Enhanced γ-aminobutyric acid and sialic acid in fermented deer antler velvet and immune promoting effects

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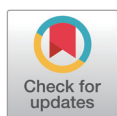
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Abstract

Deer antler velvet is widely used in traditional medicine for its anti-aging, antioxidant, and immunity-enhancing effects. However, few studies have reported on the discovery of probiotic strains for deer antler fermentation to increase functional ingredient absorption. This study evaluated the ability of probiotic lactic acid bacteria to enhance the concentrations of bioactive molecules (e.g., sialic acid and gamma-aminobutyric acid [GABA]) in extracts of deer antler velvet. Seventeen strains of *Lactobacillus* spp. that were isolated from kimchi and infant feces, including *L. sakei*, *L. rhamnosus*, *L. brevis*, and *L. plantarum*, and those that improved the life span of *Caenorhabditis elegans* were selected for evaluation. Of the 17 strains, 2 (*L. rhamnosus* LFR20-004 and *L. sakei* LFR20-007) were selected based on data showing that these strains increased both the sialic acid and GABA contents of deer antler extract after fermentation for 2 d and significantly improved the life span of *C. elegans*. Co-fermentation with both strains further increased the concentrations of sialic acid, GABA, and metabolites such as short-chain fatty acids and amino acids. We evaluated the biological effects of the fermented antler velvet (FAV) on the antibacterial immune response in *C. elegans* by assessing worm survival after pathogen infection. The survival of the *C. elegans* conditioned with FAV for 24 h was significantly higher compared with that of the control worm group fed only normal feed (non-pathogenic *E. coli* OP50) exposed to *E. coli* O157:H7, *Salmonella typhi*, and *Listeria monocytogenes*. To evaluate the protective effects of FAV on immune response, cyclophosphamide (Cy), an immune-suppressing agent was treated to *in vitro* and *in vivo*. We found that FAV significantly restored viability of mice splenocytes and immune promoting-related cytokines (interleukin [IL]-6, IL-10, inducible nitric oxide synthase [iNOS], interferon [IFN]-γ, and tumor necrosis factor [TNF]-α) were activated compared to non-fermented deer antlers. This finding indicated the protective effect of FAV against Cy-induced cell death and immunosuppressed mice. Taken together, our study suggests that immune-promoting antler velvet can be produced through fermentation using *L. rhamnosus* LFR20-004 and *L.*

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INTRODUCTION

Deer antler velvet is a non-osteolytic agent that is widely used in traditional medicine because it contains a variety of small ingredients (e.g., monosaccharides, uronic acid, free amino acids, sialic acid, gangliosides, and small peptides) [1] that promote recovery from fatigue [2], improve liver function and mitigate skeletal weakness symptoms [3,4], strengthen immunity [5], and reduce inflammation. As the elderly population is increasing, demand for health supplements and import of antlers velvets have increased rapidly in recent years. New Zealand, Russia, Canada, and China are countries importing deer antlers, with New Zealand importing greatest quantity and Russia importing the most expensive ones [6]. In Asian countries, deer antler velvets are dried and sold as slices or usually boiled with various types of herbs, botanical materials, and other ingredients in water to be consumed as medicinal soup. Despite having numerous beneficial ingredients, there are limited ways to consume deer antlers. Therefore, there have been various attempts to apply fermentation along with herb materials in order to increase the physiological efficacy of antler velvet and herbal medicine [7].

Microbial fermentation is used in the food industry for a variety of purposes, including the enhancement of flavor, preservation, and health benefits [8]. It is also used to generate useful substances through the enzymatic decomposition of organic matter. During fermentation, high-molecular weight substances are converted into low-molecular weight substances, such as organic acids and amino acids, and glycosides are broken down so that nutrients can be more easily absorbed and utilized by the body [9]. Previous studies have reported that *Bacillus subtilis*-fermented antler extract promotes hematopoiesis [10], inhibits osteoclast differentiation [11], and improves exercise endurance [12].

In this study, we focused on the optimal fermentation of deer antlers using probiotic strains, mainly the lactic acid bacteria (LAB). Fermentation with *Lactobacillus* sp. has been reported to enhance absorption of bioactive molecules, and aid in immune modulation, cholesterol homeostasis, exercise performance [13–16]. The purpose of the present study was to enhance the concentration of bioactive ingredients (e.g., sialic acid and gamma-aminobutyric acid [GABA]) in fermented antler velvet (FAV) and evaluate its effects on immune response in *Caenorhabditis elegans* and mouse models.

MATERIALS AND METHODS

Caenorhabditis elegans longevity assay

The *fer-15;ferm-1* mutants were synchronized using an alkaline hypochlorite solution. *Lactobacillus rhamnosus* GG (LGG) and *Escherichia coli* OP50 (OP50) were used as controls. Concentrated suspensions of LAB or *E. coli* OP50 (100 μL) were plated on 35-mm diameter nematode growth medium (NGM) agar plates. Day 0 was defined as the day of L4-to-adult switch. Worms were transferred, using a platinum wire, onto fresh plates daily and maintained at 25℃. The experiment was repeated three times, and survival rates were determined.

