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Anticancer and Anti-Inflammatory Activity of Probiotic *Lactococcus lactis* NK34

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The anticancer and anti-inflammatory activities of probiotic *Lactococcus lactis* NK34 were demonstrated. Treatment of cancer cells such as SK-MES-1, DLD-1, HT-29, LoVo, AGS, and MCF-7 cells with 10^6 CFU/well of *L. lactis* NK34 resulted in strong inhibition of proliferation (>77% cytotoxicity, $p < 0.05$). The anti-inflammatory activity of *L. lactis* NK34 was also demonstrated in lipopolysaccharide-induced RAW 264.7 cells, where the production of nitric oxide and proinflammatory cytokines (tumor necrosis factor-α, interleukin-18, and cyclooxygenase-2) was reduced. These results suggest that *L. lactis* NK34 could be used as a probiotic microorganism to inhibit the proliferation of cancer cells and production of proinflammatory cytokines.

Keywords: Probiotic, *Lactococcus lactis*, anticancer activity, anti-inflammatory activity, cytokine

Lactococcus lactis is used as a fermentation starter in dairy or fermented foods and is a generally recognized as safe microorganism. Recently, probiotic *L. lactis* strains were reported to possess potential antipathogenic activity and suggested as functional foods for humans or additive to animal feed [2, 5, 16]. Probiotics are used to improve the intestinal microbiota balance. The intestinal microbiota has been reported to play a fundamental role in maintaining immune homeostasis [4, 8]. Immunity describes the state of having sufficient biological defenses to avoid infection, disease, or other unwanted biological invasion. Innate immunity is the natural resistance that provides resistance through several physical, chemical, and cellular approaches. Subsequent general defenses include secretion of chemical signals (cytokines) and antimicrobial substances, fever, and phagocytic activity associated with inflammatory responses. Through these approaches, innate immunity can prevent the colonization of pathogenic bacteria and the proliferation of cancer cells.

Chronic inflammation that occurs in inflammatory bowel diseases (IBDs) induces persistent damage along the digestive tract and plays a role in the long-term development of colorectal cancer [6, 11]. Because of the involvement of inflammation in carcinogenesis, strategies to prevent many types of cancer, including colon cancer, focused on the use of nonsteroidal anti-inflammatory drugs [15]. Cytokine-expressing inflammatory cells produce large amounts of nitric oxide (NO), prostaglandin E_2 (PGE_2), and cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). NO and PGE_2 are important pro-inflammatory mediators produced by inducible NO synthase and cyclooxygenase-2 (COX-2), respectively [12]. The development of allergy has been explained through insufficient or aberrant exposure to environmental microbes. In the past, avoidance of allergens has been the standard treatment for allergies. However, induction of tolerance by exposure to antigens is an alternative to avoidance under the instructions of a doctor. Therefore, probiotics may be a safe alternative for providing the necessary microbial stimulation [13].

Lactococcus lactis NK34 has been reported as a bacteriocin producer and probiotic strain that possesses antimicrobial activity, is tolerant to artificial gastric condition, reduces DNA damage, and is resistant to antibiotics [7]. However, *L. lactis* NK34 has not been studied for its anticancer effect against various cancer cells. Therefore, we investigated the

anticancer effect against various cancer cells and anti-inflammatory effect using NO and cytokine production.

L. lactis NK34 was stored at −70°C in de Man, Rogosa, and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) supplemented with 20% glycerol [7]. *L. lactis* NK34 was grown at 37°C on MRS agar plates. Before the experiments, overnight cultures were prepared in MRS broth. Cultures were harvested by centrifugation (10,000 ×*g*, 10 min); pellets were washed three times in phosphate-buffered saline (PBS) and then resuspended in PBS at a concentration of 10^7 colony forming units (CFU)/ml.

