

Probiotics Inhibit Lipopolysaccharide-Induced Interleukin-8 Secretion from Intestinal Epithelial Cells

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Abstract

It has been suggested that probiotics could be useful for the prevention of symptomatic relapse in patients with inflammatory bowel disease (IBD). Interleukin (IL)-8 has been well recognized as one of the pro-inflammatory cytokines that could trigger inflammation and epithelial barrier dysfunction. In this study, the anti-inflammatory effects of probiotics were investigated using a human epithelial cell line (HT-29). Probiotics from infant feces and kimchi were tested for their cytotoxicity and effects on adhesion to epithelial cells. The present results show that seven strains could form 70 % adhesion on HT-29. The probiotics used in this study did not affect HT-29 cell viability. To screen anti-inflammatory lactic acid bacteria, HT-29 cells were pretreated with live and heat-killed probiotics, and lipopolysaccharide (LPS) (1 µg/mL) was then added to stimulate the cells. The cell culture supernatant was then used to measure IL-8 secretion by ELISA, and the cell pellet was used to determine IL-8 and toll-like receptor (TLR-4) mRNA expression levels by RT-PCR. Some probiotics (KJP421, KDK411, SRK414, E4191, KY21, and KY210) exhibited anti-inflammatory effects through the repression of IL-8 secretion from HT-29 cells. In particular, *Lactobacillus salivarius* E4191, originating from Egyptian infant feces, not only decreased IL-8 mRNA expression, but also decreased TLR-4 expression. These results indicate that *Lactobacillus salivarius* E4191 may have a protective effect in intestinal epithelial cells.

Key words: anti-inflammatory, probiotics, interleukin-8, inflammatory bowel disease

Introduction

Probiotics are defined as living food supplements or components of bacteria that have beneficial effects on human health (Salminen *et al.*, 1998). Most probiotics are derived from bacteria that naturally colonize the human intestine. To be considered beneficial, a probiotic bacterium must fulfill certain criteria, which include a human origin, generally regarded as safe (GRAS) status, acid and bile stability, adherence to intestinal cells, persistence for some time in the gut, the ability to produce antimicrobial substances, antagonism against pathogenic bacteria, and the ability to modulate the immune response (Dunne *et al.*, 2001). Probiotic activity has been associated with lactobacilli, bifidobacteria, streptococci, enterococci, non-pathogenic *Escherichia coli*, and *Saccharomyces boulardii* (Shanahan, 2001).

Inflammatory bowel diseases (IBDs), including Crohn's

disease and idiopathic ulcerative colitis, altered gut permeability, mucosal inflammation, and ulceration are present (Mankertz and Schulzke, 2007). The intestinal microbiota in patients with IBD seems to drive an overactive immune response, which leads to disease expression and concurrent morbidity (Shanahan and Bernstein, 2009). The potential for probiotics to modulate the intestinal microbiota, to provide beneficial immunomodulatory effectors, and to restore epithelial barrier defects suggests that a probiotic strategy might prove a viable future treatment option for patients with IBD.

The normal intestinal microflora, which is estimated to consist of 400 different bacterial species, reaches the highest concentrations in the terminal ileum and colon (Chung *et al.*, 1975). Intestinal microflora produces toxic compounds such as Gram-negative bacterial endotoxin and harmful enzymes (e.g., β -glucuronidase and trypsinase), which produce cytotoxic or carcinogenic agents (Rhodes *et al.*, 1985). Cytotoxins and endotoxins may interact on the apical intestinal surface and induce a response in intestinal epithelial cells, which produce pro-inflammatory cytokines and other mediators of inflamma-

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tory activation of the mucosal immune system via toll-like receptor (TLR) and/or cytokine receptor signaling (Jung *et al.*, 1995). Intestinal epithelia are capable of releasing some pro-inflammatory cytokines such as interleukin-8 (IL-8), and can respond to enteric pathogens and release some pro-inflammatory cytokines, which in turn direct the movement of inflammatory cells of the lamina propria (Gewirtz *et al.*, 1998). Intestinal epithelial cells were thought to be the first and most important probiotic action targets. Alterations in epithelial barrier function have been implicated in a variety of intestine-related disorders, including enteric infections, IBD, food allergy, autism, and stress (Turner, 2006). Current reviews have summarized the factors initiating and perpetuating IBD from 4 basic viewpoints: genetics, immune dysregulation, barrier dysfunction, and the role of microbial flora (Kucharzik *et al.*, 2006).