Antler velvet extract fermentation

Water extracts of deer antler, *Cervus canadensis Erxleben*, were procured from Korea RND

Nonghyup (Seoul, Korea) and stored at 4℃. To ferment with LAB and antler extract, minimal prebiotic medium with 2% antler extract (1 L standard: 20 g antler water extract, 5 g peptone, 2.5 g sodium acetate, 0.5 mL of 1 M magnesium sulfate heptahydrate, 0.5 mL of 1 M manganese sulfate tetrahydrate, 5 mL Tween 80, 1 g diammonium citrate, 1 g dipotassium phosphate, 20 g glucose, and 1 g L-glutamic acid) was prepared and autoclaved at 121℃ for 15 min. The working antler medium was aliquoted into 50-mL conical tubes, and 1% of each of the 17 LAB strains was then inoculated (>10^4 colony-forming unit [CFU]/mL). After incubation at 37℃ for 24, 48, 72, 96, and 120 h, the cultures were centrifuged at 4,000×g for 10 min at 25℃, and the resulting supernatants were filtered using 0.22-μm syringe filters (Minisart, Sartorius Stedim Biotech GmbH, Goettingen, Germany).

Total acidity, pH, and bacterial growth

The acidity of each culture (1 mL) was measured by adding 10 μL of 1% phenolphthalein and then adding 0.1 N NaOH until reaching an endpoint. The amount (mL) of 0.1 N NaOH consumed was then converted and expressed as lactic acid content (%). The culture pH was measured during fermentation using a pH meter (S20 SevenEasy pH meter, Mettler-Toledo, Columbus, OH, USA). The pH and acidity of the cultures were measured before and after each fermentation period (24, 48, 72, 96, and 120 h). Bacterial concentration was assessed using the CFU counting method. Briefly, serial diluents (100 μL) were spread on deMan, Rogosa, and Sharpe (MRS) agar plates, and CFUs (Log^{10}/mL) were counted after 2 d.

Total nitrogen, amino acid, and protein quantification

Total nitrogen content was analyzed photometrically as described previously [17], with minor modifications. Briefly, samples of each culture were diluted 25-fold, mixed with 5 mL aqueous HCl (0.2%), 20 mL ddH$_2$O, and 10 mL potassium persulfate (3% alkalinity, pH 9.7); autoclaved at 120℃ for 30 min; cooled to room temperature; and then combined with 5 mL HCl (0.06%). Finally, the absorbance of each mixture was measured at 220 nm, and nitrogen content was determined using a calibration curve generated using potassium nitrate. To measure total amino acid content, amino acids were extracted as described by Sun et al. [3], with slight modifications. Briefly, 50 μL of each culture was mixed with 2 mL ddH$_2$O, 1 mL acetic acid-sodium acetate buffer solution (pH 6.0), and 1 mL aqueous ninhydrin (2%); incubated at 100℃ for 15 min; cooled to room temperature; and then mixed with 10 mL 50% ethanol. Finally, the absorbance of each mixture was measured at 220 nm [3], and amino acid concentration was determined using a calibration curve generated using an arginine standard. The protein content of each culture was quantified using Bradford protein analysis, following the manufacturers' instructions. The concentration of each mixture was determined by measuring absorbance at 595 nm.

Gamma-aminobutyric acid analysis

GABA analysis was performed according to the method described by Lü et al. [18], with slight modifications. Briefly, 3 mL of each sample was mixed with 1.5 mL NaHCO$_3$ (0.5 M) and 0.5 mL 1-fluoro-2,4-dinitrobenzene (0.715 mg/mL), incubated at 60℃ for 1 h, cooled to room temperature, and then passed through a 0.22-μm syringe filter (PTFE, Advantec, Tokyo, Japan). The GABA content of each sample derivative (10 μL) was then analyzed using a Shimazhu 20A series HPLC system (Shimadzu, Kyoto, Japan) equipped with a Symmetry C18 column (5 μm, 3.9 × 150 mm; Waters, Milford, MA, USA). Isocratic elution was performed with 0.5% ammonium acetate aqueous solution and acetonitrile (85:15, v/v) for 20 min at a flow rate of 1 mL/min. GABA was detected based on absorption at a wavelength of 360 nm.

Sialic acid analysis

Each sample (0.1 mL) was mixed with 0.1 mL H_2SO_4 (0.2 N), hydrolyzed at 85℃ for 1 h, and then reacted with 20 μL periodate solution (0.25 M $NaIO_4$ in 9 M H_3PO_4) at room temperature for 20 min. Next, 0.1 mL arsenite reagent (10% $NaAsO_2$ in 0.1 N H_2SO_4 with 0.5 M Na_2SO_4) was added to reduce the excess periodate. Thereafter, 0.25 mL thiobarbituric acid (TBA) solution (0.6% TBA in 0.5 M Na_2SO_4) was added and color was developed at 85℃ for 15 min, followed by cooling at room temperature. Finally, each sample was mixed with 1 mL cyclohexanone and centrifuged at 900×g for 7 min. The absorbance of each supernatant was measured at 549 nm (Synergy HTX multi-mode plate reader, BioTek,Winooski, VT, USA) [19], and sialic acid content was calculated by comparison to a standard curve for N-acetyl neuraminic acid.

Metabolite analysis

The supernatants of the fermented samples were collected after centrifugation at 5,500×g for 5 min and filtered using 0.2-μm cellulose acetate syringe filters. Each sample was then mixed with ice-cold methanol, vigorously vortexed on ice for 1 min, and centrifuged at 10,000×g for 10 min at 4℃. The resulting supernatants were filtered using 0.2-μm polyvinylidene fluoride (PVDF) syringe filters (Whatman, Maidstone, UK) and completely vacuum-dried.

