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# The probiotic *Lactobacillus acidophilus* reduces cholesterol absorption through the down-regulation of Niemann-Pick C1-like 1 in Caco-2 cells

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Elevated blood cholesterol is an important risk factor associated with atherosclerosis and CHD. The search for mediators that fine tune cholesterol homeostasis has recognised probiotics as being potentially beneficial. Here, we present data describing bacterial regulation of Niemann-Pick C1-like 1 (NPC1L1), which, when weakly expressed, results in a marked reduction in intestinal absorption of cholesterol. The probiotic *Lactobacillus acidophilus* ATCC 4356 reduced NPC1L1 gene expression and inhibited the cellular uptake of micellar cholesterol in Caco-2 cells. Soluble effector molecules secreted by ATCC 4356 were shown to be responsible for the decrease in NPC1L1. Furthermore, ATCC 4356 mediated this effect partly through the liver X receptors (LXR). The role of NPC1L1 and the LXR in cholesterol metabolism underscores the basis for the use of probiotics, such as ATCC 4356, in managing hypercholesterolaemia.

***Lactobacillus acidophilus*: Cholesterol absorption: Niemann-Pick C1-like 1**

Hypercholesterolaemia is one of the risk factors for lifestyle-related diseases such as atherosclerosis and CHD. Even a 1 % reduction in serum cholesterol was found to reduce the risk of CHD by 2–3 %[1]. In addition to endogenously synthesised cholesterol, the absorption of dietary cholesterol and the reabsorption of biliary cholesterol in the small intestine contribute to the regulation of plasma cholesterol levels[2]. Moreover, reducing the intestinal absorption of dietary and biliary cholesterol decreases plasma cholesterol levels[3]. The molecular mechanism by which intestinal cholesterol absorption occurs has recently been elucidated[4]. The Niemann-Pick C1-like 1 (NPC1L1) protein has been identified as a key player in cholesterol absorption, and it is a promising target for cholesterol-lowering medication[5]. NPC1L1 is highly expressed in the small intestine, most likely at the surface of enterocytes, and it is required for intestinal cholesterol absorption[4]. NPC1L1-null mice were resistant to diet-induced hypercholesterolaemia and were completely resistant to the development of atherosclerosis when crossed with apoE-null mice[6,7]. Recent work by Duval *et al.* [8] has identified NPC1L1 as a novel target gene of the liver X receptors (LXRα and β). LXR are crucial regulators of cholesterol homeostasis and belong to the nuclear receptor superfamily[9]. The activation of LXR reduces whole-body cholesterol and decreases atherosclerosis[10,11].

In recent years, we have begun to understand the benefits of a well-composed intestinal flora, emphasising a role for pro- and prebiotics. Probiotics are live micro-organisms, which when administered in adequate amounts confer a health benefit on the host[12]. The most common and researched species belong to the genera *Lactobacillus, Bifidobacterium and Saccharomyces* [13]. Lactobacilli are considered potentially useful because of their purported ability to reduce serum cholesterol. *In vitro* studies have previously shown that cholesterol is removed by strains of lactobacilli in laboratory media[14,15]. Likewise, *in vivo* studies have reported that some lactobacilli can lower total cholesterol and low-density lipoprotein cholesterol levels[16,17]. Until now, there have been no reported studies on the use of lactobacilli to interfere with intestinal cholesterol absorption. In the present study, we have chosen to focus on *Lactobacillus acidophilus* ATCC 4356 (L.4356), which was isolated from human intestinal tracts. The primary aim of the present study, therefore, was to determine whether L.4356 decreases intestinal NPC1L1 expression and whether this reduction constitutes a possible mechanism for why L.4356 alters cholesterol absorption in Caco-2 cells.

## Experimental methods

### *Cell culture and reagents*

The human colorectal adenocarcinoma cell line Caco-2 (American Type Culture Collection (ATCC), Rockville, MD, USA) was grown and maintained according to the supplier's recommendations.

[$^3$H] Cholesterol ($178{\cdot}71 \times 10^{10}$ Bq (48·3 Ci)/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA,

**Abbreviations:** LXR, liver X receptors; L.4356, *Lactobacillus acidophilus* ATCC 4356; NPC1L1, Niemann-Pick C1-like 1; siRNA, short interfering RNA.

