

Probiotic Potential of *Enterococcus faecium* Isolated from Chicken Cecum with Immunomodulating Activity and Promoting Longevity in *Caenorhabditis elegans*^S

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Probiotics, including *Enterococcus faecium*, confer a health benefit on the host. An *Enterococcus* strain was isolated from healthy chicken cecum, identified as *E. faecium* by 16S rDNA gene sequence analysis, and designated as *E. faecium* L11. To evaluate the potential of *E. faecium* L11 as a probiotic, the gastrointestinal tolerance, immunomodulatory activity, and lifespan extension properties of the strain were assayed. *E. faecium* L11 showed >66% and >62% survival in artificial gastric juice (0.3% pepsin, pH 2.5) and simulated small intestinal juice (0.5% bile salt and 0.1% pancreatin), respectively. Heat-killed *E. faecium* L11 significantly ($p < 0.05$) increased immune cell proliferation compared with controls, and stimulated the production of cytokines (IL-6 and TNF- α) by activated macrophages obtained from ICR mice. In addition, *E. faecium* L11 showed a protective effect against *Salmonella* Typhimurium infection in *Caenorhabditis elegans*. In addition, feeding *E. faecium* L11 significantly ($p < 0.05$) extended the lifespan of *C. elegans* compared with the control. Furthermore, genes related to aging and host defense were upregulated in *E. faecium* L11-fed worms. In conclusion, *E. faecium* L11, which prolongs the lifespan of *C. elegans*, may be a potent probiotic supplement for livestock.

Keywords: *Enterococcus faecium*, immunomodulatory activity, probiotics, *Caenorhabditis elegans*, lifespan extension

Introduction

Lactic acid bacteria (LAB) are well-known probiotics possessing a variety of beneficial effects, including enhancement of immune responses and improvement of the intestinal microbial balance [1–3]. LAB strains are major commensal bacteria of the mammalian gastrointestinal tract, and their products have important roles in homeostasis and the function of innate and adaptive immune cells [4, 5]. Probiotics are proposed alternatives to antibiotics as both antimicrobial growth promoters and protectors against

infectious agents [6]. *Lactobacillus reuteri* enhances the growth performance of animals, increases the plasma immunoglobulin level, and decreases the number of gut *Escherichia coli* [7]. Similarly, *Enterococcus faecium* improves intestinal morphology [8], contributes to the regulation of intestinal innate immunity and homeostasis [9], and reduces the level of enteropathogenic *E. coli* in the intestinal contents of piglets [10]. In order to play a role as probiotics, candidate bacterial strains must be able to survive the stressful conditions in the host gastrointestinal environment [11]. In addition, the ability to enhance host resistance against

pathogens is a particularly desirable property of probiotics. Thus, the ability to inhibit *Salmonella* growth is used to select potential novel candidates to be used as probiotics in poultry [12].

Caenorhabditis elegans is a small soil nematode that feeds on bacteria, and is a natural host of some gram-negative pathogenic bacteria [13]. They are widely used as an experimental system for aging, various biological research studies, including bacterial infection, and immunological research, because of their morphological simplicity, suitability for genetic analysis, ease of maintenance in the laboratory, and short lifespan and reproductive cycle [14]. Previous reports have shown that LAB extend the lifespan of and contribute to host defense in *C. elegans* [15, 16].

The cecum of chicken has more diversity of LAB than the small intestine, and LAB isolated from poultry cecum have been reported to have higher enzyme secretion and activity than other probiotics [17, 18]. In this study, an *Enterococcus* strain, L11, was isolated from cecum of a healthy chicken and identified as *E. faecium*. To evaluate *E. faecium* L11's potential as a probiotic supplement for livestock, its digestive fluid tolerance, immunomodulatory activity, and ability to increase host resistance against pathogens and prolong the lifespan of *C. elegans* were investigated.

Materials and Methods

Isolation and Media

Enterococcus strains were isolated from healthy chicken cecum. The content of chicken cecum was serially diluted with saline. Diluted samples were spread-plated on deMan-Rogosa-Sharpe (MRS; Difco, Becton Dickinson Co., USA) agar and cultured for 48 h at 37°C in an anaerobic pouch kit (BBL GasPak, Becton Dickinson Microbiology System, USA) in a 5% CO₂ incubator. Among the 20 isolates, the colony showing the best antibacterial activity against *Salmonella* using a spot-on-lawn assay was selected (data not shown) and identified.