For gas chromatography-mass spectrometry (GC-MS) analysis, each sample was dissolved in 30 μL methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in pyridine (20 mg/mL), derivatized at 30℃ for 90 min. Samples were then trimethylsilylated by adding 50 μL *N,O-bis*(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich) and incubated at 60℃ for 30 min. GC-MS analysis was performed using a TRACE™ 1310 Gas Chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an ISQ LT single quadrupole mass spectrometer (Thermo Fisher Scientific), and separation was performed using a DB-5MS column (60 m × 0.25 mm, 0.25-um film thickness, Agilent, Santa Clara, CA, USA). Oven temperature was maintained at 50℃ for 2 min, gradually increased at 5℃/min, maintained at 180℃ for 8 min, gradually increased at 2.5℃/min to 325℃, and then maintained for 10 min. The sample was injected at 300℃, and helium was used as the carrier gas, with a flow rate of 1.5 mL/min and split ratio of 1:60. An electron ionization system with an ionization voltage of 70 eV was used for GC-MS detection, and the ion source temperature was 270℃. The mass scan range was set at 30–450 (m/z), and the acquisition rate was 5 spectra/s. Detected metabolites were identified using the NIST Mass Spectral Search Program (version 2.0; National Institute of Standards and Technology [NIST], Gaithersburg, MD, USA).

Caenorhabditis elegans killing assay

The *C. elegans* killing assay was performed as previously described [20]. L4/young adult worms were placed on conditioning NGM plates with non-fermented antler velvet (AV) medium (10 mg/mL) or FAV (10 mg/mL), and non-pathogenic *E. coli* OP50 at 25℃ for 24 h. Worms were plated at 30 per plate. Subsequently, for viability analysis, nematodes preconditioned with AV, FAV, and OP50 were transferred onto *Salmonella typhi* SL 1344, *Listeria monocytogenes* EDG-e, and *E. coli* O157:H7 EDL933 culture plates; incubated at 25℃; and examined using a microscope at 24-h intervals until all worms died. Whether worms were alive was assessed by gentle touching with a platinum wire.

Isolation of mouse splenocytes

Spleen tissues were extracted from C57BL/6 mice (24-wk-old female, 20–22 g). The tissues were disaggregated via passage through a 40 μm cell strainer in RPMI-1640 medium (SH30027, Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS), 1% antibiotics and the cells were purified by centrifugation at 1,000×g for 10 min at 4℃. Red blood cells were removed

by incubating purified cells in Red blood cell lysis buffer (A10492-0, GIBCO, Thermo Fisher Scientific) for 3 min. Splenocytes were then washed with RPMI containing 10% FBS and 1% antibiotics and centrifuged at 1,000×g for 10 min at 4℃ for 10 min.

Measurement of cell cytotoxicity and proliferation

The cell viability assay was performed using an EZ-Cytox cell viability assay kit (EZ-3000, DoGen Bio, Seoul, Korea). Splenocytes were seeded into 96-well plates (approximately 5×10^6/mL) and treated with 0, 0.1, 0.5, and 1 mg/mL of the AV or FAV samples for 48 h. Cells were co-treated with 1.6 mg/mL cyclophosphamide (Cy; C0768-1G, Sigma-Aldrich) and the antler velvet sample. Subsequently, 10 μL of EZ-Cytox reagent was added to each well and incubated for 1 h. Absorbance of the samples was then measured at 450 nm. Cell viability values were normalized to those of the cells from the untreated control groups.

Preparation of the Cy-induced immunosuppression mouse model

Ten-week-old female C57BL/6 mice (19–20 g) were housed in a room with controlled temperature (22 ± 2℃) and humidity (50 ± 5%) on a 12/12 h light/dark cycle with free access to food and water. After 1 wk of acclimatization, the mice were randomly divided into four groups (n = 5): control group, Cy group (100 mg/kg), AV 2 mg/20 g group, FAV 2 mg/20 g group. The AV or FAV-treated groups were orally administered the corresponding concentration of antler velvet for about 28 d, and the control and the Cy groups received oral administration of the same amount of saline. Mice were administered intraperitoneal injection of Cy (100 mg/kg) in a total volume of 200 μL of saline on days 21 and 24. All of the mice were administered with Cy except the control group. At the end of the experiment, mice were sacrificed, and the spleens were collected.

RNA isolation and quantitative real-time reverse-transcription-polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the spleen tissues using the total RNA Mini Kit (T2010S, New England BioLabs, Ipswich, MA, USA), as per the manufacturer's instructions. A cDNA kit (170-8891, Bio-Rad Laboratories, Hercules, CA, USA) was used to synthesize cDNA from 1 μg of total RNA, using the primers. Target gene expression was analyzed using the gene-specific primers listed in Table 1. The amplified products were subjected to electrophoresis on a 1% agarose gel and visualized with ultraviolet illumination. For qPCR, cDNA strands synthesized from the isolated total RNA were used as templates. PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA), using the SYBR Green PCR Mastermix (1725121; Bio-Rad Laboratories). The cycle threshold values were normalized against the β-actin gene expression levels.

Statistical analyses

Data were analyzed using GraphPad Prism software (Prism 9 Academic), and the results are presented as mean ± SD. Statistical analysis was performed using Student's t-test for comparisons between two groups and one-way analysis of variance for comparisons among multiple groups. $p < 0.05$ was considered significant.

Table 1. Primer sequences for quantitative real-time PCR

Gene	Sequence	Length (bp)
IL-6	F: 5'-AGTTGCCTTCTTGGGACTGA-3'	159
	R: 5'-TCCACGATTTCCCAGAGAAC-3'	
IL-10	F: 5'-GACAACATACTGCTAACCGACTCC-3'	139
	R: 5'-GCTCCTTGATTTCTGGGCCATG-3'	
iNOS	F: 5'- GCATGGACCAGTATAAGGCAAGCA-3'	222
	R: 5'-GCTTCTGGTCGATGTCATGACGAA-3'	
IFN-γ	F: 5'-TCTGGAGGAACTGGCAAAAG-3'	107
	R: 5'-GCTGATGGCCTGATTGTCTT-3'	
TNF-α	F: 5'-AGCCCCCAGTCTGTATCCTT-3'	212
	R: 5'-CTCCCTTTGCAGAACTCAGG-3'	
β-Actin	F: 5'-AGCCATGTACGTAGCCATCC-3'	228
	R: 5'-CTCTCAGCTGTGGTGGTGAA-3'	

PCR, polymerase chain reaction; IL, interleukin; IFN, interferon; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor.