RAW 264.7 cells (murine macrophage cell line, KCLB 40071), MRC-5 cells (human lung cell line, KCLB 10171), SK-MES-1 cells (human lung carcinoma cell line, KCLB 30058), DLD-1 cells (human colon adenocarcinoma cell line, KCLB 30058), HT-29 cells (human colon adenocarcinoma cell line, KCLB 30038), LoVo cells (human colon adenocarcinoma cell line, KCLB 10229), AGS cells (human stomach adenocarcinoma cell line, KCLB 21739), and MCF-7 cells (human breast adenocarcinoma cell line, KCLB 30022) were obtained from the Korean Cell Line Bank (KCLB; Seoul National University, Seoul, Korea). The cell lines were cultured in RPMI 1640 (for DLD-1, LoVo, AGS, and MCF-7 cells) or Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) (for RAW 264.7, MRC-5, SK-MES-1, and HT-29) as the strain-dependent medium containing 10% fetal bovine serum (FBS; Gibco) and 1% streptomycin/penicillin (Gibco), at 37°C in an atmosphere of 5% CO_2 and 95% air.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of *L. lactis* NK34 [12]. Cells were plated at a density of 2 × 10^5 cells/well in a 96-well plate and cultured for 24 h. The culture medium was removed and the cell monolayers were washed twice with PBS. Then, 250 µl of culture medium and 10% FBS were added to each well. The wells were inoculated with 10^5 or 10^6 CFU/well of *L. lactis* NK34 and the plates were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. After 44 h, the supernatants were removed and the cells were washed once with PBS buffer. MTT solution (0.5 mg/ml) was added to the wells and the plates were incubated for 4 h. The purple MTT formazan crystals were dissolved by adding dimethyl sulfoxide to the wells. The absorbance was measured at 570 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). The values were used to determine cell viability: Cytotoxicity = {1− [(absorbance of sample at 570 nm)/(absorbance of control at 570 nm)]} × 100.

The morphological effects of *L. lactis* NK34 were observed using a microscope (Olympus IX51 Clone; Olympus Melville, NY, USA). Cells were plated at a density of 1 × 10^5 cells/well in a 24-well plate and cultured for 24 h. The culture medium was removed and the cell monolayers were washed twice with PBS. Then, 1 ml of culture medium and 10% FBS were added to each well. The wells were inoculated with 10^6 CFU/well of *L. lactis* NK34 and the plate was inoculated at 37°C in an atmosphere of 5% CO_2 and 95% air. After 24 h, the supernatants were removed and the cells were washed once with PBS buffer.

RAW 264.7 cells (1 × 10^6 cells/well) previously cultured in DMEM were stimulated for 24 h with lipopolysaccharide (LPS) (1 µg/ml) and *L. lactis* NK34 (10^5 and 10^6 cells/well) as previously described [8]. The NO concentration was determined by measuring the amount of nitrite in the cell culture supernatant using the Griess reagent. A 100 µl aliquot of the cell culture supernatant was mixed with 100 µl of Griess reagent and the mixture was incubated for 10 min at room temperature. The absorbance was measured at 540 nm using an ELISA plate reader (Molecular Devices), and the amount of NO was estimated from a calibration curve constructed using sodium nitrate as the standard.

Total RNA was isolated from cell pellets using the EzWay total RNA isolation kit (Koma Biotech, Seoul, Korea). RT-PCRs were performed using a reverse transcription master premix (5×) (Elpis Biotech, Daejeon, Korea) on a Bioer XP Thermal Cycler (Bioer Technology, Hangzhou, China). One microliter of total RNA was reversed transcribed in a 20 µl reaction mixture containing PCR buffer, dNTP mix, primers, Taq DNA polymerase, cDNA, and nuclease-free water. Amplification was performed in a thermal cycler programmed as follows: pre-denaturation step (95°C, 15 min); 30 cycles of denaturation (95°C, 5 min), annealing (55–60°C, 1 min), and extension (72°C, 1 min); and a final extension step (72°C, 10 min).

RAW 264.7 cells were seeded at a density of 2 × 10^5 cells/well in 96-well culture plates and incubated for 24 h at 37°C in an atmosphere of 5% CO_2 and 95% air. The cells were activated by addition of 10^6 CFU/well of *L. lactis* NK34. After 24 h of incubation, the supernatants were collected. The levels of TNF-α in the supernatants were determined using commercial ELISA kits (Koma Biotech). The production of TNF-α was demonstrated after the steps of coating antibody, blocking, treatment of each sample or standard, detection antibody, enzyme conjugation, colorization, and reading at 450 nm.