Identification of *Enterococcus* Strain L11

The selected colony was identified using the API CHL50 kit (biomérieux, France), morphology test, and 16S rDNA gene sequencing. Total genomic DNA from *Enterococcus* strain L11 was extracted by a protocol of Anandharaj and Sivasankari [19]. 16S rDNA gene fragments were amplified by polymerase chain reaction (PCR) using EGE 1 (5'-AGAGTTTGATCCTGGCTCAG-3') as a forward primer and EGE 2 (5'-CTACGGCTACCTGTTACGA-3') as a reverse primer [19]. The amplification conditions were 96°C for 3 min, followed by 30 cycles of 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min, and a final elongation at 72°C for 7 min. Sequencing was performed using a BigDye Terminator cycle sequencing system (Applied Biosystems

Inc., USA) and an ABI Prism 3730xl (Applied Biosystems Inc., USA). The sequence was deposited in GenBank under the accession number KM186186, and the Basic Local Alignment Search Tool was used to find homologous sequences. The ClustalX algorithm was used for multiple sequence alignments, and the phylogenetic relationship was analyzed by the MEGA 2 software program [20].

Tolerance of *Enterococcus* Strain L11 for Simulated Gastric and Small Intestinal Juices

The effect of artificial digestive juice on the viability of *Enterococcus* strain L11 was evaluated using a method described elsewhere [21, 22]. Gastric juice was simulated using a solution consisting of 2.05 g/l NaCl, 0.60 g/l KH₂PO₄, 0.37 g/l KCl, 0.11 g/l CaCl₂, and 0.3% (w/v) pepsin from porcine gastric mucosa (Sigma, USA), adjusted to pH 2.5 with 5 M HCl. *Enterococcus* strain L11 was grown in MRS broth at 37°C for 48 h, and collected by centrifugation at 8,000 ×g for 10 min at 4°C, and the cells were washed with saline. The cells were resuspended in the simulated gastric fluid to a final concentration between 1 × 10⁷ and 1 × 10⁸ colony forming units (CFU)/ml and then cultured at 37°C for 2 h. After treatment with artificial gastric juice, pellets were obtained at 8,000 ×g for 5 min and cultured in simulated small intestinal juice containing 6.4 g/l NaHCO₃, 0.239 g/l KCl, 1.28 g/l NaCl, 0.5% (w/v) bile salts (Oxgall; Sigma, USA), and 0.1% pancreatin (Sigma, USA) for 2 h at 37°C. All steps were carried out in an orbital shaker (at 200 rpm) to simulate the peristaltic movements. The incubation of cells with artificial digestive fluids was followed by serial dilution in 0.1 M sodium phosphate buffer solution (PBS, pH 6.2) to neutralize the acidity of the broth. Aliquots of the diluted cultures were spread on MRS plates and incubated for 2–3 days at 37°C, and then viable cells were counted. The data were estimated by calculating the relative survival rate (RSR) with Eq. (1) [23]:

$$RSR = \frac{\log CFUN_t}{\log CFUN_0} \times 100 \quad (1)$$

where N_0 is the total viable count for strain L11 before treatment, and N_t is the total viable count after treatment of artificial digestive fluid.

Animals

The Korea University Institutional Animal Care and Use Committee approved the experimental protocol (Permit No. KUIACUC-2013-195). Specific pathogen-free female (6-week-old) C3H/HeNH, BALB/c, and ICR mice were supplied by Koactec Company, Korea. All mice were fed a normal diet and water was freely available.

Detection of Intestinal Immune System Modulation using Peyer's Patch Cells

Suspensions of Peyer's patch (PP) cells were prepared as reported [24] with some modifications. PP cells isolated from the small intestines of C3H/HeNH mice ($n = 16$) were maintained in

Roswell Park Memorial Institute 1640 culture medium (RPMI 1640; Hyclone, USA) supplemented with streptomycin (100 µg/ml), penicillin (100 units/ml), and 10% fetal bovine serum. Cell suspensions (180 µl at 2×10^6 cells/ml in complete medium) were added to each well of a flat-bottomed 96-well microplate (Becton, Dickinson and Company, USA), and then 20 µl of heat-killed *Enterococcus* strain L11 was added at final concentrations of 1×10^6 , 1×10^7 , and 1×10^8 CFU/ml. Heat-killed *Enterococcus* strain L11 was prepared by heating the cells at 80°C for 20 min [25]. The plates were incubated at 37°C for 5 days in atmosphere with 5% CO₂. Then, the resulting supernatant (50 µl) was cultured with 100 µl of bone marrow (BM) cells (2.5×10^5 cells/ml) from C3H/HeNH mice in medium for 6 days in 5% CO₂-95% air. BM cell proliferation was evaluated by WST test using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt reagent [26], and the absorbance was measured at 450 nm using a Spectra MAX 340PC microplate reader (Molecular Devices, USA). The intestinal immune system response regulation of heat-killed *E. faecium* L11 was presented as a relative percentage of the BM cell proliferation compared with the control (treated with PBS). The positive control was lipopolysaccharide from *Escherichia coli* (10 µg/ml) (LPS; Sigma, USA).

Mitogenic Response Assay

Splenic lymphocytes were prepared as reported previously [27] with slight modifications. The splenocytes from BALB/c mice ($n = 4$) seeded in 96-well plates at 5×10^6 cells/ml were co-cultured with the heat-killed *E. faecium* L11 (final concentrations of 1×10^6 , 1×10^7 , and 1×10^8 CFU/ml) at 37°C for 48 h. Splenocyte stimulation was performed using the WST-1 assay.