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USA). Cholesterol, sodium taurocholate, oleic acid, monoolein and phosphatidylcholine were purchased from Sigma Chemical (St Louis, MO, USA).

### *Bacteria and co-culture*

All bacterial strains, *Lactobacillus rhamnosus* GG (ATCC 53 103), *Bifidobacterium lactis* 12, L.4356 and *Bacteroides thetaiotaomicron*, were obtained from ATCC. The L.4356 was serially propagated three times in deMan, Rogosa and Sharpe medium (Difco, Detroit, MI, USA) before experimental use. A 1 % inoculum was used, and incubations were performed at 37°C for 24 h in anaerobic condition (10 % $H_2$, 10 % $CO_2$ and 80 % $N_2$). Seed cultures were taken at the end of the exponential phase of growth at cell densities of $10^7$ per ml.

Co-cultures were prepared by washing the colonic cells with warm PBS. Cells were incubated with $10^7$ per ml of L.4356 or with medium alone. The experiment was terminated by thoroughly washing the plates with ice-cold PBS.

As a control, L.4356 was heat inactivated with a 30 min incubation at 80°C. We used the same number of heat-inactivated bacteria as the number of viable bacteria in the co-culture. The bacteria were suspended in 5 ml of the respective medium, and this solution was added to the washed cells. Conditioned medium was prepared by incubating L.4356 and Caco-2 together for 6 h, followed by filtration of the medium (pore size: 0·2 μm). Culture supernatant (CS) was also collected from L.4356 grown without Caco-2 cells. Conditioned medium and CS were heat inactivated by boiling (100°C, 10 min).

### *Cholesterol uptake assay*

Caco-2 cells were incubated with viable ATCC 4356, conditioned medium or the CS of ATCC 4356 grown without Caco-2 cells, and they were then compared with the non-treated Caco-2 cells. At 1 h before harvesting the cells, 0·15 ml micellar solution containing 6·6 mM sodium taurocholate, 74 000 Bq (2 μCi) [$^3$H] cholesterol, 1 mM oleic acid, 0·5 mM monoolein, 0·1 mM unlabelled cholesterol and 0·6 mM phosphatidylcholine were added to the Caco-2 cells. At the end of the incubation, the unincorporated radiolabelled cholesterol was removed by washing the cells four times with 1·5 ml cold Dulbecco's modified eagle medium. The cellular lipids were extracted with 1·5 ml hexane–isopropyl alcohol–water (3:2:0·1, v/v/v). The radioactivity in the cellular lipid extract was estimated by counting with a Packard liquid scintillation counter.

### *Real-time quantitative PCR*

RNA was prepared using the Qiagen RNeasy Mini Kit following the manufacturer's protocol, and cDNA was synthesised using the cDNA synthesis kit from Invitrogen (Carlsbad, CA, USA) according to the protocol. Semi-quantitative SYBR Green-based (Applied Biosystems, Foster City, CA, USA) real-time PCR was used to detect transcripts. Forward and reverse primers were mixed in equal proportions and used at a final concentration of 0·2 μM. Human NPC1L1 (forward primer: 5′-TATGGTCGCCCGAAGCA-3′, reverse primer: 5′-TGCGGTTGTTCTGGAAATACTG-3′) and β-actin (forward primer: 5′-CCTGGCACCCAGCACAAT-3′, reverse primer: 5′-GCCGATCCACACGGAGTACT-3′) transcripts were measured. Each experiment was carried out with duplicate samples, and the mRNA levels of each sample were determined in triplicate. Real-time PCR was performed using the ABI 7500 System for data acquisition, and the data were analysed using the ABI 7500 System Sequence Detection software. Data are presented as mean values with standard deviations.

### *Western blot analysis*

Cells were treated according to the specifications listed in the figures and were lysed in Schindler lysis buffer (50 mM Tris (pH 8), 0·1 mM EDTA, 0·5 % NP-40, 10 % glycerol, 150 mM NaCl, 10 nM okadaic acid, 5 mM NaF, 400 μM sodium vanadate, 1 × Complete (Roche, Germany) and 1 mM phenylmethanesulphonyl fluoride). Rabbit polyclonal anti-human NPC1L1 and anti-human β-actin antibodies were purchased from Abcam (Cambridge, MA, USA). Immunodetection was carried out by using an appropriate secondary peroxidase-conjugated antibody (Pierce Biotechnology, Inc., Rockford, IL, USA), followed by chemiluminescence (enhanced chemiluminescence, Amersham, Buckinghamshire, UK).