RESULTD AND DISCUSSION

Probiotic candidates for fermentation

Previously, we had isolated approximately 200 strains of LAB, including *L. rhamnosus* and *L. plantarum* strains, from fermented Kimchi and infant feces. Among the isolates, we screened the strains having basic probiotic properties for further fermentation with antler velvet extract. Of the 200 strains isolated from kimchi and infant feces, 17 *Lactobacillus* strains (*L. sakei*, *L. brevis*, *L. rhamnosus*, and *L. plantarum*) were selected based on their superior acid resistance, bile resistance, and intestinal adhesion, as shown in Table 2. All the isolates were identified by 16S rRNA sequencing. The study of the selection of 17 strains as suitable candidates for deer antler extract fermentation is unpublished. To evaluate the activity of strains during fermentation with antler extract, 2% of velvet antler water extracts (water extract 50% w/w) containing minimal prebiotic media was used for fermentation. During the first three days of fermentation, all 17 strains maintained sufficient cell populations, lowered culture pH, and increased culture acidity (Table 3). The MR ID stains from infant feces exhibited more rapid pH lowering and higher acidity rather than LFR ID stains from Kimchi, whereas higher cell population was maintained during fermentation with Kimchi strains except for LFR20-006. There have been a few studies on the strengthening of bioactive ingredients of deer antlers via fermentation [10,12,21]. Kim et al. [21] utilized 11 *Bacillus* sp., 4 *Lactobacillus* sp., and 6 mushroom stains (*Cordyceps militaris*) to evaluate the growth in antler medium. All *Lactobacillus* sp. (*L. plantarum*, *L. mesenteroides*, *L. delbrueckii*) failed to grow in both antler extract and antler power-based media. Surprisingly, all the strains in the present study successfully exhibited high proliferation with 10^8–10^9 CFU/g, indicating that all strains were compatible with antler extract and proliferated and produced strain-specific metabolites.

We attempted to evaluate the longevity-promoting effects of all 17 selected strains using *C. elegans* to identify optimal strains for fermentation. Strains LFR20-003, LFR20-004, LFR20-007, LFR20-008, MR4, MR5, MR14, MR19, MR43, MR45, and MR50 were as efficacious if not more than the positive control LGG (Fig. 1). In our previous study, *Lactobacillus plantarum* JDFM216 was selected, and it was reported that LAB strains could mitigate immune function decline and cognitive decline due to aging [22,23]. As an important industrial application of LAB, lactic acid fermentation continues to be a highly desirable method for processing and preserving non-dairy foods owing to its low cost, low energy requirements for both processing and preparing foods for

Table 2. *Lactobacillus* strains evaluated by the present study

Strain ID[1]	Sources[2]	Oxygen[3]	Temperature	Species[4]
LFR20-001	Kimchi	Anaerobic	37℃	*L. sakei*
LFR20-002	Kimchi	Anaerobic	37℃	*L. brevis*
LFR20-003	Kimchi	Anaerobic	37℃	*L. rhamnosus*
LFR20-004	Kimchi	Anaerobic	37℃	*L. rhamnosus*
LFR20-005	Kimchi	Anaerobic	37℃	*L. sakei*
LFR20-006	Kimchi	Anaerobic	37℃	*L. rhamnosus*
LFR20-007	Kimchi	Anaerobic	37℃	*L. sakei*
LFR20-008	Kimchi	Anaerobic	37℃	*L. brevis*
LFR20-009	Kimchi	Anaerobic	37℃	*L. brevis*
MR1	Infant feces	Anaerobic	37℃	*L. plantarum*
MR4	Infant feces	Anaerobic	37℃	*L. plantarum*
MR5	Infant feces	Anaerobic	37℃	*L. plantarum*
MR14	Infant feces	Anaerobic	37℃	*L. plantarum*
MR19	Infant feces	Anaerobic	37℃	*L. plantarum*
MR43	Infant feces	Anaerobic	37℃	*L. plantarum*
MR45	Infant feces	Anaerobic	37℃	*L. plantarum*
MR50	Infant feces	Anaerobic	37℃	*L. plantarum*

[1]LFR number was provided after screening probiotic properties among 150 strains.

[2]Infant feces from 10 babies, which study was approved by Jeonju University Ethics Committee (jjIRB-210215-HB-2021-0212).

[3]Oxygen requirements.

[4]Provided only lactic acid bacteria which was identified by 16S rRNA sequencing. To amplify the V4 region from the 16S rRNA gene, 341F/805R primers were used. 16S rRNA gene amplicon sequencing was performed on the Illumina MiSeq platform at Macrogen (Seoul, Korea).