Preclinical *in vivo* and *in vitro* studies have been undertaken to delineate the mechanisms underlying the beneficial effects of probiotics in IBD. Several *in vitro* studies have identified the signaling pathways involved in the interaction between lactobacilli and epithelial cells. Probiotics have been suggested to strengthen the epithelial barrier through various mechanisms, such as induction of mucin secretion, up-regulation of cytoprotective heat shock proteins (Petrof *et al.*, 2004), enhancement of tight-junction function, and prevention of epithelial cell apoptosis (Mennigen *et al.*, 2009).

The aim of study was to screen anti-inflammatory probiotic strains by measuring IL-8 production in lipopolysaccharide (LPS)-stimulated human intestinal epithelial HT-29 cells, to develop a combination of these strains that has synergetic effects on the inflamed model cells, and confirm the findings by mechanism analysis.

Materials and Methods

Bacterial strains and culture medium

The probiotic strains used in this study were obtained from the Food Microbiology Laboratory at Korea University (Seoul, Korea). The strains were originally isolated from Korean infant feces, Egyptian infant feces, and kimchi, that are listed in Table 1. The strains were cultured in de Man, Rogosa, Sharpe (MRS) broth (Difco, USA) at 37°C for 18 h. The stock cultures were maintained at -80°C with 50% glycerol as a cryoprotectant. The strains were subcultured 3 times prior to use.

Table 1. Probiotic strains used in this study

Origin	Strain
Korean infant feces (4 strains)	<i>Lactobacillus plantarum</i> KM13
	<i>Lactobacillus plantarum</i> KM29
	<i>Lactobacillus brevis</i> KY21
	<i>Lactobacillus plantarum</i> KY210
Egyptian infant feces (5 strains)	<i>Lactobacillus salivarius</i> E11
	<i>Lactobacillus fermentum</i> E30L
	<i>Lactobacillus fermentum</i> E432L
	<i>Lactobacillus fermentum</i> E464L
	<i>Lactobacillus salivarius</i> E4191
Kimchi (10 strains)	<i>Lactobacillus salivarius</i> SWW308
	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> SKR310
	<i>Lactobacillus fermentum</i> QWW311
	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> KFM402
	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> MSK408
	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> KDK410
	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> KDK411
	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> SRK414
	<i>Lactobacillus curvatus</i> KFP419
	<i>Leuconostoc paramesenteroides</i> KJP421

Heat killed (HK) bacteria

The strains were sub-cultured 3 times in MRS broth at 37°C for 18 h. Cultured cells were harvested by centrifugation at 11,336 g for 5 min, and then washed 3 times with Phosphate Buffered Saline (PBS). These cell pellets were suspended in PBS and heat-treated at 95°C for 1 h. The bacteria were then centrifuged, resuspended in PBS, and stored. The suspended cells were frozen at -80°C overnight, and then the cells were dried under vacuum for 48 h in a freeze dryer (Ilshin LabCo, Korea). Freeze-dried cells were suspended at RPMI 1640 medium (HyClone) containing 10% fetal bovine serum (FBS) without penicillin-streptomycin, and used immediately.

Adhesion assay

HT-29 cells were seeded in 24-well plates at a concentration of 5×10^5 cells/well and cultured in 1 mL of RPMI 1640 medium (without penicillin-streptomycin) until 95% confluent. Subcultured (3 times) probiotic strains were harvested by centrifugation at 11,336 g for 5 min, and then washed 3 times with PBS. The cell pellets were resuspended in RPMI 1640 medium (without penicillin-streptomycin), and then probiotics were added at 1×10^9 CFU/mL to HT-29 cells and incubated for 2 h at 37°C in 5% CO₂. After incubation, the HT-29 cell monolayers were washed 6 times with PBS to remove non-attached bacteria. The adherent cells were released from the wells with 0.1 mL of trypsin-EDTA (HyClone, USA) and plated onto MRS agar.

