10$). After 16 weeks of treatment, the mice were sacrificed, and atherosclerotic lesions of the aortic sinus were prepared for immunohistochemistry as described below. The whole aorta or 6-μm-thick frozen sections of the aortic sinus were obtained from ApoE$^{-/-}$ mice and were stained with Oil Red O, as previously described (18). The lesion size was measured on digital microphotographs of the aortic sinus by measuring the stained surface area using ImageJ software (NIH, Bethesda, MD).

Serum lipids and lipoproteins. After the feeding period, ApoE$^{-/-}$ mice ($n = 10$/group) were fasted for 4 h and bled from the retro-orbital plexus for serum lipid analyses. Serum total cholesterol was determined with a colorimetric kit (Diagnostic Chemicals Ltd., Oxford, CT). Serum lipoproteins were separated by fast protein liquid chromatography (FPLC) gel filtration (Amersham Pharmacia Biotech AB, Piscataway, NJ) on two Superose 6 columns as described previously (19).

Intestinal absorption of cholesterol. Eight-week-old male mice were treated with *L. acidophilus* ATCC 4356 plus a Western diet (L.4356 group) ($n = 10$) or with a Western diet only as a control ($n = 10$). Cholesterol absorption studies were performed in male mice using the fecal dual-isotope ratio method (20, 21). Briefly, at the end of the experiment, the control and L.4356 group mice were intragastrically gavaged with 0.5 μCi of [^{14}C]cholesterol (Perkin-Elmer, Waltham, MA) and 1 μCi [^{3}H]sitostanol (American Radiolabeled Chemicals, St. Louis, MO) in 100 μl of soybean oil. Mice were then transferred to individual metabolic cages and kept on either the Western diet or the Western diet plus *L. acidophilus* ATCC 4356 for the next 3 days, and their feces were collected daily. The radioactive isotopes from 3 days of pooled fecal samples were dissolved in Soluene 350 (Perkin-Elmer) and counted. The percentage of cholesterol absorption was calculated as follows: [(^{14}C/^{3}H ratio in dosing mixture − ^{14}C/^{3}H ratio in feces)/(^{14}C/^{3}H ratio in dosing mixture)] × 100%.

Histological observation of the small intestines of ApoE$^{-/-}$ mice. The small intestine was dissected out and put into 4% paraformaldehyde for fixation at 4°C overnight. The tissue was paraffin embedded and then sliced (10 μm thick). Each slice was deparaffinized and stained with hematoxylin and eosin.

J774 cell culture, [^{3}H]cholesterol labeling, and cholesterol loading. J774 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum. Cells were radiolabeled with 4 μCi/ml [^{3}H]cholesterol in RPMI supplemented with 1% fetal calf serum (FCS) and cholesterol loaded with 25 μg/ml acetylated low-density lipoprotein (LDL) (Invitrogen, Carlsbad, CA). Forty-eight hours later, the cells were washed with RPMI and equilibrated for 6 h in fresh RPMI supplemented with 0.2% bovine serum albumin (BSA). Before injection into the mice, the cells were spun down and resuspended in RPMI (22).

***In vivo* reverse cholesterol transport (RCT).** Experiments were performed using 8-week-old male ApoE$^{-/-}$ mice. Each group ($n = 10$) was maintained on the Western diet or the Western diet plus *L. acidophilus* ATCC 4356 for 16 weeks. At the end of the experiment, mice were injected intraperitoneally with [^{3}H]cholesterol-labeled J774 foam cells (4.5×10^6 cells containing 1.5×10^6 count per minute) as described previously (22). Feces were collected continuously from 0 to 48 h and stored at −20°C until the cholesterol and bile acids were extracted. At 48 h, the mice were anesthetized, and blood, bile, and liver were removed and stored at −80°C until lipid extraction. Fecal cholesterol and bile acids were extracted as described previously (23). The levels of [^{3}H]cholesterol are expressed as counts per minute in the total feces by wet weight. Aliquots of plasma or bile were counted in a beta-counter. Liver cholesterol was extracted using the Folch method (24). Briefly, liver tissue (100 mg) was homogenized in 5 ml of chloroform-methanol (2:1, vol/vol) and washed two times with 1 ml of 0.36 M $CaCl_2$-methanol. Cholesterol was measured enzymatically in the organic phase using a colorimetric Diacron (Grosseto, Italy) kit according to the manufacturer's instructions.

qRT-PCR for analysis of gene expression. The livers and small intestines of the animals in each experimental group were removed. The entire small intestine was cut into three segments with length ratios of 1:3:2 (duodenum-jejunum-ileum). From the middle of each intestinal segment, 1.5 cm of the duodenal, jejunal, and ileal tissues was cut. Total RNA was isolated from the tissue samples with TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis was performed using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Transcripts were detected using SYBR green-based (Applied Biosystems, Foster City, CA) real-time PCR. The primer sequences for quantitative real-time PCR (qRT-PCR) are provided in Table 1. All analyses were performed in duplicate, and relative RNA levels were determined using β-actin as an internal control.