Table 3. Changes of pH, acidity and LAB proliferation during the fermentation of antler velvet extract

Strain ID	pH[1]			Total acidity[1] (%)			Log CFU/mL[2]		
	1d	2d	3d	1d	2d	3d	1d	2d	3d
LFR20-001	4.50	4.09	3.99	0.45	0.54	0.63	7.31 ± 0.05	7.30 ± 0.07	7.17 ± 0.05
LFR20-002	5.33	3.52	3.46	0.32	1.01	1.13	5.12 ± 0.05	8.91 ± 0.12	8.58 ± 0.34
LFR20-003	4.14	3.78	3.60	0.60	0.77	0.99	8.26 ± 0.24	7.92 ± 0.08	8.30 ± 0.30
LFR20-004	4.14	3.75	3.60	0.52	0.76	0.99	8.36 ± 0.32	8.43 ± 0.38	7.90 ± 0.07
LFR20-005	5.31	3.58	3.44	0.28	0.92	1.22	6.32 ± 0.28	9.12 ± 0.05	8.30 ± 0.00
LFR20-006	4.19	4.03	4.01	0.60	0.81	0.63	7.71 ± 0.04	6.96 ± 0.16	3.47 ± 0.12
LFR20-007	4.82	3.75	3.41	0.36	0.85	0.99	8.46 ± 0.41	9.08 ± 0.11	8.59 ± 0.11
LFR20-008	4.09	3.76	3.62	0.69	0.87	0.99	8.39 ± 0.36	8.42 ± 0.10	7.83 ± 0.06
LFR20-009	4.13	3.56	3.56	0.72	1.13	1.26	8.84 ± 0.18	9.08 ± 0.04	8.79 ± 0.10
MR1	3.77	3.42	3.57	0.72	1.15	1.08	8.94 ± 0.21	8.59 ± 0.11	5.63 ± 0.04
MR4	3.77	3.43	3.41	0.74	1.13	1.04	9.17 ± 0.10	8.72 ± 0.12	5.66 ± 0.07
MR5	3.67	3.51	3.48	0.82	1.10	1.01	8.93 ± 0.08	8.46 ± 0.15	5.22 ± 0.14
MR14	3.66	3.50	3.48	0.91	1.10	1.26	9.08 ± 0.07	8.66 ± 0.39	5.05 ± 0.25
MR19	3.66	3.47	3.48	0.86	1.04	1.22	9.12 ± 0.15	8.43 ± 0.23	5.48 ± 0.07
MR43	3.42	3.35	3.23	0.81	0.95	1.08	8.78 ± 0.26	8.26 ± 0.24	7.11 ± 0.03
MR45	3.42	3.30	3.27	0.86	0.81	1.06	8.76 ± 0.15	8.30 ± 0.30	8.00 ± 0.17
MR50	3.40	3.30	3.25	0.81	1.04	1.13	8.74 ± 0.24	8.36 ± 0.10	7.01 ± 0.15

[1]pH and total acidity were measured continuously over 1, 2, and 3 days for independent samples fermented with each strain, and are presented as average values.

[2]The probiotic water was measured three times per 1 mL of fermented product over 1, 2, and 3 days and presented as a log value.
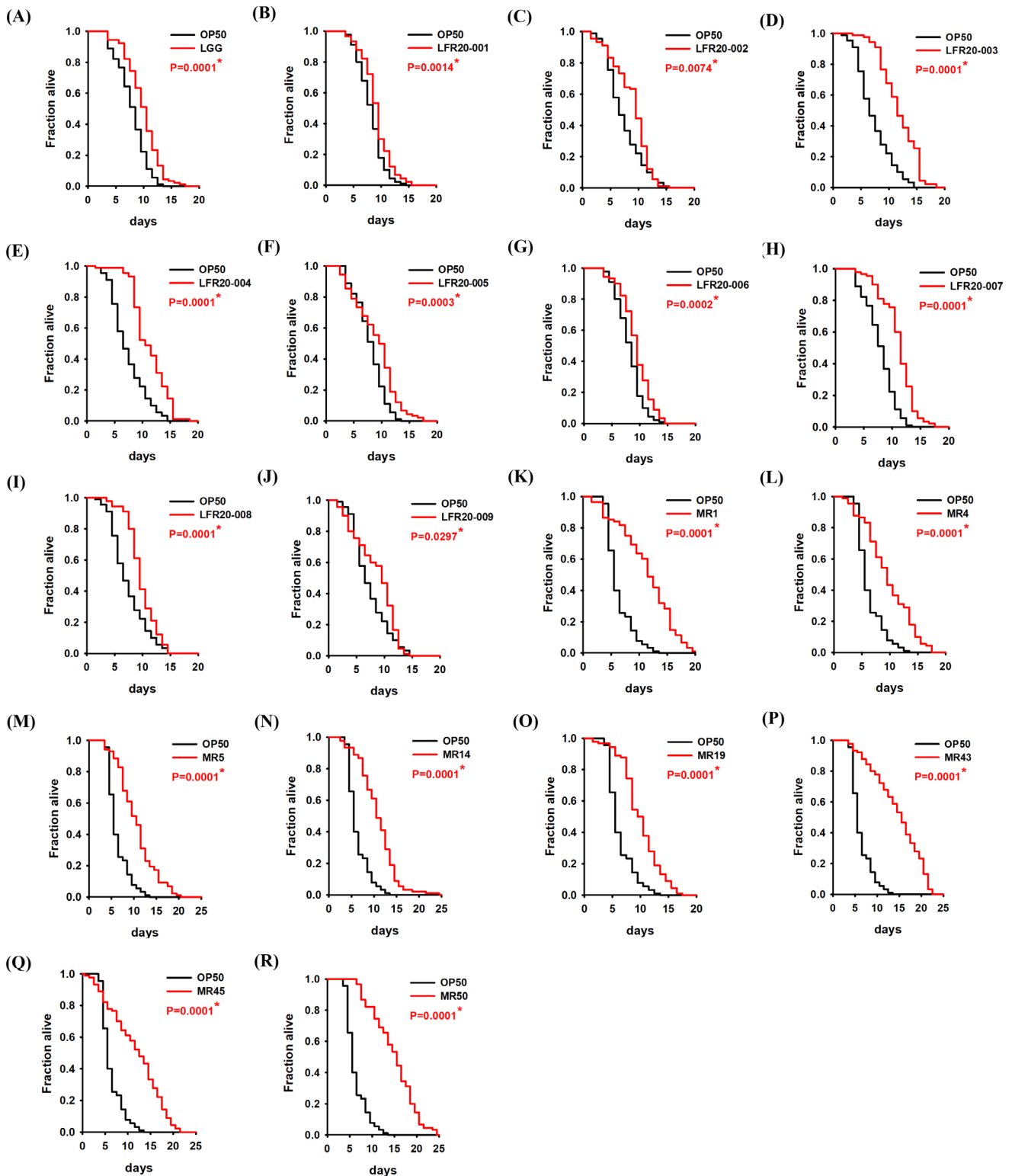
Fig. 1. Anti-aging effects of *C. elegans* by exposure to *Lactobacillus* spp. Prolonged lifespan of *C. elegans* by pre-conditioning with each strain (red line) for 24h. (A) *Lactobacillus rhamnosus* GG (LGG), (B) *Lactobacillus sakei* LFR20-001, (C) *Lactobacillus brevis* LFR20-002, (D) *Lactobacillus rhamnosus* LFR20-003, (E) *Lactobacillus rhamnosus* LFR20-004, (F) *Lactobacillus sakei* LFR20-005, (G) *Lactobacillus rhamnosus* LFR20-006, (H) *Lactobacillus sakei* LFR20-007, (I) *Lactobacillus brevis* LFR20-008, (J) *Lactobacillus brevis* LFR20-009, (K) *Lactobacillus plantarum* MR1, (L) *Lactobacillus plantarum* MR4, (M) *Lactobacillus plantarum* MR5, (N) *Lactobacillus plantarum* MR14, (O) *Lactobacillus plantarum* MR19, (P) *Lactobacillus plantarum* MR43, (Q) *Lactobacillus plantarum* MR45, (R) *Lactobacillus plantarum* MR50, (*Escherichia coli* OP50 were used for comparison).