Proliferation assay

Cell proliferation was assessed by the 3'-(4,5,-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. HT-29 cells (1×10^4 cells/well) were seeded in duplicate in 96 well plates and cultured in 200 μ L of RPMI 1640 medium for 24 h. After treatment with live and heat-killed probiotics for 24 h, 100 μ L of medium was removed from the plate, 2 mg/mL MTT solution (Thiazolyl Blue Tetrazolium Bromide, Sigma, USA) was added, and the plate was incubated at 37°C for 2 h. The optical density (OD) was measured at 540 nm using an ELISA reader (Molecular Devices, USA). Cell viability was calculated relative to untreated control cells as follows: (viability (% control) = $100 \times (\text{absorbance of treated sample}) / (\text{absorbance of control})$).

Cells culture and stimulation

A human epithelial cell (HT-29) line was obtained from the Korea Cell Line Bank (KCLB, Korea). The cells were routinely cultured at 37°C and 5% CO₂ in RPMI 1640 medium (HyClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone) and 1% penicillin/streptomycin (HyClone). For the experiments, HT-29 cells were seeded in a 6-well plate (Palcon, USA) at 1×10^6 cells/well. After 24 h, the medium was replaced with fresh RPMI 1640 medium without penicillin/streptomycin, and the cells were pre-treated with the probiotic strains for 2 h at 37°C in 5% CO₂, and then LPS (*Escherichia coli* O111:B4, Sigma, USA) was added at 1 μ g/mL and incubated for 24 h to stimulate the cells. The negative control group was not stimulated with LPS.

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from the HT-29 cells using TRIZOL™ (Invitrogen, USA), and was further purified using acidic phenol-chloroform extraction. RNA integrity was assessed by electrophoresis on a denaturing 1.5% agarose gel.

Then, 3 μ g of total RNA was reverse transcribed in a

20 μ L reaction mixture with the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). Next, cDNA was amplified using Taq DNA polymerase (GENENMED Inc., Korea). PCR was performed with a GeneAmp PCR System 2400 (Applied Biosystems, USA). For IL-8, pre-denaturation for 3 min at 95°C was followed by 30 cycles of 30 s at 95°C (denaturation), 30 s at 65°C (annealing), and 30 s at 72°C (elongation). The last cycle was followed by a 10 min final elongation at 72°C. For TLR-4, the reaction included 28 cycles of 45 s at 95°C (denaturation), 45 s at 54°C (annealing), and 1 min at 72°C (extension). Primer sequences (Bioneer, Korea) used for the amplification of gene fragments are described in Table 2.

The RT-PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide (EtBr) staining. All results were normalized to GAPDH expression.

Enzyme linked immunosorbent assay (ELISA)

IL-8 was quantified in the cell culture supernatants using the human IL-8/NAP-1 immunoassay kit (Invitrogen, USA) according to the manufacturer's instructions with an ELISA reader (Molecular Devices, USA). In these assays, the lower and upper limits of detection were 5 and 1,000 pg/mL, respectively.

Statistical analysis

The results are expressed as the mean \pm SEM. Data were analyzed statistically with SPSS software (version 12.0KO) (SPSS Inc., USA). Differences between groups were assessed for statistical significance using the Student's *t* test. Asterisks (*) indicate a significant difference at less than 0.05. Unless stated otherwise, all data presented were mean values of duplicates, obtained from two separate runs.

Results and Discussion

Adhesion abilities of probiotics

The etiology of ulcerative colitis is still unclear. How-

Table 2. Primer sequences for reverse transcription PCR

Gene name	Primer direction	Sequence (5'-3')	Size (bp)	Reference
IL-8	Forward	GTTGTGAGGACATGTGGAAGCACT	359	GenBank NM_000584
	Reverse	CACAGCTGGCAATGACAAGACTGG		
GAPDH	Forward	ATGACCACAGTCCATGCCATC	271	GenBank NM_0020463
	Reverse	CCTGCTTCACCACCTTCTTG		
TLR-4	Forward	GACGTGGAAGTGGCAGAAGAGG	502	Chang <i>et al.</i> (2006)
	Reverse	TGACGGCAGAGAGGAGGTTGAC		

ever, increased aberrant enteric microflora, including harmful and potentially harmful bacteria, and decreased normal microflora, such as bifidobacteria and lactobacilli, have been regarded as an important pathogenic factor. It is believed that abnormal interactions between the colonic mucosal immune system and aberrant enteric microflora result in altered intestinal immunological function and trigger the inflammatory disease process (Fedorak and Madsen, 2004).