Macrophage Proliferation

Macrophages were isolated as previously described [28]. ICR mice ($n = 5$) were intraperitoneally injected with 2 ml of 3% Thio-Glycollate medium (Becton, Dickinson and Company, USA). After 2 days, macrophages were isolated from the peritoneal cavity of mice and washed 3 times with 5 ml of RPMI 1640 containing antibiotics (100 µg/ml streptomycin and 100 units/ml penicillin). Then, 200 µl of macrophages (1×10^6 cells/ml) was seeded in a 96-well plate at 37°C for 2 h. The culture plate was washed with PBS to remove non-adherent cells, and 180 µl of culture medium was added to the attached macrophage layer. Heat-killed *E. faecium* L11 (final concentrations of 1×10^6 , 1×10^7 , and 1×10^8 CFU/ml) was treated to the wells and cultured at 37°C for 24 h. Macrophage proliferation was measured by colorimetric test using WST-1 reagent (Sigma-Aldrich, USA).

Assay of Cytokine Expression by Macrophages

For the cytokine assays, macrophages (1×10^6 cells/ml) isolated from ICR mice were activated with heat-killed *E. faecium* L11 at 37°C for 24 h. The cytokine concentration in macrophage culture supernatants was quantified using commercially available ELISA kits for interleukin-6 (BD OptEIA mouse IL-6 kit; BD Biosciences,

USA) and tumor necrosis factor- α (BD OptEIA mouse TNF- α kit; BD Biosciences, USA) as per the manufacturer's specification

Nematodes

Escherichia coli OP50 and *C. elegans* Bristol strain N2 (wild type) were provided by the *Caenorhabditis* Genetics Center, University of Minnesota (USA). Worms were maintained and propagated on peptone-free modified nematode growth medium (mNGM) agar plates at 25°C with standard techniques [29], using *E. coli* OP50 as the international standard feed [15]. *E. coli* OP50 was grown in Luria-Bertani medium (Becton, Dickinson and Company, USA) for 18–24 h at 37°C. Bacteria were collected by 10 min centrifugation at 3,000 $\times g$ and the cells were washed with M9 buffer solution.

Determination of Mean Lifespan of *C. elegans*

The influence of *Enterococcus* strain L11 on the longevity of *C. elegans* was determined as described previously [15, 16], with some modifications. The young adult worms were exposed to a sodium hypochlorite-sodium hydroxide solution to isolate fresh eggs. The egg suspension was spread on mNGM agar plates and then incubated overnight at 25°C to allow hatching [14]. The resulting suspension of L1 stage nematodes was centrifuged at 150 $\times g$ for 1 min, and the supernatant was removed. The remaining larvae were transferred onto fresh mNGM plates covered with *E. coli* OP50 and then incubated at 25°C for 2 days to allow the nematodes to reach the L4 stage. The 3-day-old worms (day 1 of adulthood) were transferred to mNGM/FUDR plates supplemented with 5-fluoro-2'-deoxyuridine (FUDR; Sigma Aldrich, USA) (50 µM) [30] and covered with a lawn of *Enterococcus* strain L11 or *E. coli* OP50. The worms were incubated at 25°C, and then the number of dead and live nematodes were scored every day by light microscopy (Olympus, Japan). *C. elegans* was determined as being dead when it failed to respond to a gentle touch with a dedicated picker. Each test was carried out in triplicate and repeated 3 times. The mean lifespan (MLS) of *C. elegans* was estimated using the following Eq. (2) [31]:

$$MLS = \frac{1}{N} \sum_j \frac{x_j + x_{j+1}}{2} d_j \quad (2)$$

where j is the age category (day), d_j is the number of nematodes that died in the age interval (x_j, x_{j+1}), and n is the total score of nematodes. The standard error (SE) of the mean lifespan was calculated by Eq. (3):

$$SE = \sqrt{\frac{1}{N(N-1)} \sum_j \left(\frac{x_j + x_{j+1}}{2} - MLS \right)^2 d_j} \quad (3)$$

Resistance against *Salmonella* Typhimurium Infection

After hatching, the worms were grown on *E. coli* OP50 until 3-day-old adult worms and then were allocated to a control group that was seeded with *E. coli* OP50 continually or a test group that was seeded with *E. faecium* L11 for 4 days. The 7-day-old worms were transferred to NGM agar plates with *Salmonella* Typhimurium

lawns and incubated at 25°C. The scores of dead and live worms were checked every day [15]. Each test was assessed with at least 100 nematodes.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Nematodes fed *E. coli* OP50 or *E. faecium* L11 for 24 h were harvested and washed twice with sterile M9 solution, and total mRNA was isolated using Trizol following previously published methods [32, 33]. Total RNA was converted to cDNA using the ReveritAid First Strand cDNA Synthesis kit system (Thermo Scientific, USA), followed by quantitative real time PCR using the SYBR Green (KAPA Biosystems, USA) and RT-PCR system (Quantstudio 6 flex; Applied Biosystems, USA). The primer sequences used in this study are shown in Table S1, and were designed using the Primer 3 software program [34]. The control gene *act-2* was used to normalize gene expression. The assays were carried out 3 times independently, and relative expression levels were estimated using the $2^{-\Delta\Delta CT}$ method [35].

