J. Microbiol. Biotechnol. (2016), 26(2), 402–407
http://dx.doi.org/10.4014/jmb.1507.07086

jmb

Regulation of the *Lactobacillus* Strains on HMGCoA Reductase Gene Transcription in Human HepG2 Cells via Nuclear Factor-κB

Kun Chen[1]*, Shaocong Li[2], Fang Chen[1], Jun Li[1], and Xuegang Luo[3]

[1]*College of Pharmaceutical, Liaocheng University, Shandong 252059, P.R. China*
[2]*Department of Gynaecology and Obstetrics; Liaocheng People's Hospital, Shandong 252000, P.R. China*
[3]*Key Laboratory of Industrial Microbiology, Ministry of Education, University of Tianjin Science and Technology, Tianjin 300457, P.R. China*

Received: July 28, 2015
Revised: October 29, 2015
Accepted: October 31, 2015

First published online
November 3, 2015

**Corresponding author*
Phone: +86-635-8239680;
Fax: +86-635-8239680;
E-mail: chenkun97411@163.com

pISSN 1017-7825, eISSN 1738-8872

Lactic acid bacteria have been identified to be effective in reducing cholesterol levels. Most of the mechanistic studies were focused on the bile salt deconjugation ability of bile salt hydrolase in lactic acid bacteria. However, the mechanism by which *Lactobacillus* decreases cholesterol levels has not been thoroughly studied in intact primate cells. 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) is the vital enzyme in cholesterol synthesis. To confirm the effect of probiotic *Lactobacillus* strains on HMGCR level, in the present study, human hepatoma HepG2 cells were treated with *Lactobacillus* strains, and then the HMGCR level was illustrated by luciferase reporter assay and RT-PCR. The results showed that the level of HMGCR was suppressed after being treated with the live *Lactobacillus* strains. These works might set a foundation for the following study of the antihyperlipidemic effects of *L. acidophilus*, and contribute to the development of functional foods or drugs that benefit patients suffering from hyperlipidemia diseases.

Keywords: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase, cholesterol, *Lactobacillus*, nuclear factor κB

Introduction

Lactic acid bacteria (LAB) are regarded as safe microorganisms [1]. The main reported functions of LAB are related to promotion of the development of microflora, antidiabetic and antihyperlipidemic effects, health enhancement through the inhibition of carcinogenesis, anticolitic effect, and nonspecific activation of the host immune system [3].

The plasma cholesterol concentration can be regulated by the biosynthesis of cholesterol from saturated fat, removal of cholesterol from the circulation, absorption of dietary cholesterol, and excretion of cholesterol via bile and feces [6].

Cholesterol homeostasis in blood is vital for the prevention of cardiovascular and related diseases. Some mechanisms of serum cholesterol reduction by probiotics had been studied but the details remained unclear. The previous work had indicated that cholesterol assimilation was completed by bile salts, and cholesterol removal from the medium was decided by the concentration of bile salts [11]. However, Dambekodi and Gilliland [4] had shown no relationship between the amount of cholesterol removal in vitro and the degree of bile salt deconjugation, which led to another hypothesis that cholesterol removal may be related to assimilation of cholesterol. Experiments showed that strains that could remove cholesterol in vitro were also able to reduce the level of cholesterol in vivo. Cholesterol assimilation by LAB strains may be associated with bacterial growth and viability [9]. Nevertheless, the effects of LAB on the synthesis of cholesterol remain to be thoroughly studied.

3-Hydroxy-3-methylglutaryl-coenzyme A reductase HMGCR is the vital enzyme in cholesterol synthesis in human cells. Thus, could LAB or its products be involved in cholesterol synthesis network through HMGCR?

This study investigated the effects of LAB isolated from human intestine on cholesterol lowering in vitro through

the influence of LAB on the HMGCR promoter transcription activity regulated by the NF-κB family.