To evaluate whether the anti-inflammatory effect of probiotics is a result of a secreted factor, or if it requires contact between probiotics and cells, adhesion ability was assessed after 2 h of co-culture. *Lactobacillus rhamnosus* GG was used as positive control. Seven of the probiotic strains (*Leuconostoc paramesenteroides* KJP421, *Lactobacillus salivarius* E4191, *Leuconostoc mesenteroides* subsp. *mesenteroides* KFM402, *Leuconostoc mesenteroides* subsp. *mesenteroides* KDK411, *Leuconostoc mesenteroides* subsp. *mesenteroides* SRK414, *Lactobacillus brevis* KY21, and *Lactobacillus plantarum* KY210) were selected for their strong interaction with HT-29 cells, with adhesion values of greater than 70% of the inoculated bacteria. In contrast, *Lactobacillus salivarius* SWW308 showed the lowest adhesion capacity at 45.7% (Fig. 1).

Effect of live and heat-killed probiotics on cell proliferation

Most studies of the immunomodulatory effects of lactic acid bacteria have focused on viable microorganisms. However, heat-killed lactic acid bacteria not only possess immunomodulatory functions (Chuang *et al.*, 2007; Pochard *et al.*, 2002; Sashihara *et al.*, 2006), but also pro-

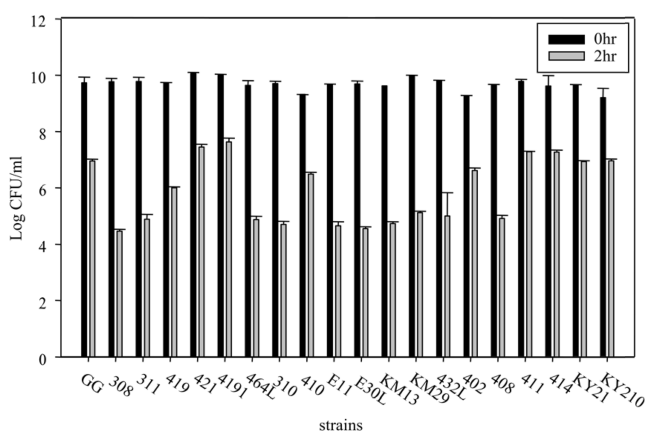


Fig. 1. Adherence of probiotic strains to intestinal epithelial HT-29 cells. 1×10^9 CFU/mL of each strain were cultured with a mono layer of HT-29 cells for 2 h. After washing 6 times with PBS, adherent bacteria were plated on de Man, Rogosa, Sharpe (MRS) agar.

vide the advantages of a longer product shelf life, easier storage, and more convenient transportation.

This study attempted to establish appropriate conditions for heat killed probiotics with immunomodulatory functions. Before measuring IL-8 secretion from HT-29, any possible toxicity of both the live and heat-killed probiotics was investigated. The MTT assay results indicated that cell viability was not affected by the lactic acid bacteria used in this study (Fig. 2).

Lipopolysaccharide (LPS)-induced IL-8 up-regulation

Neutrophil infiltration into inflammatory sites is a hallmark of acute inflammation. Locally produced chemotactic factors are thought to mediate the sequence of events leading to infiltration at inflammatory sites. IL-8, a novel leukocyte chemotactic activating cytokine (chemokine), is produced by various cell types in response to inflammatory stimuli, and exerts various effects on leukocytes, particularly neutrophils *in vitro* (Harada *et al.*, 1994).