Statistical Analysis

All results are indicated as the mean \pm standard deviation (SD). Statistical analysis was performed using SPSS ver. 10 (SPSS Inc., USA), and the difference between each sample and the control was evaluated for statistical significance using the Student's *t*-test. The *C. elegans* survival rate was estimated by the Kaplan-Meier method, and the significance of the differences in survival was evaluated using the log-rank test [36]. Differences were considered to be statistically significant when the *p* value was less than 0.05.

Results

Identification of *Enterococcus* Strain L11

The morphological and biochemical results of *Enterococcus* strain L11 are shown in Table S2. The characteristics showed that strain L11 was a gram-positive (G+) facultatively anaerobic coccus, which was oxidase and catalase negative. The isolate was identified as *Enterococcus faecium* by partial 16S rDNA gene sequence analysis (Fig. S1), and was designated as *E. faecium* L11.

Viability of *E. faecium* L11 in Simulated Gastric and Small Intestinal Juices

The ability of probiotic bacteria to endure the stressful conditions of the gut is very important [11]. The influences of artificial digestive juices on the viability of *E. faecium* L11 were investigated. The relative viability of *E. faecium* L11 to acidic condition containing 0.3% pepsin was $95.5 \pm 5.2\%$ and $66.8 \pm 3.3\%$ after 1 and 2 h exposure, respectively, compared with the viability at 0 h (Fig. 1). In simulated small intestinal juice containing bile salts and pancreatin,

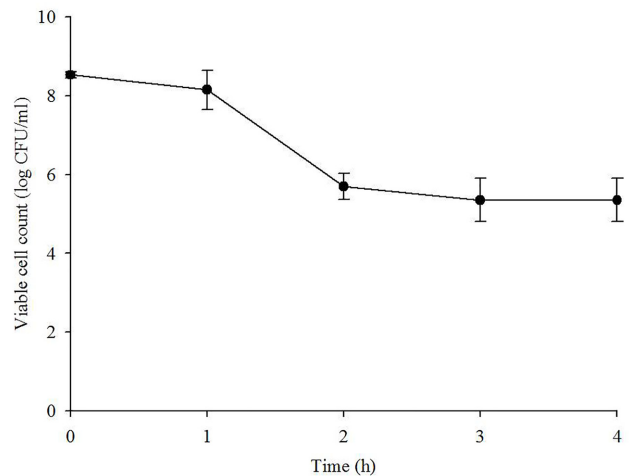


Fig. 1. Effect of artificial digestive fluid on the viability of *Enterococcus faecium* L11.

The cells were treated with artificial gastric juices for 2 h and then artificial small intestinal juice for 2 h and the viability was determined.

the relative viability was $62.7 \pm 6.0\%$ and $62.8 \pm 5.9\%$ after 1 and 2 h exposure, respectively, compared with the control (0 h). The data presented that *E. faecium* L11 is capable of surviving in digestive enzyme and low pH conditions.

Peyer's Patch-Mediated Intestinal Immune System Modulating Activity

The in vitro effect of heat-killed *E. faecium* L11 on PP cell-mediated intestinal immunoregulation was measured in C3H/HeNH mice. The results indicated that proliferation of BM cells significantly ($p < 0.05$) increased in the sample treated with the supernatant of PP cell culture with a high level of *E. faecium* L11 (1×10^8 CFU/ml) compared with the control (Fig. 2A). The proliferation of BM increased 1.14-fold compared with the control. The supernatants of PP cell cultures with lower levels of *E. faecium* L11 (1×10^6 and 1×10^7 CFU/ml) did not improve the stimulation of BM cells.

Direct Mitogenic Stimulation

To evaluate the effect of heat-killed *E. faecium* L11 on mitogenic stimulation in BALB/c mice, the proliferation of splenocytes was also examined by cell viability test in vitro. As shown in Fig. 2B, the proliferation of splenocytes treated with *E. faecium* L11 was significantly ($p < 0.05$) increased compared with the control (2.0-fold). The stimulation of splenocytes treated with *E. faecium* L11 (1×10^8 CFU/ml) was significantly ($p < 0.05$) increased compared with LPS (10 μ g/ml)-treated cells as well.