Materials and Methods

Microorganism and Related Preparations

Lactobacillus acidophilus TCCC 11036 (LA), *Lactobacillus rhamnosus* GG TCCC 11274 (LGG), *Lactobacillus casei* TCCC 13003 (LC), *Lactobacillus reuteri* TCCC 13353 (LR), and *Lactobacillus sakei* TCCC 11152 (LS) were gifts from the TCCC (Tianjin, China) and grown in MRS broth at 37°C. Gram-positive *Staphyloccocus aureus* was cultured in LB broth. The following protocols were modified from the previous work [3].

(i) *Lactobacillus acidophilus* (LIVE). *L. acidophilus* cells were suspended in MRS broth and cultured anaerobically at 37°C for 24 h. After centrifugation at 5,000 rpm for 15 min, the bacterial cells were resuspended at the designated concentration in HepG2 cell medium, and used immediately.

(ii) Heat-killed *L. acidophilus* (DEAD). Bacterial cells were heated at 80°C for 10 min, and then the cells were centrifuged and resuspended as described above.

(iii) *L. acidophilus* broth culture medium (SN) and soluble fraction (DC). *L. acidophilus* was cultured for 24 h. The supernatant was separated by centrifugation at 5,000 rpm for 15 min and monitored by 1 ml SN equivalent to 1×10^9 cells cultured. SN was saved at −20°C until use. The precipitated bacteria were washed once and resuspended in PBS and boiled for 30 min at 100°C, and then centrifuged at 12,000 rpm for 10 min at 4°C. Then the soluble fraction was released to liquid. DC was also saved at −20°C until use. Serial dilutions of SN and DC were used in the experiments.

(iv) *S. aureus* (TCCC11048) (SAL) and heat-killed *S. aureus* (SAD). The bacteria in culture broth were centrifuged at 4,000 rpm for 10 min, and then resuspended at the set concentration in HepG2 cell medium. *S. aureus* was heated at 80°C for 10 min and then centrifuged and resuspended.

(v) Genome DNA of *L. acidophilus* (GDNA). The genomic DNA of *L. acidophilus* was extracted following the protocol described by Sambrook [12].

Cell Culture

The human HepG2 cells were obtained from American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (Sigma) with 10% (v/v) fetal bovine serum at 37°C in a 5% CO_2 humidified incubator [1]. Lipopolysaccharide (LPS; 100 ng/ml) (*E. coli* O55:B5; Sigma) was used as a positive control [3].

Plasmids, Transient Transfections, and Luciferase Reporter Assay

The human HMGCR promoter reporter plasmid (HMGCR-Luc) contains an nt -1439 to +20 bp of 5′-flanking DNA linked to the luciferase reporter gene. pCMV2 constructs containing cDNAs for human NF-κB1 (p50) and RelA (p65) were generous gifts from Dr. Marty Mayo of the University of North Carolina, USA [8]. pNFκB-Luc (Clontech) was a commercial vector. All constructs were verified by DNA sequencing (Invitrogen). DNA transfections were carried out in 12-well plates by using TurboFect Transfection Reagent (Fermentas) according to the manufacturer's instructions. pEGFP-N1, a plasmid containing a promoter-driven green protein gene, was used as the control for transfection efficiency. Cells were lysed and the luciferase activity was then assayed with a Biotek Gen5 Microplate Reader.

RT-PCR Analysis

RNA was isolated by the Trizol method and reverse transcribed with moloney murine leukemia virus reverse transcriptase (Promega), and then amplified by RT-PCR using the following: human GAPDH primers (sense, 5′-ATTCAACGGCACAGTCAAGG-3′; and anti-sense, 5′-GCAGAAGGGGCGGA GATGA-3′) and human HMGCR primers (sense, 5′-TTCTTGCCAACTACT TCGTGTT-3′; and anti-sense, 5′-GCTGCCAAATTGGACGACC-3′). The PCR temperatures used were 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min, followed by extension of 5 min at 72°C. The amplified products were electrophoresed on a 2% agarose gel.