### *RNA interference*

Short interfering RNA (siRNA) specific for LXRα and β (SMART pool siRNA) and non-silencing control siRNA were purchased from Dharmacon. Transfections were carried out according to the manufacturer's protocol using the DharmaFECT Reagent 4.

### *Statistical analysis*

Data are expressed as means and standard deviations. The statistical significance of the difference between two means was evaluated using Student's *t* test. For analysing multiple mean values, Dunnett's test was used after ANOVA. In these tests, values of $P < 0{\cdot}05$ were considered significant.

## Results

### Lactobacillus acidophilus *ATCC 4356 inhibits Niemann-Pick C1-like 1 expression in Caco-2 cells*

The colon carcinoma cell line Caco-2 was used to elucidate the effects of probiotics on NPC1L1 expression in colonocytes. Cells were stimulated for 6 h with L.4356, *L. rhamnosus* GG, *B. lactis 12* or *B. thetaiotaomicron* (Fig. 1(a)). The two lactobacilli (L.4356 and *L. rhamnosus* GG) generated a substantial downregulation of NPC1L1 expression, while *B. lactis 12* induced a modest decrease. In contrast, the commensal *B. thetaiotaomicron* was unable to inhibit NPC1L1 expression. Subsequent experiments were performed with L.4356. ATCC 4356 was able to downregulate NPC1L1 in both a dose- and time-dependent manner (Fig. 1(b) and (c)). Downregulation of NPC1L1 expression was confirmed by evaluating its protein levels in extracts from cells stimulated for 6 h (Fig. 1(d)).

