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-\(\alpha\), 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
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, SK-MES-1, DLD-1, HT-29, LoVo, and MCF-7 cells were inoculated with 10^5 L. lactis NK34 and 1% streptomycin/penicillin (Gibco), 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 Bior Ex Thermal Cycler (Bior Technology, Hangzhou, China). One microliter of total RNA was reversely 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 10^5 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.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cytotoxic effect (%)</th>
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<tr>
<td></td>
<td>10^5 CFU/well</td>
</tr>
<tr>
<td>Normal cell line</td>
<td></td>
</tr>
<tr>
<td>MRC-5 (human lung)</td>
<td>0 ± 0.00^a</td>
</tr>
<tr>
<td>Carcinoma cell line</td>
<td></td>
</tr>
<tr>
<td>SK-MES-1 (human lung)</td>
<td>95.07 ± 0.00^b</td>
</tr>
<tr>
<td>DLD-1 (human colon)</td>
<td>35.71 ± 0.01^bc</td>
</tr>
<tr>
<td>HT-29 (human colon)</td>
<td>28.47 ± 0.03^bc</td>
</tr>
<tr>
<td>LoVo (human colon)</td>
<td>97.45 ± 0.00^d,e</td>
</tr>
<tr>
<td>AGS (human stomach)</td>
<td>69.02 ± 0.00^d,e</td>
</tr>
<tr>
<td>MCF-7 (human breast)</td>
<td>58.59 ± 0.00^d,e</td>
</tr>
</tbody>
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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.
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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