The anticancer activity of probiotic bacteria has been demonstrated in *in vivo* and *in vitro* systems [13]. The cytotoxicity of *L. lactis* NK34 was evaluated in various

Table 1. Cytotoxic effect of *L. lactis* NK34 against normal and cancer cells.

Cell lines	Cytotoxic effect (%)	
	10^5 CFU/well	10^6 CFU/well
Normal cell line		
MRC-5 (human lung)	0 ± 0.00^{a}	11.11 ± 0.00^{a}
Carcinoma cell line		
SK-MES-1 (human lung)	95.07 ± 0.00^{e}	96.71 ± 0.00^{b}
DLD-1 (human colon)	35.71 ± 0.01^{bc}	77.23 ± 0.00^{b}
HT-29 (human colon)	28.47 ± 0.03^{b}	97.05 ± 0.00^{b}
LoVo (human colon)	97.45 ± 0.00^{e}	97.64 ± 0.00^{b}
AGS (human stomach)	69.02 ± 0.00^{de}	82.07 ± 0.00^{b}
MCF-7 (human breast)	58.59 ± 0.00^{cd}	97.99 ± 0.00^{b}

Values are represented as the mean ± SD. Mean values followed by different letters in the same column are significantly different ($p < 0.05$).

cancer cells and normal cells using the MTT assay and morphology observation (Table 1 and Fig. 1). Proliferation of normal MRC-5 cells was inhibited by 11.11%, and therefore, this strain was considered as a low cytotoxic substance. Treatment of cancer cells with 10^6 CFU/well of *L. lactis* NK34 resulted in strong inhibition of proliferation. Proliferation of DLD-1, HT-29, and LoVo cells was inhibited by 77.23%, 97.05%, and 97.64%, respectively ($p < 0.05$). The anticancer activity was proportional to the cell concentration. The results from the MTT assay and morphological changes revealed that *L. lactis* NK34 can inhibit proliferation of cancer cells. Dextran produced by *Leuconostoc mesenteroides* B-1149 has been shown to inhibit the proliferation of cervical cancer cells (HeLa) and colon cancer cells (HT-29) [14]. However, their proliferation was inhibited by < 45% at the tested concentrations. *Lactobacillus acidophilus* KFRI342 inhibited 37.9% proliferation of human colon cancer cells, SNU-4 cells [3]. *Bacillus polyfermenticus* KU3, isolated from *kimchi*, inhibited 90% proliferation of LoVo, HT-29, AGS, and MCF-7 cells in 10^6 CFU/well treatment [9].

LPS is a major component of the outer membrane of gram-negative bacteria and elicits strong immune responses