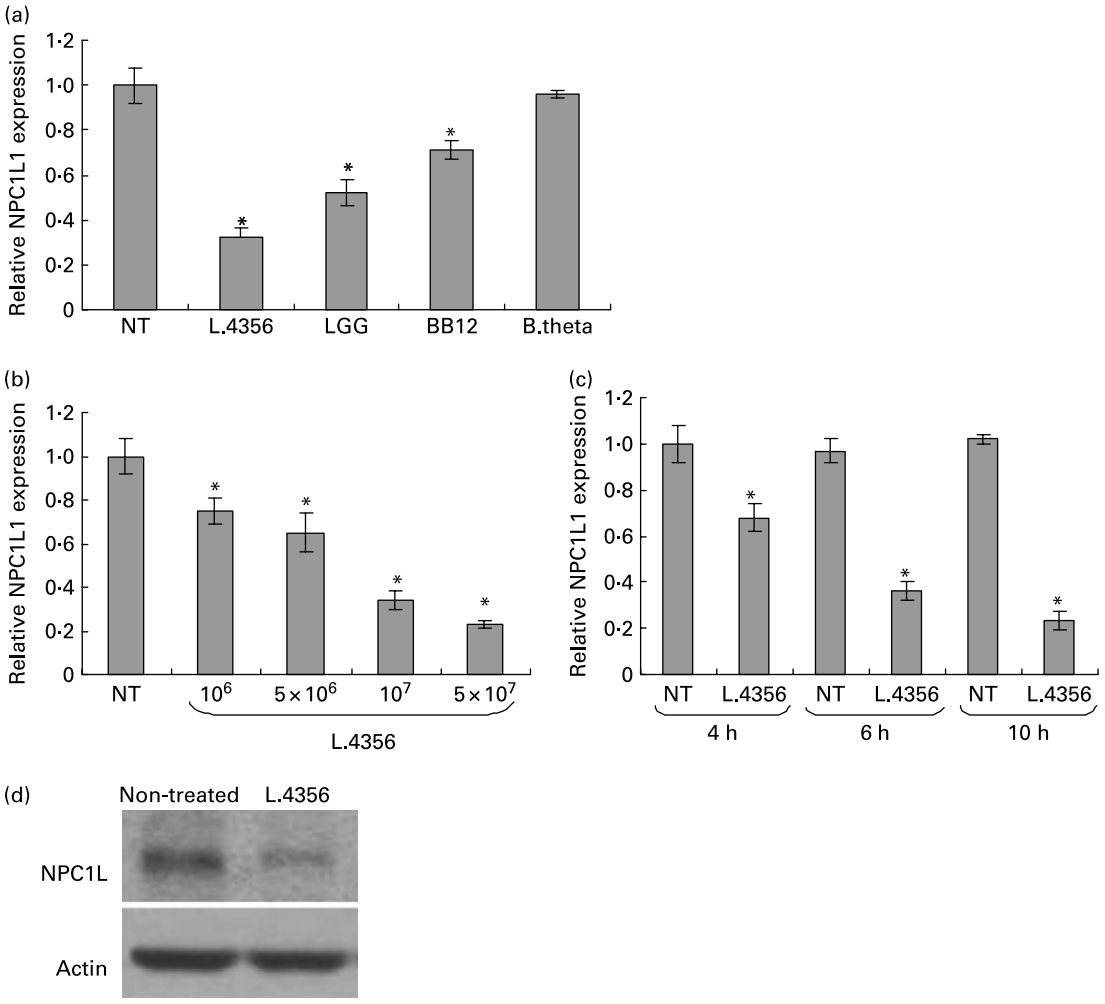


**Fig. 1.** Probiotics can downregulate Niemann-Pick C1-like 1 (NPC1L1) in Caco-2 cells. (a) Real-time PCR of NPC1L1 expression in Caco-2 cells co-cultured with *Lactobacillus acidophilus* ATCC 4356 (L.4356; $10^7$ per ml), *Lactobacillus rhamnosus* GG (LGG; $10^7$ per ml), *Bifidobacterium lactis* (BB12; $10^7$ per ml) and *Bacteroides thetaiotaomicron* (*B. theta*; $10^7$ per ml), respectively, for 6 h was compared with the non-treated (NT) control. Data are presented as means and standard deviations. *$P<0{\cdot}05$ compared with the NT control (by a Dunnett's test for multiple comparisons). (b) Expression analysis of NPC1L1 after 6 h of stimulation with L.4356 at different concentrations in Caco-2 cells. Data are presented as means and standard deviations. *$P<0{\cdot}05$ compared with the NT control (by a Dunnett's test for multiple comparisons). (c) Time course of the effects of L.4356 ($10^7$ per ml) on NPC1L1 mRNA expression in Caco-2 cells. Data are presented as means and standard deviations. *$P<0{\cdot}05$ compared with the NT control (by a Student's *t* test). (d) Western blot of NPC1L1 in Caco-2 cells treated with L.4356 for 6 h.

To elucidate the mechanism of NPC1L1 inhibition, potential components of the bacteria–cell interaction were separated and studied individually. Heat-killed ATCC 4356 was unable to inhibit NPC1L1 expression. Interestingly, conditioned media from the bacterial interaction with Caco-2 cells, even when heat inactivated, were sufficient to inhibit NPC1L1 expression (Fig. 2(a)). The need for bacteria–cell contact for the production of stimulatory molecules was also addressed. Here, we saw that CS from ATCC 4356 grown without Caco-2 cells (CS) and heat-inactivated CS were virtually as effective as conditioned media (CM) in which bacteria–cell contact has occurred (Fig. 2(b)).

### Lactobacillus acidophilus *ATCC 4356 decreases micellar cholesterol uptake*

To address whether L.4356 interferes with the uptake of micellar cholesterol solution, Caco-2 cells were incubated with viable ATCC 4356, heat-killed ATCC 4356, conditioned media or supernatants from ATCC 4356 grown without cells and compared with a non-treated control. At 1 h before harvesting the cells, 0·15 ml of a micellar solution was added to the medium. The amount of cholesterol taken up by the cells was then measured. The results are shown in Fig. 3. The uptake of cholesterol by cells incubated with viable ATCC 4356 was approximately twofold lower than the uptake by the non-treated control. Cells incubated with conditioned media or CS had decreased uptake of micellar cholesterol compared with the non-treated control, whereas cells incubated with heat-killed ATCC 4356 did not.