consumption, high yield, and diversified flavors as compared to fermentation with mold or *Bacillus* sp. [24].

Effect of fermentation on bioactive molecule contents

On the second day after fermentation with antler velvet, the amounts of total nitrogen, amino acid, proteins, and bioactive substances were measured. In most fermented extracts, the amounts of total nitrogen, protein, and amino acids decreased (Table 4). In general, *Lactobacillus* has the ability to hydrolyze proteins present in the medium, and this proteolytic activity produces a variety of peptides, metabolites with biological activity, as well as free amino acids required by bacteria. [25,26]. In this experiment, it was found that even for the same *Lactobacillus* species, the protein utilization rate was variable. This result indicates the diversity in mechanisms involved in the longevity-promoting properties in *C. elegans*.

LAB producing and releasing GABA, a neurotransmitter, have been reported, and their mechanisms were reviewed [27–29]. Although there are reports of the effect of deer antler extract [30] and bone extract [31] on memory improvement in scopolamine-induced memory impaired mice, the production of GABA in deer antler extract after fermentation has not yet been investigated. The GABA content of the antler velvet was increased six times when fermented by MR43, MR45, and MR50, which were all isolated from infant feces, and was increased three and two times when fermented by LFR20-007 and LFR20-004, respectively, which were isolated from kimchi (Fig. 2A). In addition, the amount of ganglioside, a major bioactive component of deer antlers, was measured. Sialic acid in antler velvet has been considered a biomarker among bioactive molecules. Various extraction methods utilizing enzymes and fermentation, amongst others, have been developed to improve the recovery of sialic acid [32–34]. All the selected strains increased the sialic acid content; however, fermentation with LFR20-006, LFR20-007, and LFR20-008 increased the sialic acid content by approximately eight-fold (Fig. 2B). Interestingly, after

Table 4. Effect of fermentation (48 h) with probiotic *Lactobacillus* strains on the nutrient content of antler velvet extract

| Strain	Total N (µg/mL)	Protein (µg/mL)	Amino acid (mg/mL)
Control	881.016 ± 30.504	537.000 ± 0.003	4.216 ± 0.072
LFR20-001	222.798 ± 15.882	380.629 ± 0.017	3.291 ± 0.041
LFR20-002	283.474 ± 12.182	345.615 ± 0.020	3.016 ± 0.050
LFR20-003	247.888 ± 3.236	419.414 ± 0.008	3.763 ± 0.100
LFR20-004	306.900 ± 29.207	341.844 ± 0.011	3.482 ± 0.014
LFR20-005	394.585 ± 23.576	306.292 ± 0.007	2.950 ± 0.027
LFR20-006	204.877 ± 18.244	516.376 ± 0.002	3.608 ± 0.023
LFR20-007	720.238 ± 38.258	356.389 ± 0.009	2.945 ± 0.033
LFR20-008	177.355 ± 14.795	478.130 ± 0.013	3.896 ± 0.053
LFR20-009	244.048 ± 9.798	351.002 ± 0.009	3.597 ± 0.040
MR1	322.261 ± 8.140	405.408 ± 0.007	3.103 ± 0.045
MR4	333.781 ± 28.079	322.452 ± 0.008	3.019 ± 0.029
MR5	291.539 ± 10.199	473.282 ± 0.025	2.666 ± 0.014
MR14	285.394 ± 22.301	405.947 ± 0.011	2.912 ± 0.023
MR19	322.901 ± 24.770	412.950 ± 0.017	2.865 ± 0.024
MR43	449.757 ± 40.967	476.514 ± 0.010	3.305 ± 0.080
MR45	462.558 ± 30.657	421.030 ± 0.017	3.640 ± 0.082
MR50	915.323 ± 33.944	444.193 ± 0.004	3.185 ± 0.065