Results

Effect of LAB on Transcription of the HMGCR Gene

The four lactobacilli strains and SA (as a negative control) were examined for their abilities to influence the HMGCR promoter reporter gene in human HepG2 cells. After 24 h culture, the cells of LG, LR, LA, LC, and SA were centrifuged at 5,000 rpm for 15 min, and then the sediment was resuspended at the designated concentration in HepG2 medium and used immediately.

In this paper, a putative NF-κB binding site (GGGACA CTCC) at −265 bp was found by DNA BLAST. Thus, we deduced that the NF-κB family and cholesterol metabolism have some relations. To investigate the role of the LAB in HMGCR regulation, we tested the effects of live LAB cells on HMGCR transcription. For this purpose, the human HepG2 cells were transfected with the HMGCR-Luc and its NF-κB binding site mutation plasmid HMGCR-mutNFκB-Luc, respectively.

As shown in Fig. 1, the expression of luciferase was dramatically suppressed by the treatment with the four LAB separately in the HepG2 cells transfected with HMGCR-Luc. In contrast, in the HepG2 cells transfected with HMGCR-mutNFκB-Luc, as illustrated in Fig. 2, the luciferase levels had little response to the various LAB (Fig. 2).

NF-κB is Responsible for the Regulation of HMGCR

To test the specific effects of NF-κB on the transcription

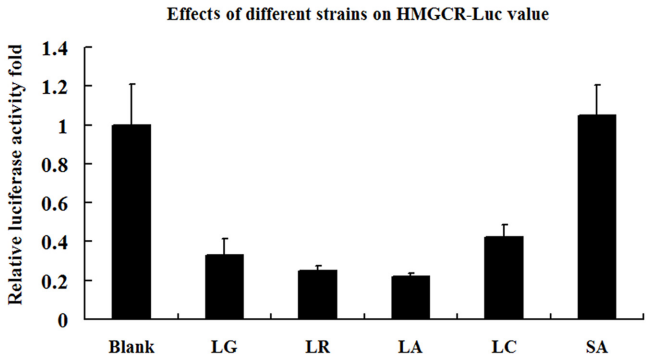

Fig. 1. Effect of various bacterial strains on HMGCR-Luc value. The HepG2 cells were transfected with the luciferase reporter construct for 12 h. Live *Lactobacillus* strains (LG, LR, LA and LC) or SA were then added at a concentration of 1×10^7 CFU/l, respectively. Cells were harvested 24 h post-transfection, and the luciferase activity was measured. The Blank represents cells left untreated with any stimulus.

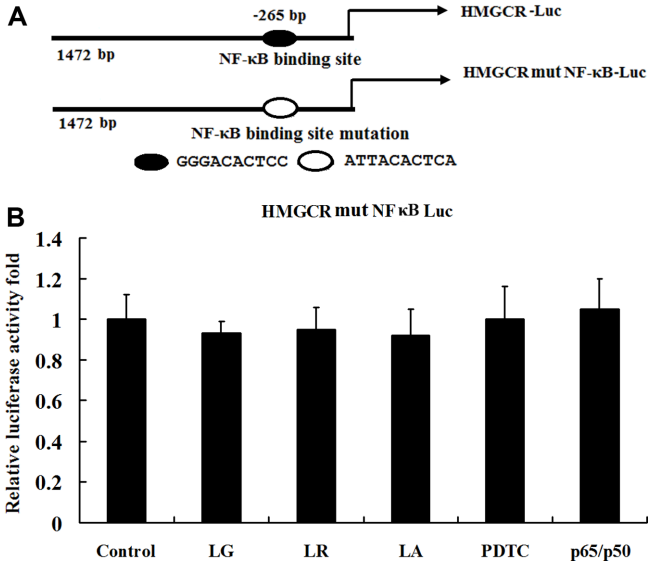

Fig. 2. Mutation of HMGCR-Luc at the NF-κB binding site. (**A**) HMGCR-mutNFκB-Luc is the mutation of HMGCR-Luc at the NFκB binding site. (**B**) Effects of *L. acidophilus* preparations on the promoter-luc activity. The HepG2 cells were transfected with the luciferase reporter construct for 12 h, respectively. Cells were then stimulated with LG, LR, LA, and p65/p50. PTDC was used as a positive stimulus. Cells were harvested 24 h post-transfection, and the luciferase activity was measured.