Western blotting. The small intestines were homogenized in a 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$, 0.25 M sucrose, and a protease inhibitor cocktail (Sigma). From small intestinal lysates, 100 μg of protein per lane was fractionated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Following the protein transfer step, the membrane was blocked and then immunoblotted with a polyclonal anti-mouse ABCG5/G8 antibody (Novus Biologicals, Littleton, CO), a polyclonal anti-mouse ABCA1 antibody, a polyclonal anti-mouse PPARα and LXRα antibody, or a polyclonal anti-mouse NPC1L1

TABLE 2 Sequences of primers used for detection of bacteria

Target	Primer sequences	Product size (bp)	Reference
Lactobacillus acidophilus ATCC 4356	Forward, CTT CGG TGA TGA CGT TGG GA; reverse, CCA ATG TGG CCG ATC AGT CT	254	48
Lactobacillus	Forward, CGA TGA GTG CTA GGT GTT GGA; reverse, CAA GAT GTC AAG ACC TGG TAA G	186	49
Bifidobacterium	Forward, CTC CTG GAA ACG GGT GG; reverse, GGT GTT CTT CCC GAT ATC TAC A	549	50
Escherichia coli	Forward, GTT AAT ACC TTT GCT CAT TGA; reverse, ACC AGG GTA TCT AAT CCT GTT	340	51

antibody. Bound primary antibody was detected with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, Danvers, MA) and enhanced chemiluminescence (ELC plus; Amersham International). After the membranes were stripped, β-actin was immunoblotted as a loading control. The optical densities of the bands were quantified with ImageJ software (National Institutes of Health, USA).

qRT-PCR for analysis of bacteria. Bacterial DNA was extracted from the digesta in the duodenum, jejunum, ileum, colon, and feces of ApoE$^{-/-}$ mice using a QIAamp DNA stool minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time PCRs were performed in duplicate using the LightCycler 480 system with a SYBR green I master kit (Roche, Auckland, New Zealand) according to the method described by Han et al. (25). The primers used to quantify the different bacterial groups, including *Lactobacillus*, *Bifidobacterium*, and *Escherichia coli* strains, are listed in Table 2. The reaction mixture consisted of 10 μl of SYBR green I master, 1 μl of DNA sample, and each primer (10 pmol/μl) in a final volume of 20 μl. An initial DNA denaturation step at 95°C for 10 min was followed by 30 amplification cycles (95°C for 15 s, 55°C for 25 s, and 72°C for 20 s) and cooling at 4°C. The standard curve was constructed by plotting the Cp values against serial dilutions of DNA solutions extracted from each bacterial group using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Real-time monitoring was achieved by measuring the fluorescence at the end of the elongation phase.

Statistical analysis. Statistical analyses were performed using non-parametric tests and analysis of variance (ANOVA) with GraphPad software. The results are presented as the mean ± standard error of the mean (SEM). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

***L. acidophilus* ATCC 4356 inhibits atherosclerotic lesion formation in ApoE$^{-/-}$ mice.** To investigate whether *L. acidophilus* strains could suppress the progression of atherosclerosis *in vivo*, we examined the effects of *L. acidophilus* ATCC 4356 and *L. acidophilus* ATCC 4962 on atherosclerotic lesion formation in ApoE$^{-/-}$ mice. Quantification of an Oil Red O-stained *en face* preparation of the aorta revealed a dramatic reduction in atherosclerotic lesions in the L.4356 group mice compared to the control group mice, whereas the L.4962 group exhibited no effects on atherosclerotic lesions ($P < 0.01$) (Fig. 1A and B). We then analyzed aortic sinus sections from mice of three groups. Quantification of the lesions after Oil Red O staining revealed a significant reduction in lesion areas in the L.4356 group mice compared to the control group mice, but the L.4962 treatment had no significant effect on lesion areas ($P < 0.01$) (Fig. 1C and D). Therefore, the effects on atherosclerosis development are specific to *L. acidophilus* ATCC 4356.

***L. acidophilus* ATCC 4356 reduces atherogenic plasma lipoproteins in ApoE$^{-/-}$ mice.** Because the progression of atherosclerosis is influenced by lipid metabolism, we determined whether *L. acidophilus* ATCC 4356 reduced the plasma lipid content in ApoE$^{-/-}$ mice. Compared to control mice, *L. acidophilus* ATCC 4356-treated mice exhibited a 37% reduction in total cholesterol levels (923 ± 44 versus 581 ± 18 mg/dl; $P < 0.05$), which could be entirely accounted for by a reduction in their non-high-