### *The liver X receptors-signalling pathway may play a role in the inhibition of NPC1L1 expression and cholesterol absorption by* L. acidophilus *ATCC 4356*

ATCC 4356 appears to be able to upregulate the expression of LXR in a dose- and time-dependent manner (Fig. 4(a) and (b)). When LXR are depleted by siRNA in Caco-2 cells,

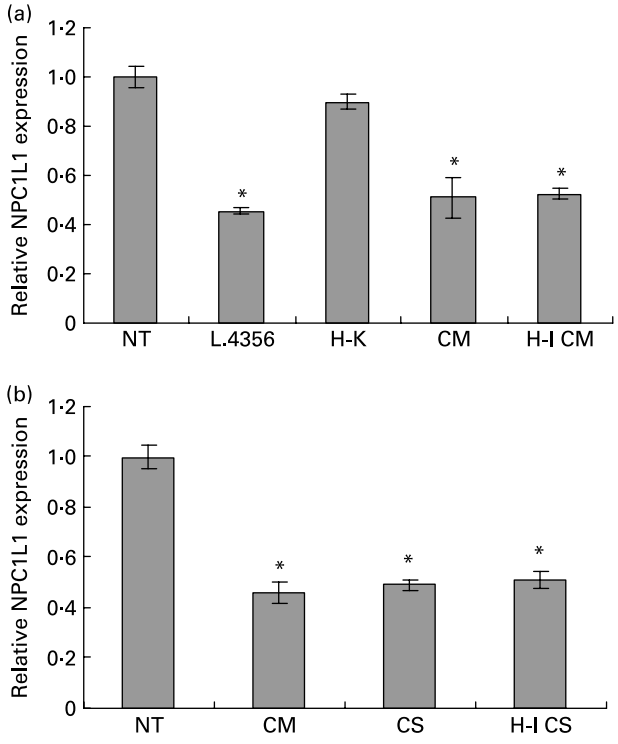


**Fig. 2.** Niemann-Pick C1-like 1 expression is regulated by secreted factors from *Lactobacillus acidophilus* ATCC 4356 (L.4356). (a) Real-time PCR of Caco-2 cells stimulated for 6 h by viable or heat-killed L.4356 (H-K) as well as fresh (conditioned media (CM)) or heat-inactivated CM of 4356 (H-I CM), compared with the non-treated (NT) control. (b) Real-time PCR of Caco-2 cells stimulated for 6 h by CM, L.4356 culture supernatant (CS) or H-I CS, compared with the NT control. Bars signify means and standard deviations. Statistically significant differences *v.* the NT control were determined by a Dunnett's test (*$P<0{\cdot}05$).

NPC1L1 expression is no longer decreased by ATCC 4356 (Fig. 4(c)). Furthermore, ATCC4356 could not significantly reduce micellar cholesterol uptake in Caco-2 cells in which LXR were depleted by siRNA (Fig. 4(d)).

## Discussion

High levels of serum cholesterol have been associated with an increased risk of CHD. The use of probiotic bacteria to reduce serum cholesterol levels has attracted much interest in recent years[18]. We have investigated the regulation of NPC1L1 by specific probiotics in an attempt to characterise possible mechanisms of action on cholesterol absorption for these beneficial strains. We show that NPC1L1 expression can be decreased by L.4356 as well as other probiotic strains, as opposed to the common commensal *B. thetaiotaomicron*. The lactobacilli ATCC 4356 and *L. rhamnosus* GG were better inhibitors of NPC1L1 than the *Bifidobacterium B. lactis 12*, demonstrating distinctions among probiotic strains as well.

In order to address the mechanism of action for ATCC 4356, we monitored NPC1L1 expression in response to the different components of the bacteria–Caco-2 cell interaction. Inability of the heat-killed L.4356 to generate a response allowed us to exclude the bacterial wall components as stimuli. CS of ATCC 4356 grown without Caco-2 cells reduced NPC1L1 expression, as did conditioned medium from ATCC 4356 co-cultured with cells. These results indicate that soluble factors generated from bacteria alone, but not dependent on contact between bacteria and cells, suppress NPC1L1 expression. Other studies have reported similar observations, demonstrating that soluble factors secreted by probiotics are sufficient to beneficially modulate host cell functions[19,20]. Further characterisation of these soluble factors is important to exploit their beneficial effects more efficiently. Interestingly, heat inactivation did not alter the inhibitory effects of the CS on NPC1L1 expression. Taken together, these data suggest that probiotics, which secreted heat stable molecules, e.g. small oligopeptides or lipids, could be responsible for the inhibition.