of the HMGCR gene, the RelA and p50 expression plasmids were co-transfected with the HMGCR-Luc vector into HepG2 cells. LPS is known to induce NF-κB activity

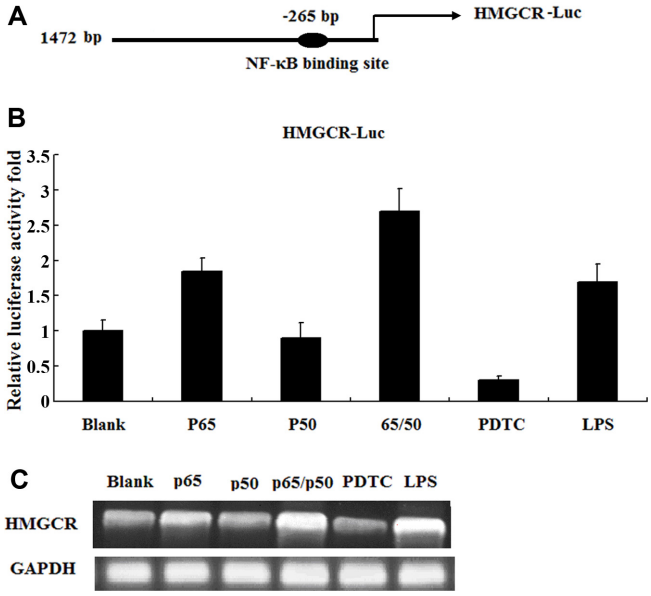

Fig. 3. NF-κBs were responsible for the regulation of HMGCR. (**A**) The luciferase construct of the HMGCR promoter; the NF-κB binding site is marked. (**B**) NF-κBs were responsible for the regulation of HMGCR-Luc. The HepG2 cells were transfected with the luciferase HMGCR promoter-reporter construct for 12 h. Some cells were also co-transfected with p65 or p50 alone or cotransfected with both p65 and p50. Cells were then stimulated or not stimulated with PDTC (10 μM) or LPS (100 ng/ml). Cells were harvested 24 h post-transfection and the luciferase activity was measured. The Blank bar stands for cells transfected with HMGCR-Luc alone. (**C**) RT-PCR analysis of the transcriptional level of the HMGCR gene. The HepG2 cells were transfected with p65 or p50 alone or cotransfected with p65 and p50. In contrast, cells were then treated with PDTC (10 μM) or LPS (100 ng/ml). Cells were harvested 24 h post-transfection, and the mRNA was extracted and RT-PCR was performed as described above. The Blank stands for cells neither transfected with NF-κB nor treated with PDTC or LPS. GAPDH was used as an internal control.

[14] and pyrrolidine dithiocarbamate (PDTC) is an antioxidant that has been widely used as a chemical inhibitor of NF-κBs [7]. From transfection assays with HepG2 cells, the NF-κB heterodimer p65-p50 remarkably upregulated the expression of the reporter gene, higher than p65 and p50. PDTC significantly attenuated the reporter gene activity. In this experiment, we found that RelA and p50 play an important role in the regulation of HMGCR expression.

Semiquantitative RT-PCR was carried out to evaluate the effects of NF-κBs on the endogenous HMGCR expression (Fig. 3). In agreement with the transfection results, the HMGCR expression was regulated by NF-κBs.