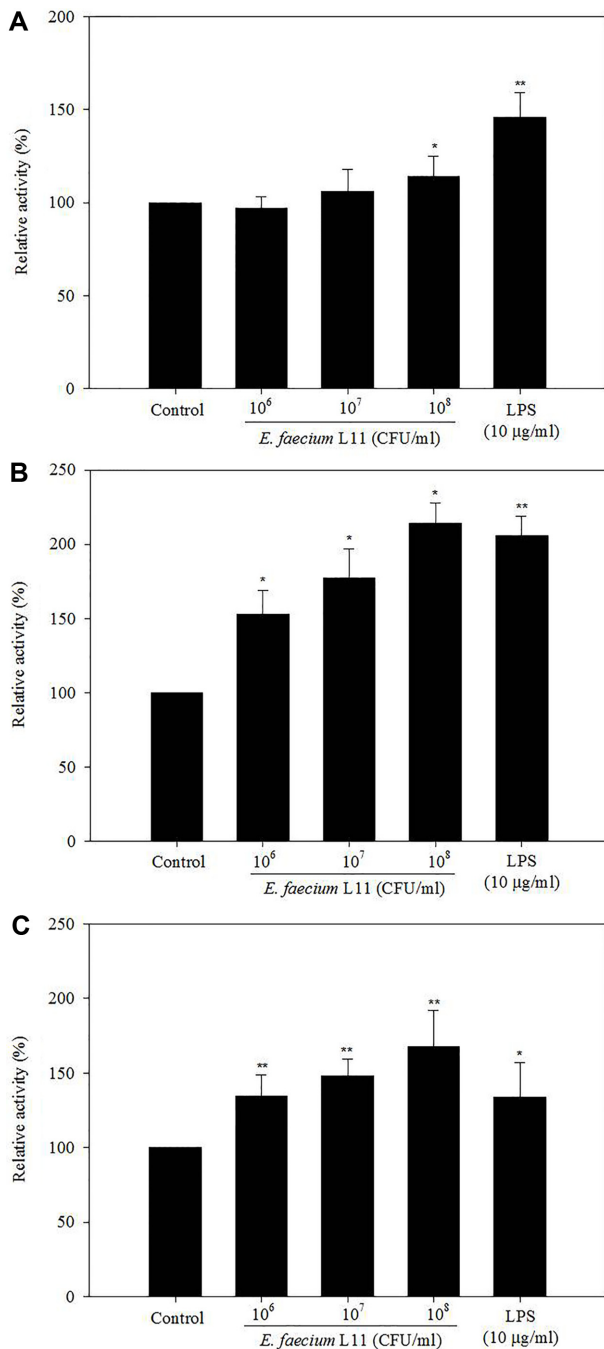


Fig. 2. Effect of heat-killed *E. faecium* L11 on the immune cell proliferation.

(A) Proliferation of bone marrow cells, which were obtained from Peyer's patch, was evaluated after treatment with heat-killed *E. faecium* L11. (B) Splenocytes stimulation was evaluated after treatment with heat-killed *E. faecium* L11. (C) Macrophages proliferation was measured after treatment with heat-killed *E. faecium* L11. The proliferation of these cells was assessed using WST-1 test. The control was treated with PBS. LPS was used as a positive control. * $p < 0.05$, ** $p < 0.01$ compared with the negative control.

Macrophage Proliferation

To evaluate the effect of heat-killed *E. faecium* L11 on mouse peritoneal macrophages, the stimulation of macrophages was determined in vitro. The proliferation of macrophages was significantly ($p < 0.01$) increased dose-dependently to $134.7 \pm 14.3\%$, $147.9 \pm 11.3\%$, and $167.8 \pm 23.9\%$ following treatment of heat-killed *E. faecium* L11 at the concentrations of 1×10^6 CFU/ml, 1×10^7 CFU/ml, and 1×10^8 CFU/ml, respectively (Fig. 2C).

Cytokine Expression by Macrophages

To assess the ability of *E. faecium* L11 to induce cytokine production, the concentrations of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) from stimulated macrophages were determined in vitro. In the sample treated with *E. faecium* L11 (1×10^6 , 1×10^7 , and 1×10^8 CFU/ml), the concentrations of IL-6 were 36.2 ± 32.7 , 253.5 ± 10.3 , and 1063.5 ± 14.7 ng/ml, respectively (Fig. 3A). Heat-killed *E. faecium* L11 significantly ($p < 0.05$) increased IL-6 production in a dose-dependent manner. The measurement of IL-6 in sample treated with the highest level of *E. faecium* L11 showed a 37-fold increase compared with the control (28.6 ± 21.8 ng/ml) treated with PBS. The formation of TNF- α by macrophages treated with *E. faecium* L11 was also significantly ($p < 0.05$) increased compared with the control. The concentrations of TNF- α in macrophage cultures treated with heat-killed *E. faecium* L11 (1×10^7 and 1×10^8 CFU/ml) and LPS (10 μ g/ml) were 119.7 ± 34.3 , $1,998.1 \pm 327.6$, and $1,748.4 \pm 245.7$ ng/ml, respectively, whereas the control and macrophage cultures treated with the lowest level of *E. faecium* L11 (1×10^6 CFU/ml) did not produce TNF- α (Fig. 3B).