Previous studies reported that lactobacilli are capable of removing cholesterol *in vitro* via various mechanisms, such as assimilation, binding to the surface of cells, incorporation into the cellular membrane and co-precipitation with deconjugated bile[15,21]. In contrast to previous studies, the results of the present study indicate that soluble factors in supernatants produced by ATCC 4356 in a medium containing bile salt and cholesterol were able to reduce cholesterol absorption by inhibiting NPC1L1 gene expression in Caco-2 cells.

It has been reported that the expression of NPC1L1 gene was downregulated by LXR activators in the intestine[8]. We hypothesised that LXR may be involved in our observed NPC1L1 suppression. Here, we show that L.4356 influenced NPC1L1 expression through the LXR-mediated signal transduction pathway. Upon stimulation of Caco-2 cells with L.4356, the expression of the LXR was significantly increased in Caco-2 cells. When LXR are depleted by siRNA in Caco-2 cells, NPC1L1 expression is no longer decreased by ATCC 4356, and no reduction in micellar cholesterol uptake was observed. This implies that ATCC 4356 induces NPC1L1 suppression via the LXR pathway.

In conclusion, ATCC 4356 inhibits the expression of NPC1L1, which results in decreased cholesterol uptake in Caco-2 cells. These findings suggest the intriguing possibility that it may be possible to modify NPC1L1, a central player

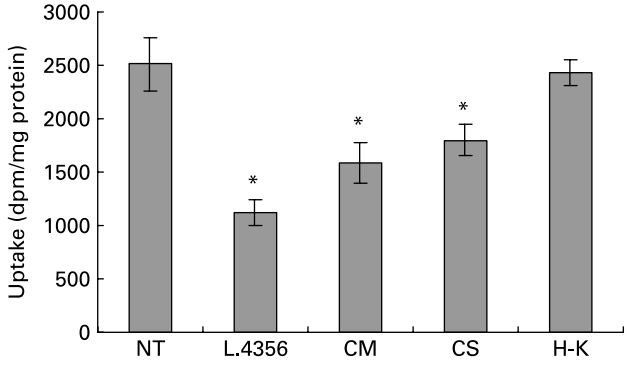


**Fig. 3.** *Lactobacillus acidophilus* ATCC 4356 inhibits cholesterol uptake in Caco-2 cells. Caco-2 cells were co-cultured with viable ATCC 4356 or heat-killed ATCC 4356 (H-K) as well as conditioned media (CM) or ATCC 4356 culture supernatant (CS). At 1 h before harvesting the cells, the medium was supplemented with 0·15 ml of the micellar solution. At the end of the incubation, the cells were washed thoroughly and cellular lipids were extracted. The radioactivity in the cellular lipid extract was then measured. The values shown are means and standard deviations of three independent experiments. Statistically significant differences *v.* the non-treated (NT) control were determined by a Dunnett's test (*$P<0{\cdot}05$).