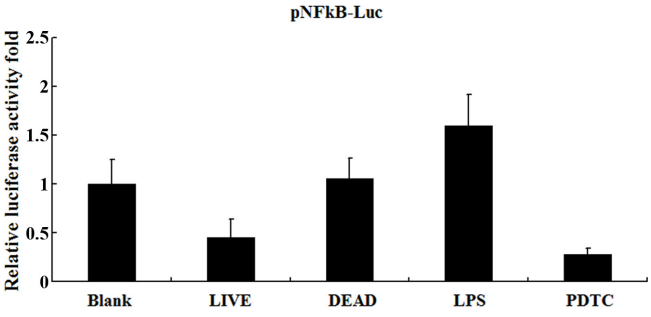

Fig. 4. Effects of LAB on the transcriptional activity of NF-κB.
The HepG2 cells were transfected with pNF-κB-Luc for 12 h and then stimulated with LA (Live or Dead), PDTC (10 μM), or LPS (100 ng/ml), respectively. Cells were harvested 24 h post-transfection, and luciferase activity was measured. The Blank represent cells untreated with any stimulus.

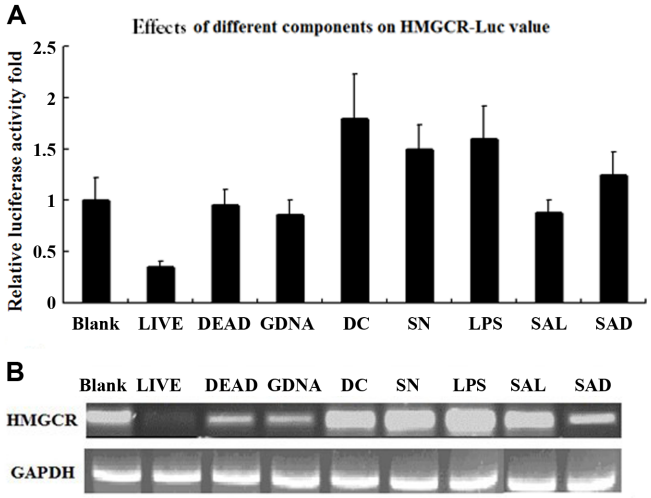

Fig. 5. Effects of *L. acidophilus* and related products on HMGCR.
(**A**) Effect on HMGCR-Luc value. The HepG2 cells were transfected with the luciferase HMGCR promoter reporter construct for 12 h. The following components were added. Cells were harvested 24 h post-transfection, and luciferase activity was measured. LIVE: live LA; DEAD, heat-killed LA; GDNA, genomic DNA of LA; DC, sediment of LA; SN, supernatant of fermented broth of LA; LPS, lipopolysaccharide; SAL, live *S. aureus*; SAD, heat-killed *S. aureus*. The Blank represents cells left untreated with any stimulus. (**B**) RT-PCR. Cells were cultured in 6-well plates and then were treated as described. After 24 h, cells were harvested and mRNA was extracted. RT-PCR was performed as described above. The Blank represents cells untreated with any stimulus.

In the following experiment, the effects of LAB on NF-κB expression were investigated with the pNFκB-Luc construct. As shown in Fig. 4, the live *L. acidophilus* remarkably downregulated the expression of the reporter gene. It was indicated that live LAB could repress HMGCR expression by imposing influence on the NF-κB family in HepG2 cells.

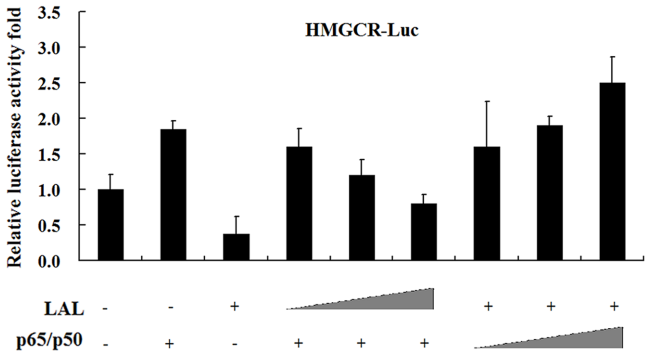

Fig. 6. Dose-response of live *Lactobacillus* on HMGCR-Luc value.
The HepG2 cells were transfected with the luciferase HMGCR promoter-reporter construct for 12 h. Different concentrations of Live LA (1×10^5, 1×10^6, and 1×10^7 CFU/ml) and p65/p50 plasmids were used to determine the dose response on HMGCR-Luc value. The LAL "+" represents the concentration of 1×10^7 CFU/ml. Cells were harvested 24 h post-transfection and luciferase activity was measured.