Assessment of *C. elegans* Viability

To examine the beneficial effects of the newly isolated *E. faecium* L11 strain on resistance to pathogens, survival assays were performed. The lifespan of worms fed *E. faecium* L11 increased markedly compared with nematodes fed *E. coli* OP50 (Table 1). The mean lifespan of worms fed *E. faecium* L11 was 14.1% higher than that of the control ($p < 0.001$). The percentage of living worms fed *E. faecium* L11 was higher than that of living nematodes fed *E. coli* OP50 after 13 days (Fig. 4A). The effect of *E. faecium* L11 on pathogen resistance of worms against *S. Typhimurium* infection was higher than nematodes fed *E. coli* OP50 (Table 2, Fig. 4B). *S. Typhimurium* infection killed approximately 96% of *C. elegans* fed *E. coli* OP50 within 9 days after the worms were transferred to a *Salmonella* lawn plate, whereas 26% of worms fed *E. faecium* L11 survived ($p < 0.001$).

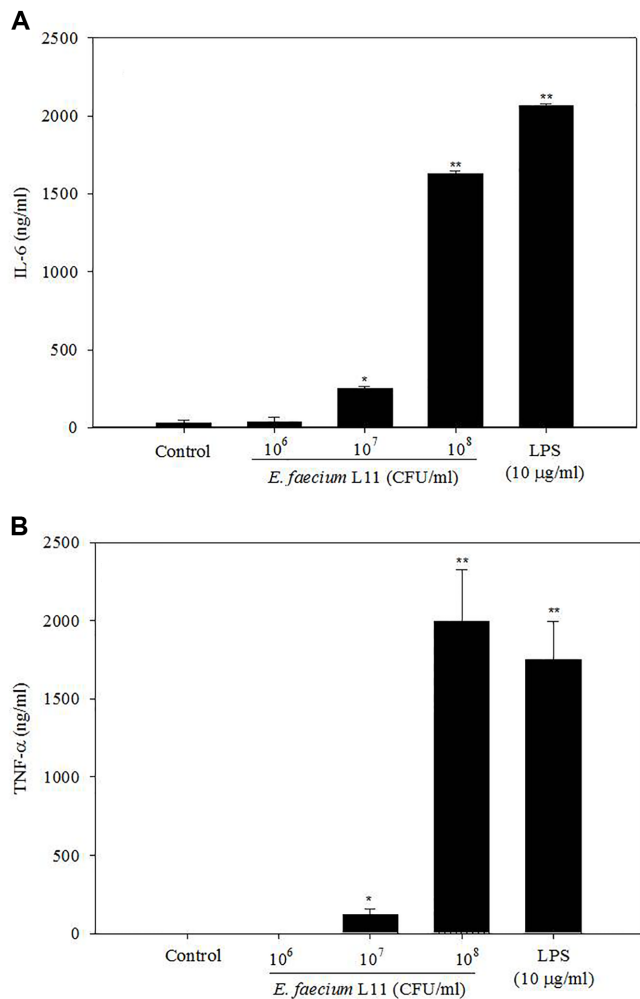


Fig. 3. Effect of heat-killed *E. faecium* L11 on the production of cytokines from mouse peritoneal macrophages. Macrophages were activated with heat-killed *E. faecium* L11. The level of each cytokine from stimulated macrophages was determined by ELISA kits. The control was treated with PBS. LPS was used as a positive control. (A) Interleukin-6, (B) Tumor necrosis factor- α . * $p < 0.05$, ** $p < 0.01$ compared with the negative control.

Gene Expression in *C. elegans*

Expression levels of genes related to lifespan extension, such as *pmk-1*, *sek-1*, *dbl-1*, *sma-3*, *daf-2*, and *daf-16*, were measured. The expression in *C. elegans* fed *E. faecium* L11 of

Table 1. Mean lifespan of *C. elegans* (N2) fed *E. coli* OP50 or *E. faecium* L11.

	Bacterial strain	MLS (day) \pm SE (N)
<i>C. elegans</i> (N2)	<i>E. coli</i> OP50	15.73 \pm 0.31 (135)
	<i>E. faecium</i> L11	17.94 \pm 0.61*** (170)

MLS, mean lifespan; SE, standard error. *** $p < 0.001$.