(A)

(B)

(C)
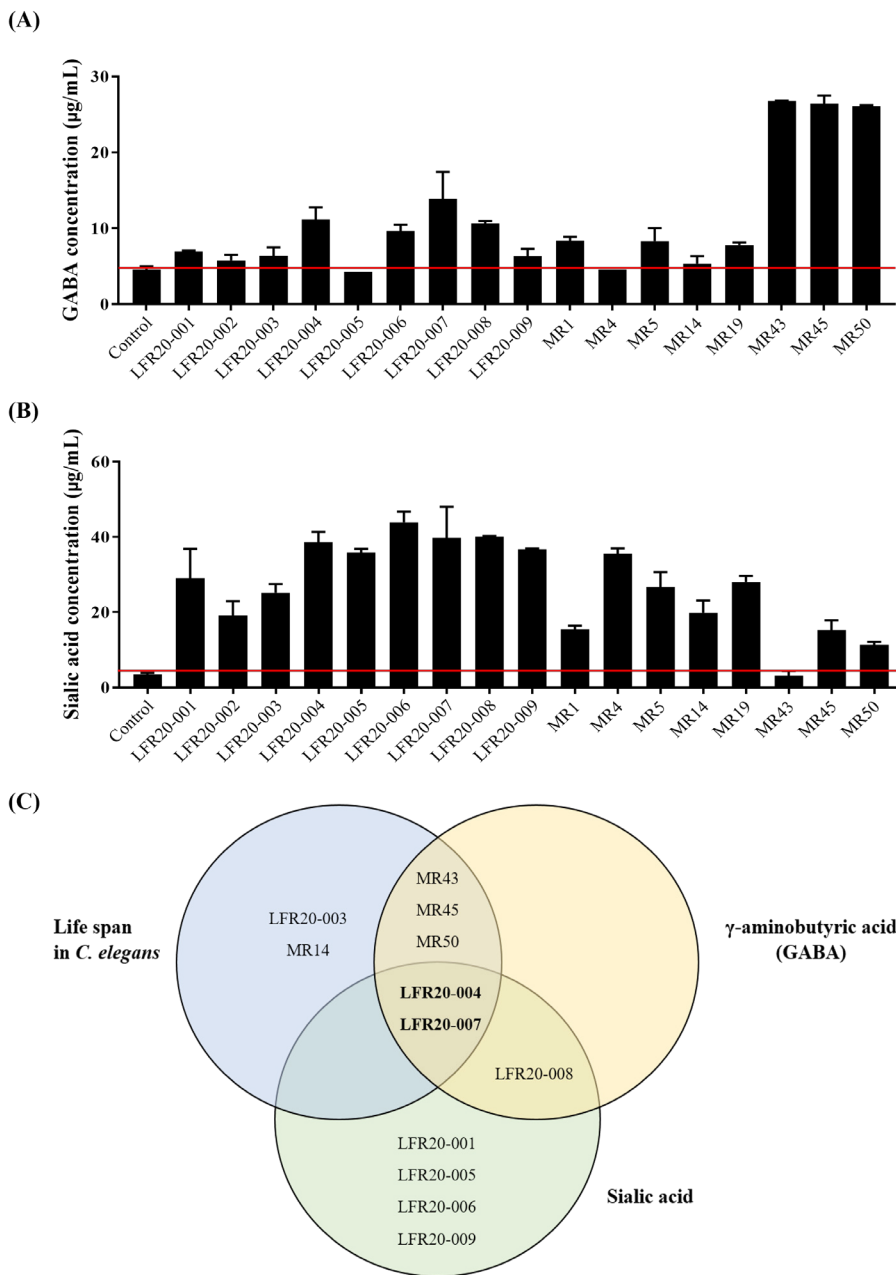
Fig. 2. Changes for amounts of bioactive ingredients and optimal selection strains for fermented antler velvet. (A) GABA content, (B) Sialic acid content, (C) Venn Diagram produced by the online software Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html) to select optimal strains for FAV. *C. elegans*, *Caenorhabditis elegans*; GABA, gamma-aminobutyric acid.

fermentation, the levels of sialic acid, rather than GABA, in most fermented extracts were increased dramatically. Park et al. [10] reported that the sialic acid content was highest, exhibiting a four-fold increase approximately in *Bacillus subtilis*-FAV extract on the fourth day. The 11 LAB strains in this study (LFR20-001, -003, -004, -005, -006, -007, -008, -009, MR4, MR5, and MR19) exhibited greater ability to produce sialic acid in fermented deer antler.

Effect of FAV extract on *C. elegans* infected with pathogens

We then compared the biological activity of FAV with AV. We utilized two strains, LFR20-004 and LFR20-007, among the strains that increased the longevity in *C. elegans* (10 strains), GABA production (6 strains), and sialic acid content (10 strains) for the fermentation (Fig. 2C). GC-MS analysis of FAV revealed that free amino acid, short-chain fatty acid, and monosaccharide contents were changed significantly (Table 5). This profile may include metabolites derived from two strains and ingredients used by LAB or not used in AV samples.