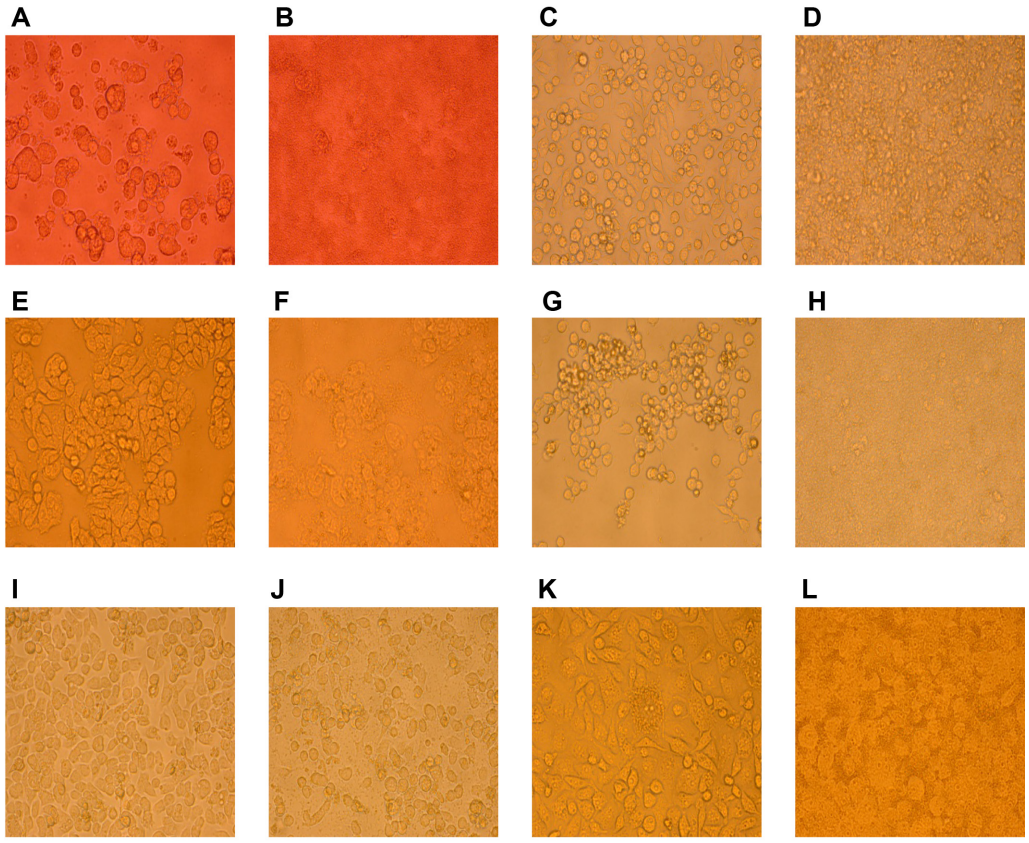

Fig. 1. Morphological change of cancer cells by *L. lactis* NK34.
(**A**) SK-MES-1, (**B**) SK-MES-1 with *L. lactis* NK34, (**C**) DLD-1, (**D**) DLD-1 with *L. lactis* NK34, (**E**) HT-29, (**F**) HT-29 with *L. lactis* NK34, (**G**) LoVo, (**H**) LoVo with *L. lactis* NK34, (**I**) AGS, (**J**) AGS with *L. lactis* NK34, (**K**) MCF-7, (**L**) MCF-7 with *L. lactis* NK34.

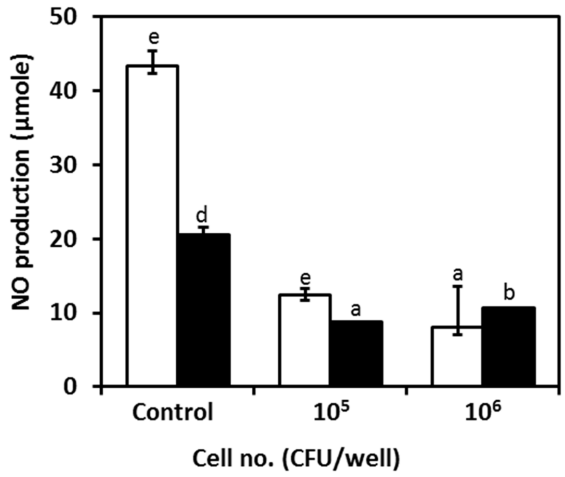

Fig. 2. Inhibitory effect of *L. lactis* NK34 in the NO production of RAW 264.7 cells induced by lipopolysaccharide.
□ LPS-stimulated RAW 264.7 cells, ■ non-LPS-stimulated RAW 264.7 cells. The values represent the mean ± SD. Mean values followed by different letters are significantly different ($p < 0.05$).