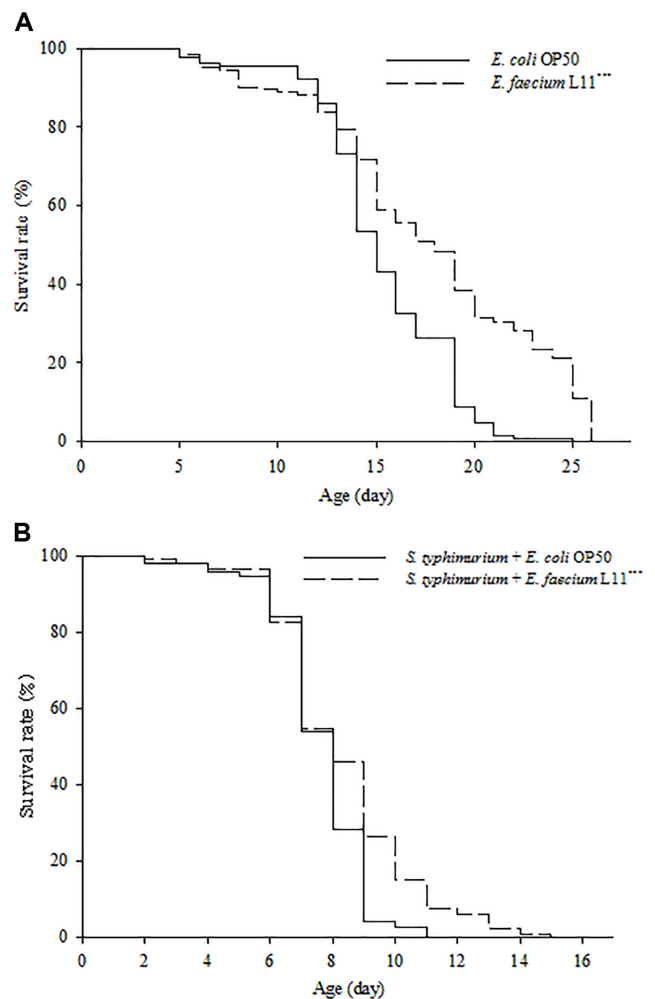


Fig. 4. Effect of *E. faecium* L11 on the lifespan of *C. elegans*. Worms were fed with *E. coli* OP50 for 3 days after hatching and then transferred to a diet of the indicated strains. (A) Worms were fed with *E. coli* OP50 (—) or *E. faecium* L11 (---). (B) Effect of feeding *E. faecium* L11 on the survival of *C. elegans* infected with *S. Typhimurium*. Worms were fed *E. coli* OP50 and *S. Typhimurium* (—) or *E. faecium* L11 and *S. Typhimurium* (---). Each assay was carried out in triplicate and repeated three times. Data were analyzed by log-rank to determine statistical significance. *** $p < 0.001$ compared with *E. coli* OP50.

these genes was significantly ($p < 0.01$) upregulated compared with the *E. coli* OP50-fed group (Fig. 5). Expression levels of *pmk-1* and *sek-1* increased 1.51- and 1.39-fold, respectively,

Table 2. Resistance of *C. elegans* (N2) against *S. Typhimurium* infection when fed *E. faecium* L11.

	Bacterial strain	MLS (day) \pm SE (N)
<i>C. elegans</i> (N2)	<i>E. coli</i> OP50	8.09 \pm 0.37 (147)
	<i>E. faecium</i> L11	8.81 \pm 0.44*** (154)

MLS, mean lifespan; SE, standard error. *** $p < 0.001$.

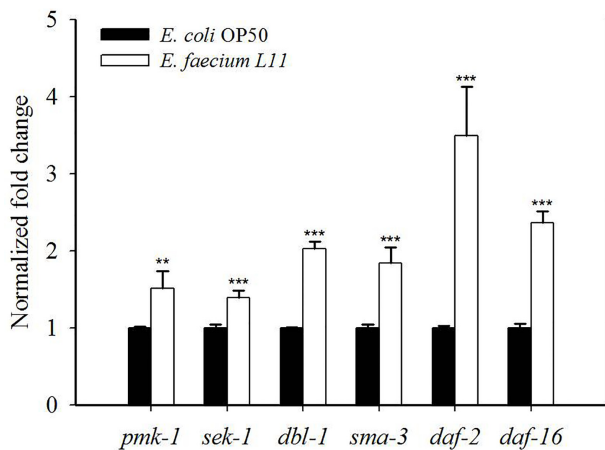


Fig. 5. Gene expression in *C. elegans* (N2) when fed *E. faecium* L11 was compared with that in the worm fed with *E. coli* OP50 (control).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

in nematodes fed *E. faecium* L11 compared with controls. These genes are related to the p38 MAPK (p38 mitogen-activated protein kinase) pathway. The expression levels of *dbl-1* and *sma-3* increased 2.02- and 1.83-fold, respectively, compared with the worms fed *E. coli* OP50. The aging-related genes *daf-2* and *daf-16* were upregulated 3.48- and 2.36-fold, respectively, in *C. elegans* fed *E. faecium* L11 compared with controls.

Discussion

An important property for probiotics is the ability to survive passage through the digestive tract, which means that they must be able to resist gastric juices and endure the presence of bile in the small intestine [37]. Probiotics showing a survival rate higher than 60% through artificial digestive fluid transit are considered potentially able to reach the colon [22]. *E. faecium* L11 in simulated gastric juice showed a relatively high survival rate and over 60% survived after sequential exposure for 2 h in simulated small intestinal juice containing bile salts and pancreatin, which suggests that the strain possesses an important characteristic for probiotics.