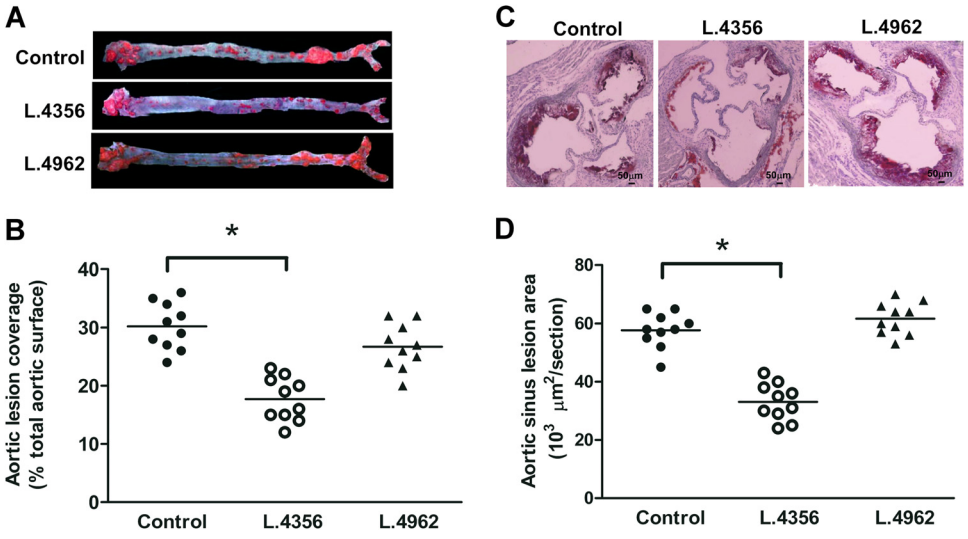

FIG 1 *Lactobacillus acidophilus* ATCC 4356 (L.4356) inhibits the development of atherosclerosis in ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice ($n = 10$/each group) were treated with *L. acidophilus* ATCC 4356 for 16 weeks, and an atherosclerosis study was then performed as described in Materials and Methods. (A) Aorta *en face* lesion coverage and representative Oil Red O staining from ApoE$^{-/-}$ mice fed with or without *L. acidophilus* ATCC 4356. (B) Quantitative analysis of the atherosclerotic surface area in the entire aorta. Data are expressed as a percentage of the total aortic area. (C) Representative Oil Red O-stained aortic sinus sections from ApoE$^{-/-}$ mice fed with or without *L. acidophilus* ATCC 4356. (D) Quantitative analysis of lesion areas in the aortic sinus. *, $P < 0.05$ versus control.

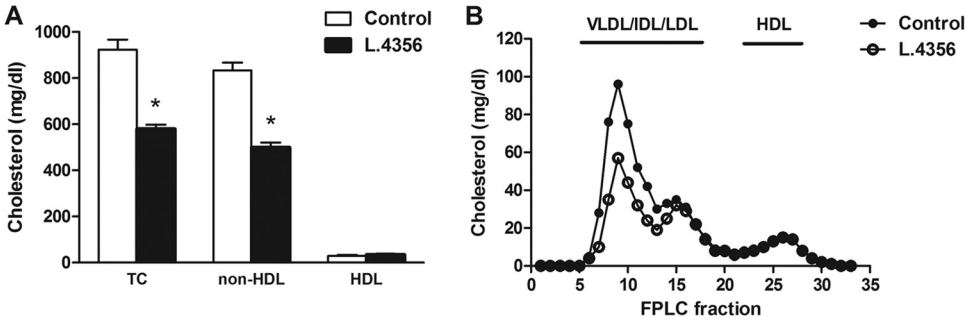

FIG 2 *Lactobacillus acidophilus* ATCC 4356 (L.4356) decreases the concentration of non-HDL-cholesterol-containing lipoproteins in ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were treated with or without L.4356 for 16 weeks. (A) Plasma cholesterol concentrations in the control and *L. acidophilus* ATCC 4356-treated mice. Data are presented as the mean ± SEM ($n = 10$ for each group). *, $P < 0.05$ versus controls. (B) Plasma lipoproteins were separated by fast protein liquid chromatography and were measured in the collected fractions.

density lipoprotein (non-HDL) cholesterol concentrations (833 ± 35 versus 500 ± 22 mg/dl; $P < 0.05$). In contrast, the HDL cholesterol levels were comparable in the *L. acidophilus* ATCC 4356-treated and control mice (30 ± 5 versus 35 ± 4 mg/dl) (Fig. 2A). The FPLC lipoprotein profile revealed that in the *L. acidophilus* ATCC 4356-treated mice, the decrease in plasma cholesterol was largely attributable to a decrease in the very-low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) levels, with no observable difference in the HDL cholesterol levels (Fig. 2B). These data indicate that *L. acidophilus* ATCC 4356 treatment of ApoE$^{-/-}$ mice results in an atheroprotective plasma lipoprotein profile.

***L. acidophilus* ATCC 4356 decreases intestinal cholesterol absorption without altering the epithelial structure of the small intestines of ApoE$^{-/-}$ mice.** To gain insight into the mechanism underlying the reduced total cholesterol levels in the sera of *L. acidophilus* ATCC 4356-treated mice, intestinal cholesterol absorption was determined. *L. acidophilus* ATCC 4356 treatment led to a 44% reduction in cholesterol absorption efficiency ($P < 0.05$) (Fig. 3). To investigate whether *L. acidophilus* ATCC 4356 treatment had any impact on the morphology of the small intestine, we stained intestinal sections with hematoxylin and eosin. As shown in Fig. 4, *L. acidophilus* ATCC 4356 did not influence the morphology of the small intestine. All specimens had intact villi, epithelia, enterocytes, and brush borders. Therefore, *L. acidophilus* ATCC 4356 treatment does not affect gross small intestinal morphology.