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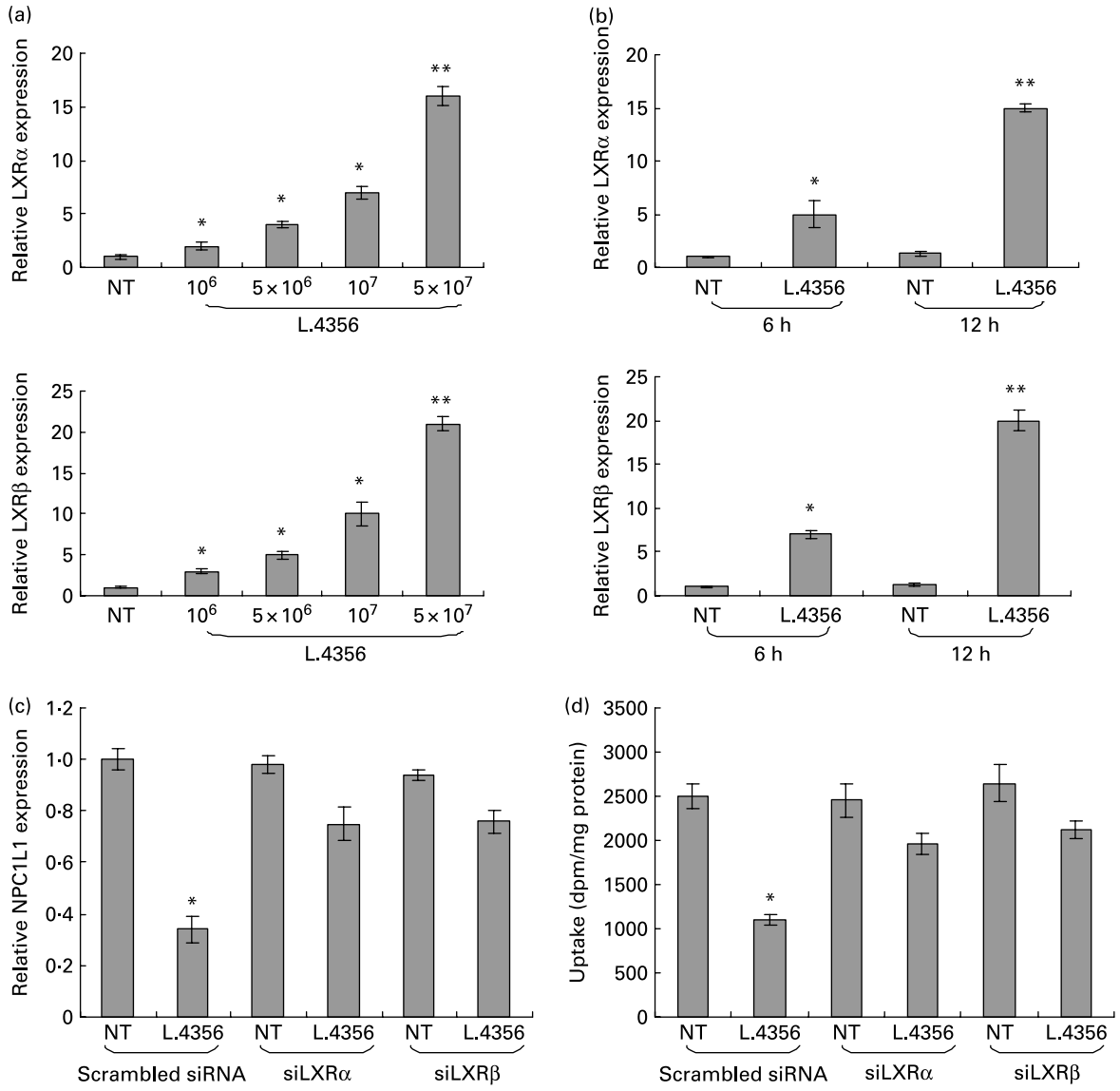


**Fig. 4.** *Lactobacillus acidophilus* ATCC 4356 (L.4356) inhibits Niemann-Pick C1-like 1 (NPC1L1) gene expression and cholesterol absorption, which may be mediated through liver X receptors (LXR). (a) Expression analysis of LXR in Caco-2 cells after 6 h of stimulation with increasing concentrations of ATCC 4356. Data are presented as means and standard deviations. *$P<0{\cdot}05$ compared with the non-treated (NT) control (by Dunnett's test for multiple comparisons) (b) The expression of LXR in Caco-2 cells co-cultured with ATCC 4356 ($10^7$ per ml) for 6 and 12 h compared with the NT. (c) Inhibition of NPC1L1 expression in Caco-2 cells after 6 h incubation with L.4356, by transfecting short interfering RNA (siRNA) for LXR compared with scramble control transfections. (d) After the inhibition of LXR expression using siRNA, Caco-2 cells were incubated with L.4356 for 6 h. At 1 h before harvesting the cells, the medium was supplemented with 0·15 ml of the micellar solution. At the end of the incubation, the cells were washed thoroughly, and cellular lipids were extracted. The radioactivity in the cellular lipid extract was then estimated. The values shown are means and standard deviations of three independent experiments. Statistically significant differences *v.* the NT control were determined by a Student's *t* test (*$P<0{\cdot}05$; **$P<0{\cdot}01$).

in cholesterol homeostasis, by manipulating the gut microbiota. We hypothesise that ATCC 4356, possibly via LXR signalling, could be a useful tool in cholesterol-lowering treatments.

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The authors have no conflicts of interest to declare.

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