The spleen plays an important role in immune function. Splenocytes consist of various immune cell populations, including B and T lymphocytes, dendritic cells, and macrophages, which are associated with the post-infection immune system [38]. The proliferation of splenocytes treated with heat-killed *E. faecium* L11 was significantly enhanced compared with the control, indicating activation of the

immune cells. The activation of macrophages plays an essential role in the innate immune response, including phagocytosis of pathogens and production of cytokines [39, 40]. Activated macrophages produce various cytokines, including IL-1, IL-6, IL-12, and TNF- α [41]. Among these cytokines, IL-6 and TNF- α are related to adaptive immune responses [42]. IL-6 is a pivotal immune and inflammatory mediator, regulating the proliferation and differentiation of B and T lymphocytes [43]. TNF- α , which is produced by activated macrophages, plays a role in immunoregulation and defence against infection [44]. The proliferation of mouse peritoneal macrophages and expression levels of IL-6 and TNF- α in macrophages treated with heat-killed *E. faecium* L11 increased dose-dependently compared with the PBS control. The results obtained in this study showed that *E. faecium* L11 induces primary macrophage maturation and is a potent inducer of IL-6 and TNF- α . Therefore, *E. faecium* L11 has immunostimulatory activity on spleen cells and macrophages, which might enhance the host defence system.

Nutrition affects both aging and immunity. It has been reported that *C. elegans* fed lactobacilli and bifidobacteria have an increased average lifespan and immunity [14, 15]. The mean lifespan of worms fed *E. faecium* L11 was significantly longer than controls. *Lactobacillus rhamnosus* extends the lifespan of nematodes through modulation of the DAF-2/DAF-16 signaling pathway [45], whereas *Bifidobacterium infantis* extends the lifespan of the worms via a p38 MAPK-dependent pathway, not through DAF-16 [14]. The *C. elegans* innate immune system is regulated by three signaling pathways; the p38 MAPK pathway, transforming growth factor-beta (TGF- β) pathway, and DAF-2/DAF-16 pathway. These pathways are also interconnected at the molecular level [46]. MAPK pathways are considered to be the most general signal transduction cascades in immunity and three MAPK cascades are involved in immunity. DAF-2 shortens the lifespan of *C. elegans* via the inhibition of DAF-16. DAF-16 regulates genes involved in longevity in adults, stress resistance, and antimicrobial response [47]. MAPK/ERK (extracellular signaling-regulated kinase) signaling contributes to promote the longevity of *C. elegans* through DAF-16 [48]. The genes *daf-2* and *daf-16* were upregulated in worms fed *E. faecium* L11, which suggests that *E. faecium* L11 might extend the lifespan of nematodes not via the DAF-2/DAF-16 pathway but by *daf-16* activation. Worms fed *E. faecium* L11 showed upregulated expression of genes related to the TGF- β pathway (*dbl-1* and *sma-3*) and genes related to the MAPK pathway (*pmk-1* and *sek-1*). These results suggest that *E. faecium* L11 might promote the longevity of

C. elegans through signaling pathways involved in the innate immune system.

Resistance against *Salmonella* infection depends upon the *nsy-1-sek-1-pmk-1* pathway [49]. The lysozymes (LYS-1, LYS-7, and LYS-8) and lectins are antimicrobial peptides that contribute to nematode defense against infection. The lysozyme LYS-8 is under the control of *dbl-1* and *sma-3* [47]. In this study, worms fed *E. faecium* L11 had significantly increased resistance to *S. Typhimurium* compared with nematodes fed *E. coli* OP50. Expression levels of the *pmk-1*, *sek-1*, *dbl-1*, and *sma-3* genes were significantly increased in worms fed *E. faecium* L11 compared with worms fed control. Thus, these genes might contribute to the resistance against *Salmonella* infection in worms fed *E. faecium* L11.

Major strains of the LAB used as probiotics are *Lactobacillus* and *Bifidobacterium*; however, there have been many studies to use genus *Enterococcus* as a probiotic. Although *E. faecium* has not yet acquired generally-recognized-as-safe status, it can be used for the treatment and prevention of human and animal diseases such as diarrhea caused by antibiotics and relieving symptoms of irritable bowel syndrome [50]. In fact, some strains of *Enterococcus* have been used commercially; for instance, *E. faecium* SF68, a probiotic active ingredient for helping in the prevention and treatment of intestinal disorders for human and animal applications (Cerbios-Pharma SA, Switzerland); *E. faecalis* in inactive form, an ingredient of bacteria-containing medicine for gentle immune modulation (Symbio Pharm, Germany). Further studies on the safety of *E. faecium* L11 are needed before its commercialization.

In conclusion, *E. faecium* L11 isolated from chicken cecum demonstrates digestive fluid tolerance, which is a prerequisite for probiotics. *E. faecium* L11 also activates the intestinal immune system and increases the expression of genes related to aging and innate immunity in *C. elegans*, resulting in the enhancement of host defense response and increase of lifespan. Therefore, *E. faecium* L11 may be used as a probiotic agent, especially as a feed additive for livestock.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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