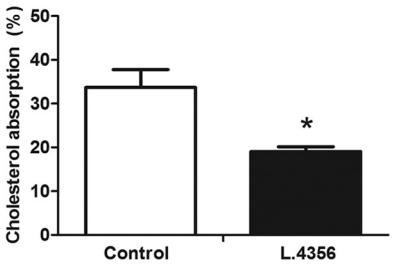

FIG 3 *Lactobacillus acidophilus* ATCC 4356 (L.4356) decreases intestinal cholesterol absorption in ApoE$^{-/-}$ mice. Intestinal cholesterol absorption was measured using the fecal dual-isotope method as described in Materials and Methods. It was reduced by 44% in the L.4356 group compared to the control group. Data are presented as the mean ± SEM ($n = 10$ in each group) *, $P < 0.05$ versus controls.

***L. acidophilus* ATCC 4356 inhibits intestinal NPC1L1 expression in ApoE$^{-/-}$ mice.** To address the mechanism underlying *L. acidophilus* ATCC 4356-mediated inhibition of cholesterol absorption, NPC1L1 mRNA levels in the small intestines were measured (Fig. 5A). In the duodenal and jejunal segments, the NPC1L1 mRNA levels detected in the *L. acidophilus* ATCC 4356-treated mice were significantly lower than those detected in the control mice. These decreases in NPC1L1 levels in the *L. acidophilus* ATCC 4356-treated mice were also confirmed at the protein level (Fig. 5B). In contrast, no significant differences in NPC1L1 mRNA levels were observed in the ileal segment.

***L. acidophilus* ATCC 4356 promotes macrophage RCT *in vivo*.** A previous report demonstrated that the suppression of cholesterol absorption from the intestine increases macrophage reverse cholesterol transport (RCT) (26). To investigate whether *L. acidophilus* ATCC 4356 administration promotes RCT *in vivo*, cholesterol-loaded/[^{3}H]cholesterol labeled J774 macrophages were injected intraperitoneally into either control mice or ApoE$^{-/-}$ mice that had been treated with *L. acidophilus* ATCC 4356 for 16 weeks. The transfer of the tracer to the plasma, liver, bile, and feces was then evaluated and expressed as a percentage of the injected dose (Fig. 6). In the *L. acidophilus* ATCC 4356-treated

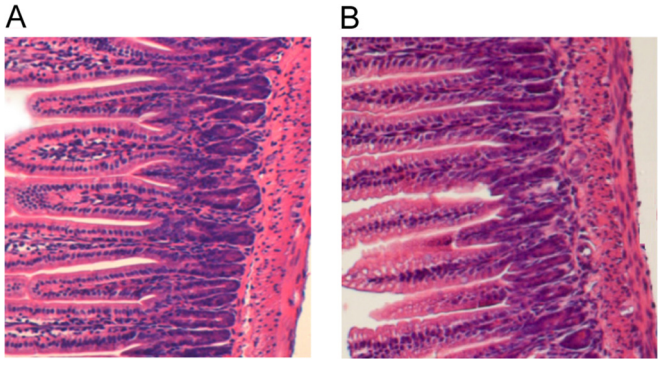

FIG 4 *Lactobacillus acidophilus* ATCC 4356 treatment does not change the morphology of the mouse small intestine. The small intestine was dissected out and placed in 4% paraformaldehyde for fixation overnight. The tissue was then sliced (10 μm thick). Each slice was deparaffinized and stained with hematoxylin and eosin. The morphology was observed under a microscope (magnification, ×100). (A) Small intestine from the control mice; (B) small intestine from the *L. acidophilus* ATCC 4356-treated mice.