IL-8 production has been observed *in vitro* in a wide variety of cells, including monocytes, T lymphocytes, neutrophils, vascular endothelial cells, dermal fibroblasts, keratinocytes, hepatocytes, and human gastric cancer cells (Yasumoto *et al.*, 1992). Moreover, although IL-8

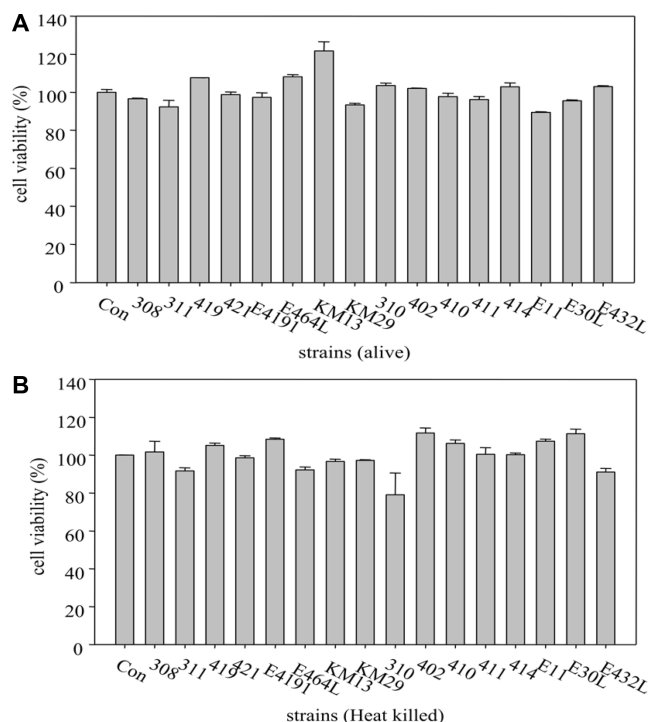


Fig. 2. Effects of live and heat-killed probiotics on cell viability. (A) Live probiotic, (B) Heat-killed probiotic. 1×10^6 bacteria were co-cultured with 1×10^4 cells at 37°C for 24 h, and then the MTT assay was performed.

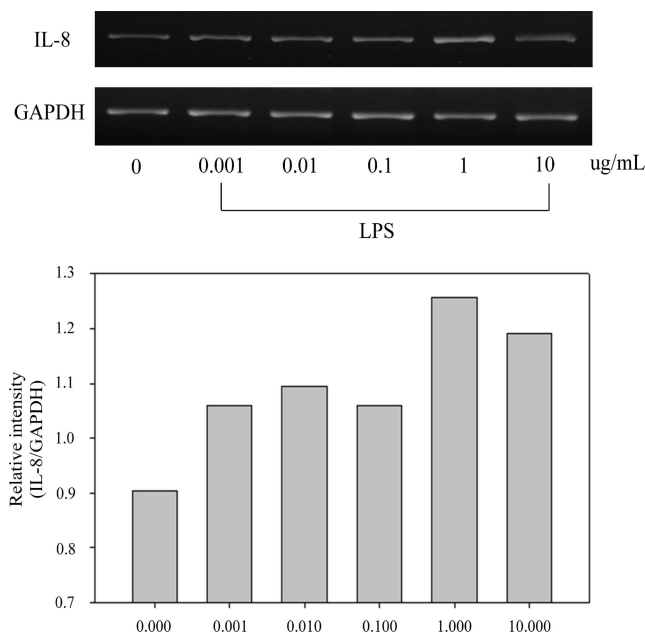


Fig. 3. Lipopolysaccharide induced interleukin (IL)-8 secretion from HT-29 cells. HT-29 cells were stimulated with 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 µg/mL, and 10 µg/mL LPS for 24 h. Expression of IL-8 was measured by RT-PCR.

production is not constitutive, it usually occurs in the presence of inflammatory stimuli such as LPS, IL-1, and tumor necrosis factor (TNF). In fact, IL-8 is not detected in normal adult plasma, but intravenous injection of LPS induces a massive elevation of plasma IL-8 (Martich *et al.*, 1991).