Effect of Different Bacterial Preparations on HMGCR

To identify HMGCR-Luc transfected into HepG2 cells in response to various forms of LAB, live *L. acidophilus* (LIVE), dead *L. acidophilus* (DEAD), genomic DNA of *L. acidophilus* (GDNA), boiled *L. acidophilus* (DC), supernatant of *L. acidophilus* fermented broth (SN), and live (SAL) or dead (SAD) *S. aureus* was added 12 h after transfection, respectively. The luciferase activity was measured 12 h later. As can be observed in Fig. 5, expression of the reporter gene was actually suppressed by living *L. acidophilus* but induced by boiled *L. acidophilus* (DC) and supernatant of *L. acidophilus* fermented broth (SN). Notably, the genomic DNA of *L. acidophilus* (GDNA) and live *S. aureus* (SAL) had relatively little influence on HMGCR-Luc.

The semiquantitative RT-PCR results were consistent with those obtained by HMGCR-Luc data.

Dose-Response of Live *Lactobacillus* Concentrations on HMGCR-Luc Value

Different concentrations of the live *L. acidophilus* (0, 1×10^5, 1×10^6, 1×10^7, 2×10^7, and 5×10^7 cells/ml) were used to test the dose response of live strain on reporter gene expression (data was not shown). As shown in Fig. 6, at a concentration of 1×10^5, 1×10^6, or 1×10^7 cells/ml, live *L. acidophilus* significantly inhibited luciferase gene expression and enhanced it when the amounts were higher.

Discussion

NF-κB/Rel is a family of ubiquitously expressed transcription factors, which bind a common DNA sequence (5′-GGGRNWYYCC-3′, where R represents any purine, Y is for pyrimidine, N is for nucleotide, and W is for adenine or thymidine) [13]. NF-κB is the central mediator of the inflammatory response and can be activated by a variety of stimuli, such as microbial and viral products, cytokines, DNA damage and noxious chemicals. Activated NF-κB then turns on many downstream target genes to mediate innate and adaptive immune responses, development of the immune system, cell survival, cell proliferation, and cell migration [16]. NF-κB p50:p65 and p50:p50 are the most ubiquitous and abundant NF-κB dimers.

The HMGCR catalyzes the conversion of HMG-CoA to mevalonic acid in cholesterol synthesis, representing the rate-limiting step. Recent experiments had suggested that the HMGCR mRNA level in mononuclear cells was lower in subjects consuming a high-fat diet compared with those consuming a high-carbohydrate diet [15]. It is reported that the expression of HMGCR was modified by the cholesterol and fat levels in the diet [1]. From the above progress, it appears that there is coordinated regulation of HMGCR genes and cholesterol level.

The previous work suggested that some components of LABs, such as LPS, cell wall-associated polysaccharides, proteins, and lipoteichoic acids, could stimulate some related genes in the cell signal pathway [5]. This study provided novel evidence that the transcription for HMGCR in HepG2 cells was affected by the addition of live *Lactobacilli* strains.

The data presented in this study demonstrated that NF-κB p50:p65 and p50:p50 played a central role in LAB-induced downregulation of HMGCR gene expression in HepG2 cells through a NF-κB binding site (GGGACA CTCC) at nt −265 bp, which enables cholesterol synthesis in vitro, possibly in response to NF-κBs. To gain insight into the mechanism underlying the repressed HMGCR transcription level in the absence of LAB, we assessed the effects of several LAB or various states of LAB on HMGCR-Luc and mRNA in human hepatoma HepG2 cells. Based on our findings, we speculate that a combination of dietary LAB would have cumulative inhibitory effects on cholesterol synthesis in humans because LAB would putatively lower the relative amount of HMGCR gene expression.