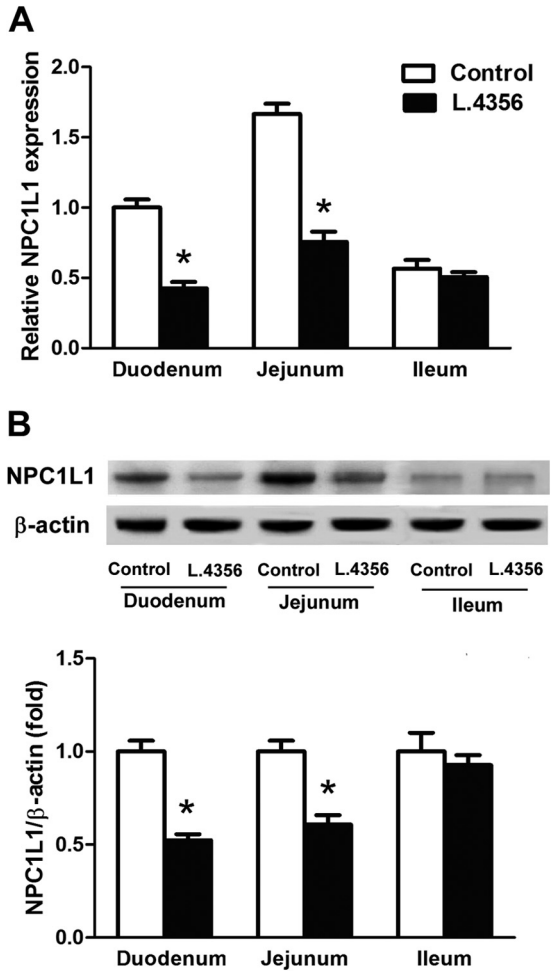

FIG 5 *Lactobacillus acidophilus* ATCC 4356 (L.4356) decreases Niemann-Pick C1-like 1 (NPC1L1) expression in the small intestines of ApoE$^{-/-}$ mice. (A) Real-time PCR of NPC1L1 mRNA in the small intestines of *L. acidophilus* ATCC 4356-treated mice was compared to that in the small intestines of the controls. The signal obtained from the control mice was set to a normalized value of 100 arbitrary units. β-Actin was used as an internal control. The data are presented as the mean ± SEM. *, $P < 0.05$ versus controls. (B) Protein expression of NPC1L1 in the small intestines of *L. acidophilus* ATCC 4356-treated mice was compared to that in the small intestines of the control group. Bar graphs show the relative quantification of NPC1L1 (normalized to β-actin; $n = 3$). *, $P < 0.05$ versus controls.

group, the macrophage-derived radioactivity remained unchanged in the plasma (Fig. 6A) and in the liver (Fig. 6B) compared to the control group. Of note, in the *L. acidophilus* ATCC 4356-treated group, the biliary and fecal [^{3}H]cholesterol levels were markedly elevated (Fig. 6C), whereas no significant differences were observed in the minute amounts of radioactivity found in the secreted bile acids (Fig. 6D) compared with the control group. These results strongly suggest that in the *L. acidophilus* ATCC 4356-treated mice, decreased cholesterol absorption rates lead to an increase in macrophage RCT. This increase in macrophage RCT likely explains the atheroprotective effect of *L. acidophilus* ATCC 4356.

Expression of genes related to cellular cholesterol transport. To search for potential mechanisms mediating the effect of *L. acidophilus* ATCC 4356 on RCT, we used quantitative RT-PCR to determine the mRNA expression of PPARα, PPARδ, and LXRα, which are major regulators of cholesterol homeostasis, and of their downstream cholesterol transporter target genes that are important for RCT (27). Gene profiles were analyzed in small intestines and livers derived from the control mice and the ApoE$^{-/-}$ mice treated with *L. acidophilus* ATCC 4356. Compared to the control mice, the mice treated with *L. acidophilus* ATCC 4356 had significantly upregulated PPARα and LXRα expression levels in the small intestine, whereas no differences were found in PPARδ expression (Fig. 7B and C). Similarly, no statistically significant differences in the mRNA levels of the hepatic LXRα, the hepatic ABCG5/G8 heterodimer, or the hepatic SR-BI and 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA-S) were found between the control and *L. acidophilus* ATCC 4356-treated mice (Fig. 7A). The expression levels of ABCG5/G8 and ABCA1, which favor cholesterol export from absorptive cells into the lumen or the basolateral compartment (27), respectively, were significantly increased (Fig. 7B and C). Overall, these results indicate that *L. acidophilus* ATCC 4356 promotes RCT, most likely via a coordinated effect of PPARα and LXRα.

Colonization by *L. acidophilus* ATCC 4356 in the ApoE$^{-/-}$ mouse intestinal tract. As shown in Table 3, *L. acidophilus* ATCC 4356 was not detected in the duodenum, jejunum, ileum, colon, and feces of ApoE$^{-/-}$ mice at day 0. However, it was detected in the duodenum, jejunum, ileum, colon, and feces after gavage at week 16. Counts of the strain in the ileum, colon, and feces were significantly ($P < 0.05$) higher than those in the duodenum and jejunum. At week 18, after stopping administration of bacteria for 2 weeks, the strain was still detected in the small intestine, colon, and feces. The numbers of the bacteria had slightly declined.

Microbial populations in the feces. Table 4 summarizes the microbial population composition of the fecal samples of the *L. acidophilus* ATCC 4356-treated and control groups, as determined by real-time PCR. Significant increases in the total lactobacillus and bifidobacterium populations were observed in the fecal samples of ApoE$^{-/-}$ mice fed *L. acidophilus* ATCC 4356 compared to those of the control group. Conversely, for the *L. acidophilus* ATCC 4356-treated group, the counts of *Escherichia coli* in the ApoE$^{-/-}$ mice feces significantly decreased at week 16. After 16 weeks of administration, the counts of *Escherichia coli* remained stable until the end of 18 weeks.