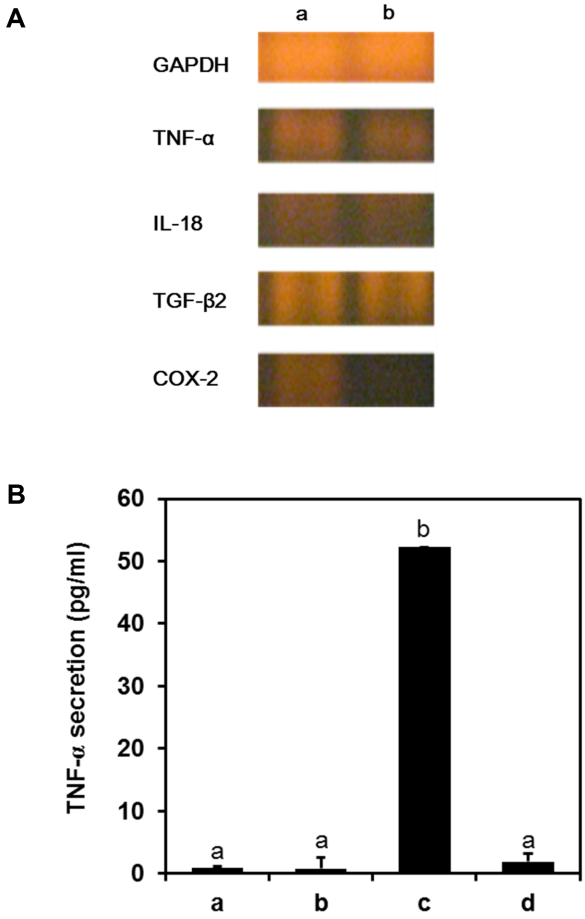

Fig. 3. Anti-inflammatory properties of *Lactobacillus lactis* NK34.
(**A**) Cytokine expression of GAPDH, TNF-α, IL-18, TGF-β2, and COX-2. a, LPS-stimulated RAW 264.7 cells; b, *L. lactis* NK34-treated LPS-stimulated RAW 264.7 cells. (**B**) Secretion of TNF-α in RAW 264.7 cells. a, non-LPS-stimulated RAW 264.7 cells; b, *L. lactis* NK34-treated non-LPS-stimulated RAW 264.7 cells; c, LPS-stimulated RAW 264.7 cells; d, *L. lactis* NK34-treated LPS-stimulated RAW 264.7 cells. The values represent the mean ± SD. Mean values followed by different letters are significantly different ($p < 0.05$).

[1]. In addition, microbial imbalance between gut microbiota with gram-negative bacteria and LPS produced by the latter ones play a key role in the pathogenesis of IBD. The anti-inflammatory activity of *L. lactis* NK34 was evaluated using LPS as the inflammatory mediator in RAW 264.7 cells (Fig. 2). Following stimulation with LPS, NO production was reduced in all groups treated with *L. lactis* NK34 compared with cells not treated with *L. lactis* NK34. Therefore, we conclude that *L. lactis* NK34 has no pro-inflammatory properties, which suggests that it is safe for use in humans.

Anti-inflammatory properties of probiotics have been demonstrated *in vitro*, in animal models, and even in clinical trials. Fig. 3 shows the variation in the values of TNF-α, IL-18, TGF-β2, and COX-2 as inflammatory biomarkers. Treatment with *L. lactis* NK34 reduced the secretion of pro-inflammatory cytokines TNF-α, IL-18, and COX-2 in LPS-stimulated RAW 264.7 cells (Fig. 3A). However, anti-inflammatory cytokine TGF-β2 was not influenced noticeable by treatment with *L. lactis* NK34.

Fig. 3B shows the amounts of TNF-α produced in RAW 264.7 cells. Control and *L. lactis* NK34-treated cells produced 0.869 pg/ml and 0.812 pg/ml of TNF-α, respectively. LPS-stimulated and *L. lactis* NK34-treated LPS-stimulated RAW 264.7 cells produced 52.209 pg/ml and 1.838 pg/ml of TNF-α, respectively ($p < 0.01$). Inhibition of an inflammatory response by the probiotic strain after stimulation with LPS suggests that *L. lactis* NK34 interacts with LPS, possibly by preventing interactions of LPS with cells. LPS induced the production of TNF-α, which was reversed by *L. lactis* NK34. Intestinal bacterial pathogens such as *Escherichia coli* and *Enterococcus faecalis* induced the production of the pro-inflammatory cytokine TNF-α [10].

In conclusion, probiotic *L. lactis* NK34 was cytotoxic against SK-MES-1, DLD-1, HT-29, LoVo, and AGS cancer cells, but not normal MRC-5 cells, using MTT assay. The anti-inflammatory effect of *L. lactis* NK34 was demonstrated by decreases of NO production and pro-inflammatory cytokines. These results suggest that *L. lactis* NK34 could

be used as a probiotic microorganism for its anticancer and anti-inflammatory effects.