Our experiments measured IL-8 production in LPS-stimulated human intestinal epithelial HT-29 cells. To determine the optimal dose of LPS for IL-8 production, LPS (1 ng/mL, 10 ng/mL, 100 ng/mL, 1 µg/mL, and 10 µg/mL) was incubated with HT-29 cells for 24 h. At a concentration of 1 µg/mL, LPS significantly induced IL-8 production (Fig. 3). This concentration was then used in all subsequent experiments.

Live and heat-killed probiotics inhibited LPS-induced up-regulation of IL-8

To assess the anti-inflammatory properties of the probiotic strains, IL-8 production was evaluated by incubating HT-29 cell monolayers with LPS in the presence of the probiotic strains listed in Table 1.

Inhibition of IL-8 production was most effective when the ratio of cells to lactic acid bacteria was 1:10. To screen the anti-inflammatory lactic acid bacteria, 1×10^7 CFU of bacteria were added to 1×10^6 HT-29 cells. After a 2-h incubation, the cells were stimulated with 1 µg/mL LPS

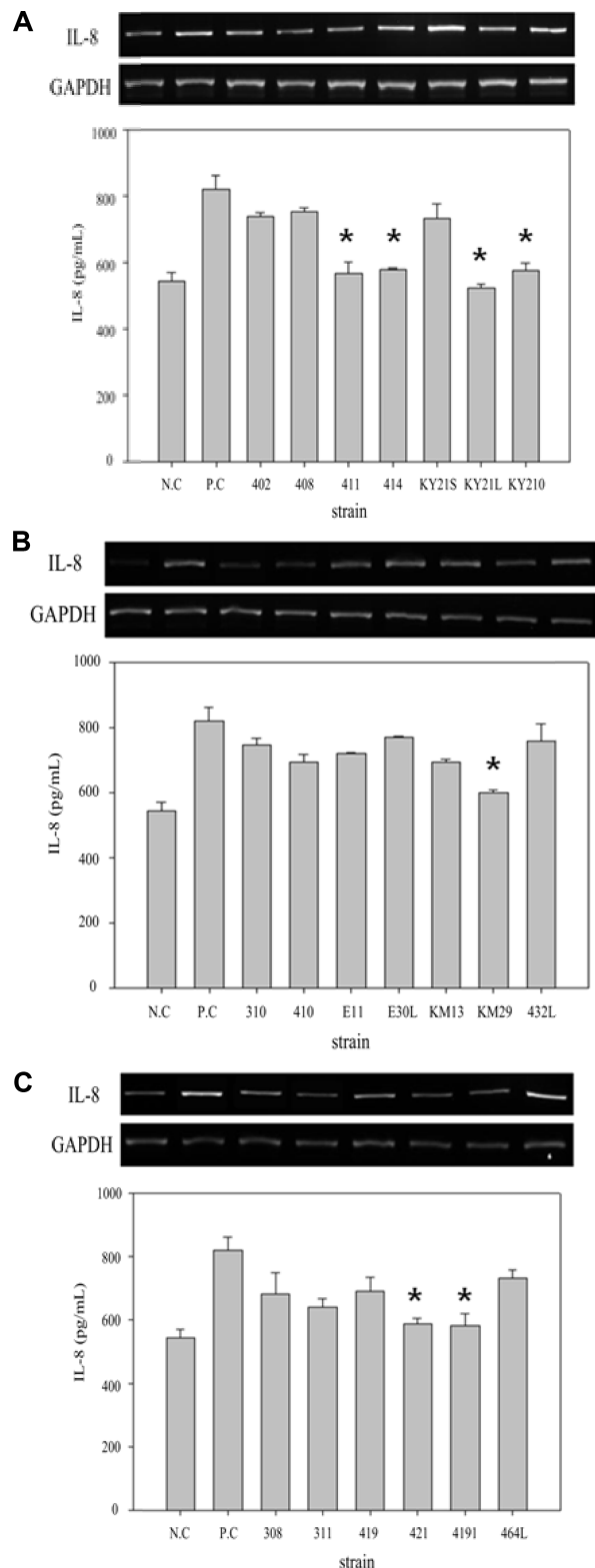


Fig. 4. Live probiotic strains induce IL-8 secretion from human intestinal epithelial cells (A, B, C). HT-29 cells (1×10^6) were pre-treated with 1×10^7 bacteria at 37°C for 2 h, and then 1 µg/mL of LPS was added and incubated for 24 h. The supernatant was used for ELISA, and the cells were used for RT-PCR to measure IL-8 expression. * $p < 0.05$, LPS treatment group (P.C.) versus LPS untreated group (N.C.).

for 24 h.