DISCUSSION

Since the early studies by Mann (28), ever-increasing interest has been focused on the hypocholesterolemic effects exerted by LAB. The present study demonstrates that *L. acidophilus* ATCC 4356 likely exerts an atheroprotective effect in ApoE$^{-/-}$ mice by lowering circulating atherogenic cholesterol-containing lipoproteins. Interestingly, the pronounced effect of *L. acidophilus* ATCC 4356 treatment on the plasma cholesterol concentration coincides with a decrease in intestinal cholesterol absorption. Decreased cholesterol absorption is associated with reduced intestinal gene expression of NPC1L1. Indeed, NPC1L1 has recently been identified as a target of the hypocholesterolemic drug ezetimibe, which regulates intestinal cholesterol absorption (8, 29–32) In our present study, lactobacillus and bifidobacterium counts in the small intestines of mice fed *L. acidophilus* ATCC 4356 were significantly greater than those in the small intestines of mice fed solely the Western diet. Moreover, NPC1L1 mRNA levels in the duodenal and jejunal segments of mice fed *L. acidophilus* ATCC 4356 were lower than those

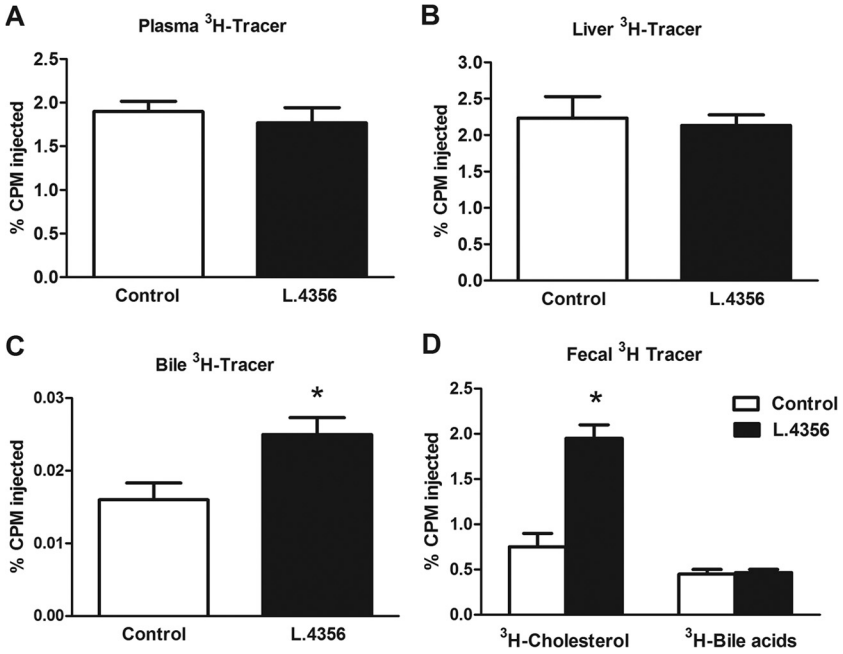

FIG 6 Effect of *Lactobacillus acidophilus* ATCC 4356 (L.4356) on macrophage reverse cholesterol transport (RCT) *in vivo*. Macrophage-derived [^{3}H]cholesterol contents in plasma (A), liver (B), bile (C), and feces (D) were measured by liquid scintillation counting. *L. acidophilus* ATCC 4356 induces macrophage-mediated RCT, resulting in augmented fecal cholesterol removal. The results are shown as the mean ± SEM ($n = 10$ per group) and are expressed as the percentage of radioactivity injected. *, $P < 0.05$ versus controls.

in the duodenal and jejunal segments of the control group. The present results indicate that *L. acidophilus* ATCC 4356 is able to reduce cholesterol absorption by inhibiting the intestinal expression of NPC1L1, predominantly in the duodenal and jejunal segments of the small intestine, where most of the cholesterol absorption takes place. In line with our findings, previous studies examining the effect of a potent inhibition of cholesterol absorption on atherosclerosis formation have consistently shown a strong atheroprotective effect. For example, Davis et al. (8) demonstrated that in ApoE$^{-/-}$ mice, knocking out NPC1L1 resulted in a 77% decrease in the cholesterol absorption rates and in absolute protection against atherosclerosis. Furthermore, Greenberg et al. (26) demonstrated that even a modest reduction of 41% in the intestinal cholesterol absorption led to a 70% decrease in atherosclerosis formation.

Previous studies (33, 34) reported that the anticholesterolemic effect of some probiotics is based on their bile salt hydrolase activity, which acts on bile salts, releasing less soluble deconjugated bile salts. These are eliminated in feces instead of being reabsorbed, leading to synthesis of new bile salts from cholesterol, which in turn decreases cholesterol levels. In contrast to previous studies, the results of the present study indicate that *L. acidophilus* ATCC 4356 was able to reduce cholesterol levels by inhibiting cholesterol absorption. We hypothesized that the ability of *L. acidophilus* ATCC 4356 to attenuate atherosclerosis may be associated with its effect on cholesterol absorption.