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References

1. Baumgart DC, Thomas S, Przesdzing I, Metzke D, Bielecki C, Lehmann SM, *et al*. 2009. Exaggerated inflammatory response of primary human myeloid dendritic cells to lipopolysaccharide in patients with inflammatory bowel disease. *Clin. Exp. Immunol.* **157:** 423-436.
2. Commane D, Hughes R, Shortt C, Rowland I. 2005. The potential mechanisms involved in the anti-carcinogenic action of probiotics. *Mut. Res.* **591:** 276-289.
3. Chang JH, Shin YY, Chae SK, Chee KM. 2010. Probiotic characteristics of lactic acid isolated from *kimchi*. *J. Appl. Microbiol.* **109:** 220-230.
4. Festen EAM, Szperl AM, Weersma RK, Wikmenga C, Wapenaar MC. 2009. Inflammatory bowel disease and celiac disease: overlaps in the pathology and genetics, and their potential drug targets. *Curr. Drug Targets Immune Endocr. Metab. Disord.* **9:** 199-218.
5. Heo WS, Kim YR, Kim EY, Bai S, Kong IS. 2013. Effects of dietary probiotic, *Lactococcus lactis* subsp. *lactis* I2, supplementation on the growth and immune response of olive flounder (*Paralichthys olivaceus*). *Aquaculture* **376-379:** 20-24.
6. Lakatos L, Lakatos PL. 2006. Is the incidence and prevalence of inflammatory bowel diseases increasing in Eastern Europe? *Postgrad. Med. J.* **82:** 332-337.
7. Lee NK, Noh JE, Choi GH, Park E, Chang HI, Yun CW, *et al*. 2007. Potential probiotic properties of *Lactococcus lactis* NK34 isolated from *jeotgal*. *Food Sci. Biotechnol.* **16:** 843-847.
8. Lee NK, Kim SY, Han KJ, Eom SJ, Paik HD. 2014. Probiotic potential of *Lactobacillus* strains with anti-allergic effects from *kimchi* for yogurt starters. *LWT Food Sci. Technol.* **58:** 130-134.
9. Lee NK, Son SH, Jeon EB, Kim GH, Lee JY, Paik HD. 2015. The prophylactic effect of probiotic *Bacillus polyfermenticus* KU3 against cancer cells. *J. Funct. Foods* **14:** 513-518.
10. Marcinkiewicz J, Ciszek M, Bobek M, Strus M, Heczko PB, Kurnyta M, *et al*. 2007. Differential inflammatory mediator response *in vitro* from murine macrophages to lactobacilli and pathogenic intestinal bacteria. *Int. J. Exp. Pathol.* **88:** 155-164.
11. O'Sullivan GC, Kelly P, O'Halloran S, Collins C, Collins JK, Dunne C, Shanahan F. 2005. Probiotics: an emerging therapy. *Curr. Pharm. Des.* **11:** 3-10.
12. Otte JM, Mahjurian-Namari R, Brand S, Werner I, Schmidt WE, Schmitz F. 2009. Probiotics regulate the expression of COX-2 in intestinal epithelial cells. *Nutr. Cancer* **61:** 103-113.
13. Ouwehand AC. 2011. Allergenic effects of probiotics. *J. Nutr.* **137:** 794S-797S.
14. Shukla R, Iliev I, Goyal A. 2014. *Leuconostoc mesenteroides* NRRL B-1149 as probiotic and its dextran with anticancer properties. *J. Biosci. Biotechnol.* **3:** 79-87.
15. Westbrook AM, Szakmary A, Schiestl RH. 2010. Mechanisms of intestinal inflammation and development of associated cancers: lessons learned from mouse models. *Mutat. Res.* **705:** 40-59.
16. Zhou X, Wang Y, Li W. 2010. Inhibition ability of probiotic, *Lactococcus lactis*, against *A. hydrophila* and study of its immunostimulatory effect in tilapia (*Oreochromis niloticus*). *Int. J. Eng. Sci. Technol.* **2:** 73-80.