As shown in Fig. 4, *Leuconostoc paramesenteroides* KJP 421, *Lactobacillus salivarius* E4191, *Leuconostoc mesenteroides* subsp. *mesenteroides* KDK 411, *Leuconostoc mesenteroides* subsp. *mesenteroides* SRK 414, *Lactobacillus brevis* KY21, and *Lactobacillus plantarum* KY210 reduced the secretion of IL-8 by 71.6% (587.3 ± 17.3 pg/mL), 71% (582.3 ± 37.9 pg/mL), 69.2% (567.2 ± 32.6 pg/mL), 70.6% (579 ± 2.2 pg/mL), 63.7% (522.6 ± 12.4 pg/mL), and 70.4% (577.6 ± 20 pg/mL), respectively. These lactic acid bacteria were used for further study. In contrast, most heat-killed lactic acid bacteria did not show any effects on IL-8 secretion except *Lactobacillus salivarius* E4191 (Fig. 5).

Toll-like receptors (TLRs), which serve as a major link between the innate and adaptive mucosal immune responses, act as transmembrane co-receptors with CD14 in the cellular response to LPS (Ingalls *et al.*, 1999). TLR-4 is the primary mediator of LPS signaling (Cario and Pldolsky,

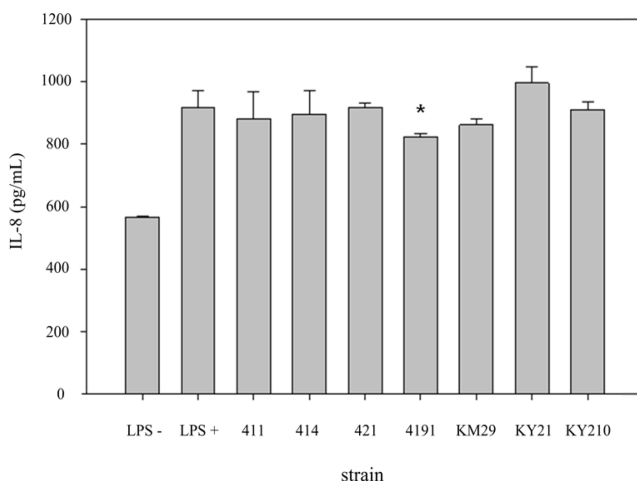


Fig. 5. Effect of heat-killed probiotic strains on IL-8 secretion from HT-29 cells. HT-29 cells (1×10^6) were pre-treated with 1×10^7 heat-killed bacteria at 37°C for 2 h, and then $1 \mu\text{g/mL}$ of LPS was added and incubated for 24 h. The supernatant was used for ELISA, and the cells were used to measure IL-8 expression by RT-PCR.

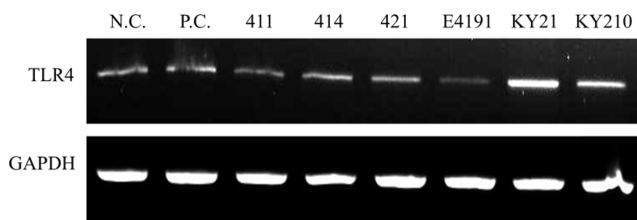


Fig. 6. Effects of probiotics on TLR4 expression in LPS-stimulated HT-29 cells. HT-29 cells were pre-treated for with each probiotic for 2 h before stimulation with LPS for 24 h. LPS treatment group (P.C.); LPS untreated group (N.C.).

2000). As shown in Fig. 6, live *Lactobacillus salivarius* E4191 also decreased TLR-4 expression. These data show that lactic acid bacteria directly affect intestinal epithelial cells, and the adhesion ability previous shown supports this conclusion.

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