Atherosclerosis is a chronic lipid-driven inflammatory disease of arteries (35). The causal relationship between the blood cholesterol level and atherosclerosis is widely accepted (36). Our current results, together with those of previous studies (8, 26), suggest that a reduction in cholesterol absorption exerts an atheroprotective effect. However, one study provided conflicting results regarding the hypocholesterolemic role of *L. acidophilus* ATCC 4356. Chen et al. (37) showed that the administration of *L. acidophilus* ATCC 4356 to ApoE$^{-/-}$ mice attenuated atherosclerosis lesion formation but did not have any effect on the lipid levels in ApoE$^{-/-}$ mice. The discrepancies between that study and the present study might be partly attributable to the different length of the feeding period (12 weeks versus 16 weeks). They also showed that *L. acidophilus* ATCC 4356 treatment inhibited oxidative stress and suppressed inflammatory status. In contrast to their studies, the results of the present study indicate that *L. acidophilus* ATCC 4356 was able to attenuate the atherosclerotic progression by inhibiting cholesterol absorption. Until now there have been no reported studies addressing the effect of probiotics on atherogenesis by reducing intestinal cholesterol absorption.

PPARs and LXRs are major regulators of intestinal cholesterol homeostasis via transcription of various target genes in the enterocyte. PPARδ activation in mice has been found to decrease intestinal expression of NPC1L1 and to promote RCT (30). In fact, PPARδ was also shown to stimulate a nonbiliary transintestinal cholesterol efflux (TICE) pathway that contributes to the total fecal neutral sterol excretion without involving NPC1L1 (38, 39). In contrast, in the present study, the intestinal upregulation of PPARα, but that not of PPARδ, resulted in NPC1L1 repression, thus impairing the intestinal reabsorption of macrophage-derived cholesterol. In line with our findings, fenofibrate-induced PPARα activation has been found to decrease the expression of NPC1L1 in the small intestines of mice, at both the mRNA and protein levels (40). Interestingly, the intestinal NPC1L1 gene is also an LXRα target gene, both in mice *in vivo* and in a human enterocyte cell line *in vitro* (41). In addition, it was recently found that intestinal-specific, but not hepatic-specific, LXRα activation decreases intestinal cholesterol absorption, thereby increasing RCT *in vivo* (42).

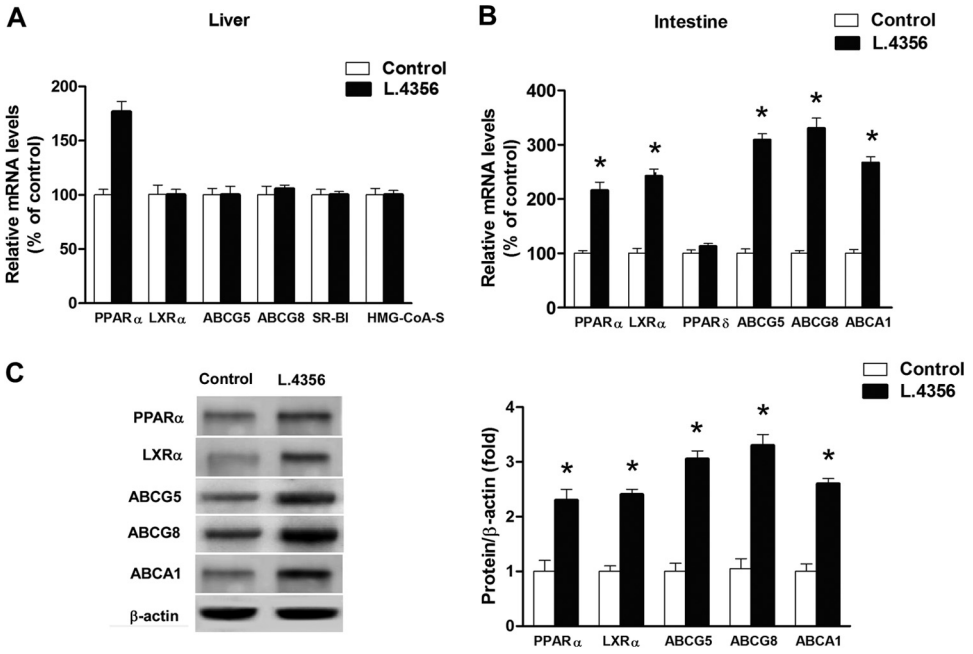

FIG 7 Analysis of gene expression in the small intestines and livers of ApoE$^{-/-}$ mice. (A and B) Relative mRNA expression of peroxisome proliferator-activated receptor transcription factors PPARα, PPARδ, and liver X receptor (LXRα), ATP-binding cassette transporters (A1/G5/G8), scavenger receptor-BI (SR-BI), and 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA-S) was evaluated by quantitative real-time PCR in the livers (A) and small intestines (B) derived from the control and *L. acidophilus* ATCC 4356-treated ApoE$^{-/-}$ (L.4356) mice ($n = 10$ mice per group). The signal obtained from the control mice was set to a normalized value of 100 arbitrary units. β-Actin was used as an internal control. *, $P < 0.05$. (C) Total proteins from small intestines of ApoE$^{-/-}$ mice treated with or without *L. acidophilus* ATCC 4356 were assessed for their PPARα, LXRα, ABCA1, ABCG5, and ABCG8 levels by Western blotting ($n = 10$ mice per group). The bar graph shows the relative quantification of PPARα, LXRα, ABCA1, ABCG5, and ABCG8 in the control and L.4356 groups (normalized to β-actin; $n = 3$). *, $P < 0.05$ versus controls.