In conclusion, we identified a NF-κB binding site at nt −265 bp of the HMGCR gene, and this site has turned out to be one of the major regulative elements in cholesterol synthesis in HepG2 cells. In addition, NF-κB is crucial for LAB-mediated HMGCR gene regulation, and the NF-κB activation pathway may be a potential therapeutic target in HMGCR signaling.

Acknowledgments

Funding for this work was provided by doctoral scientific research funds of Liaocheng University and National Natural Science Foundation of China (No. 31401799 and No. 81402512).

References

1. Boucher P, de Lorgeril M, Salen P, Crozier P, Delaye J, Vallon JJ, *et al*. 1998. Effect of dietary cholesterol on low density lipoprotein-receptor, 3-hydroxy-3-methylglutaryl-CoA reductase, and low density lipoprotein receptor-related protein mRNA expression in healthy humans. *Lipids* **33:** 1177-1186.
2. Brown MS, Goldstein JL. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science* **232:** 34-47.
3. Chen K, Li J, Luo X, Liao X, Gao J, Hu X, *et al*. 2012. Induction of nitric oxide synthesis of a *Lactobacillus acidophilus* strain in human HepG2 cells. *Adv. Mater. Res.* **365:** 194-198.
4. Dambekodi PC, Gilliland SE. 1998. Incorporation of cholesterol into the cellular membrane of *Bifidobacterium longum*. *J. Dairy Sci*. **81:** 1818-1824.
5. Kim DW, Cho SB, Yun CH, Jeong HY, Chung WT, Choi CW, *et al*. 2007. Induction of cytokines and nitric oxide in murine macrophages stimulated with enzymatically digested *Lactobacillus* strains. *J. Microbiol.* **45:** 373-378.
6. Lin SY, Ayres JW, Winkler W Jr, Sandine WE. 1989. *Lactobacillus* effects on cholesterol: in vitro and in vivo results. *J. Dairy Sci.* **72:** 2885-2899.
7. Liu SF, Ye X, Malik AB. 1999. Inhibition of NF-kappaB activation by pyrrolidine dithiocarbamate prevents in vivo expression of proinflammatory genes. *Circulation* **100:** 1330-1337.
8. Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS Jr, Mayo MW. 2000. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-κB. *Mol. Cell. Biol.* **20:** 1626-1638.
9. Pereira DIA, Gibson GR. 2002. Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. *Appl. Environ. Microbiol.* **68:** 4689-4693.
10. Powell EE, Kroon PA. 1994. Low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in human mononuclear leukocytes is regulated coordinately and parallels gene expression in human liver. *J. Clin. Invest.* **93:** 2168-2174.

11. Rasic JL, Vujicic IF, Skrinjar M, Vulic M. 1992. Assimilation of cholesterol by some cultures of lactic acid bacteria and bifidobacteria. *Biotechnol. Lett*. **14:** 39-44.
12. Sambrook J. 2003. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.
13. Smale ST. 2011. Hierarchies of NF-κB target-gene regulation. *Nat. Immunol.* **12:** 689-694.
14. Spitzer JA, Zheng M, Kolls JK, Vande Stouwe C, Spitzer JJ. 2002. Ethanol and LPS modulate NF-kappaB activation, inducible NO synthase and COX-2 gene expression in rat liver cells in vivo. *Front. Biosci.* **7:** a99-a108.
15. Vidon C, Boucher P, Cachefo A, Peroni O, Diraison F, Beylot M. 2001. Effects of isoenergetic high-carbohydrate compared with high-fat diets on human cholesterol synthesis and expression of key regulatory genes of cholesterol metabolism. *Am. J. Clin. Nutr.* **73:** 878-884.
16. Wan F, Lenardo MJ. 2010. The nuclear signaling of NF-κB: current knowledge, new insights, and future perspectives. *Cell Res*. **20:** 24-33.