Moreover, chronic administration of a synthetic LXR agonist to mice significantly increases PPARα expression levels in the small intestine but not in the liver (43). Collectively, these studies support the notion that the small intestine has an important regulatory role in the RCT pathway and strongly suggest the involvement of a complex transcriptional cross talk between PPARα and LXRα in determining the intestinal cholesterol excretion in response to *L. acidophilus* ATCC 4356.

The human gut microbiota is a complex community that provides important metabolic functions for the host. Consequently, alterations in the gut microbiota have been associated with the pathogenesis of several human diseases associated with metabolic disturbances, particularly those that have been increasing in incidence over the last several decades, including obesity, diabetes, and atherosclerosis (44). Effective interventions to change the gut microbiota composition with probiotic supplementation may improve health and prevent the onset of certain diseases. Stepankova et al. (45) found that the absence of gut microbiota (germfree conditions) accelerated atherosclerosis in ApoE$^{-/-}$ mice fed a standard low-cholesterol diet. In this study, we found that supplementation with *L. acidophilus* ATCC 4356 increased the counts of lactobacilli and bifidobacteria, suggesting that *L. acidophilus* ATCC 4356 can successfully tolerate gastric acid and bile salts, ultimately facilitating the proliferation of lactobacilli and bifidobacteria. Chen et al. (37) also found that *L. acidophilus* ATCC 4356 supplementation increased the bifidobacterial population in rats. However, the fecal *Escherichia coli* levels were significantly decreased in the *L. acidophilus* ATCC 4356-treated mice. These results are consistent with those of Liong and Shah, who reported that decreased fecal *Escherichia coli* counts result from probiotic feeding in rats (46). The colonization of the intestinal mucosa with such probiotic bacteria provides a barrier effect against pathogens through a variety of mechanisms, including the occupation of niches, competition for nutrients, and the production of antimicrobials (47). Considering the relationship between gut microbiota and metabolic disease, we hypothesized that the ability of *L. acidophilus* ATCC 4356 to attenuate atherosclerosis may be associated with its effect on intestinal microflora; however, further

TABLE 3 *Lactobacillus acidophilus* ATCC 4356 counts in the intestinal tracts of ApoE$^{-/-}$ mice as measured by quantitative real-time PCR

Wk	*L. acidophilus* ATCC 4356 counts ($\log_{10}$ 16S rRNA gene copies per g of sample)[a] in:				
	Duodenum	Jejunum	Ileum	Colon	Feces
0	0	0	0	0	0
16	2.45 ± 0.02 c	2.52 ± 0.02 c	3.76 ± 0.03 b	5.48 ± 0.04 a	5.41 ± 0.04 a
18	2.36 ± 0.02 c	2.43 ± 0.02 c	3.50 ± 0.02 b	5.36 ± 0.03 a	5.27 ± 0.02 a

[a] Mean values within the same row with different letters differ significantly ($P < 0.05$). The data are shown as the mean ± standard deviation.

TABLE 4 Effects of *L. acidophilus* ATCC 4356 feeding on the fecal bacterial population

Treatment group[a]	Wk	Log_{10} 16S rRNA gene copies per g of feces[b]		
		Lactobacilli	Bifidobacteria	Coliforms
Control	0	9.15 ± 0.04 a	7.84 ± 0.03 a	6.80 ± 0.03 a
	16	9.21 ± 0.03 a	7.89 ± 0.03 a	6.74 ± 0.03 a
	18	9.18 ± 0.02 a	7.79 ± 0.02 a	6.76 ± 0.02 a
L.4356	0	9.13 ± 0.02 b	7.82 ± 0.04 b	6.74 ± 0.03 a
	16	9.81 ± 0.03 a	8.55 ± 0.03 a	4.21 ± 0.02 b
	18	9.62 ± 0.02 a	8.42 ± 0.03 a	4.37 ± 0.02 b

[a] Control, Western diet only; L.4356, Western diet plus *L. acidophilus* ATCC 4356.
[b] Mean values within the same column with different letters differ significantly ($P < 0.05$). The data are shown as the mean ± standard deviation.

research is required to test the validity of this hypothesis and investigate the possible mechanisms involved.

In summary, we demonstrated that *L. acidophilus* ATCC 4356 inhibits the development of atherosclerosis in ApoE$^{-/-}$ mice. We also showed that a decrease in cholesterol absorption is attributable to decreased plasma cholesterol levels and increased RCT from peripheral macrophages. The mechanism of the *L. acidophilus* ATCC 4356-induced RCT response in the intestine appears to involve a complex cross talk between PPARα and LXRα. We have demonstrated a previously unknown role for *L. acidophilus* ATCC 4356 in modulating atherosclerotic lesion progression, and intestinal microbiota might participate in these effects through the mediation of *L. acidophilus* ATCC 4356. Our work provides new insight into the antiatherosclerotic properties of *L. acidophilus* ATCC 4356.

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