We evaluated the biological effects of FAV on the antibacterial immune response in *C. elegans* by assessing whether the FAV and AV with probiotics influenced the host's survival after infection by pathogens (*E. coli* O157:H7, *Salmonella typhi*, and *Listeria monocytogenes)*. The survival of the *C. elegans* conditioned with FAV or AV for 24h was significantly higher compared with that of the control worm group fed only normal feed (non-pathogenic *E. coli* OP50) when exposed to two pathogens (*E. coli* O157:H7 and *Listeria monocytogenes*) (Figs. 3A and B). The worms conditioned with FAV exhibited significant resistance to infection by *S. typhi*, while worms conditioned with AV were not different from those fed normal feed (Fig. 3C). There have been few studies on the antibacterial efficacy of deer extracts against pathogens. To our knowledge, this study is the first to report that non-fermented deer antlers increase resistance of *C. elegans* to *E. coli* O157:H7 and *Listeria monocytogenes*. Dai et al. [35] reported that antler velvet extracts stimulated anti-inflammatory cytokines in splenocytes and phagocytes and play a protective role in *Staphylococcus aureus*-infected mice. However, fermentation products are known to have excellent antibacterial efficacy owing to the production of short-chain fatty acids such as lactic acid and play an important role in the safe storage of fermented foods [36–39]. Our results showed that the FAV increased resistance of *C. elegans* to pathogenic bacteria.

Table 5. Changed metabolites during the fermentation of antler velvet extract

	PC1[1]	*p*-value	FC[2]
Metabolites increased by fermentation			
D-Lactic acid	0.990	< 0.001	287.20
L-Proline	0.984	0.005	+
Mevalonic lactone	0.979	0.006	+
Succinic acid	0.979	0.007	+
Glutaric acid	0.968	0.01	+
Glycine	0.955	0.009	6.091
D-Mannitol	0.945	0.013	+
Propanoic acid	0.937	0.028	+
Phenylalanine	0.920	0.040	+
Aminomalonic acid	0.907	0.053	+
Metabolites reduced by fermentation			
d-Galactose	−0.908	0.040	3.712
Mannose	−0.928	0.010	1.835
D-(−)-Tagatose	−0.971	< 0.001	-
d-Galactose	−0.983	0.005	1.735

[1]It was listed in the order of high absolute loadings on PC1 generated by principal component analysis (PCA).

[2]The numerical value is fold change (FC), the plus means metabolites detected in FAV and the minus means metabolites not-detected in FAV, but in AV.
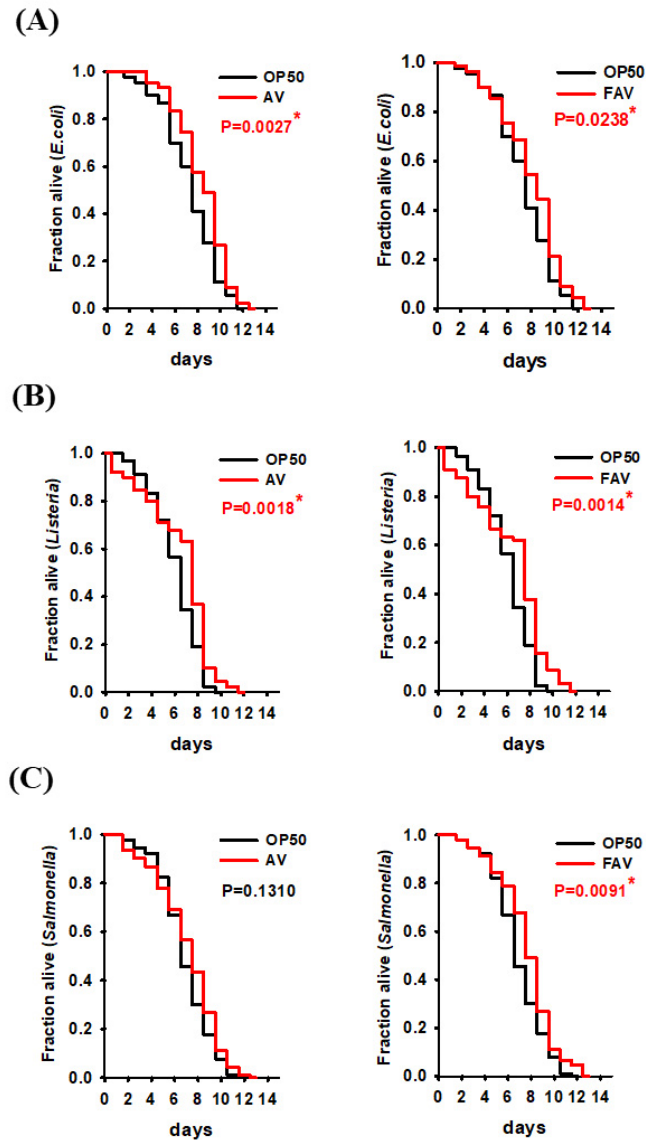
Fig. 3. Killing assays of *C. elegans* worms fed with the FAV. *C. elegans* preconditioned with AV (10 mg/mL), FAV (10 mg/mL), and OP50 for 24 h were transferred onto each pathogen containing solid medium (A) *E. coli* O157: H7 EDL933 (ATCC 700927), (B) *Listeria monocytogenes* EGD-e (ATCC BAA-679) and (C) *Salmonella typhi* SL1344 (ATCC 14028). *C. elegans*, *Caenorhabditis elegans*; FAV, fermented antler velvet; AV, non-fermented antler velvet; OP50, *Escherichia coli* OP50; *E. coli*; *Escherichia coli*.

Cytoprotective effect of FAV on mouse splenocytes

To evaluate the effects of FAV and AV on cytotoxicity of Cy, an immune-suppressing agent, to primary splenocytes, cells isolated from spleens of mice were incubated with various concentrations of AV or FAV (0, 0.1, 0.5, or 1 mg/mL) and Cy (1.6 mg/mL) for 48 h. Surprisingly, we found that FAV completely restored cell viability at 0.1 mg/mL, which was the lowest concentration. However, the viability of splenocytes treated with AV remained reduced by Cy (Fig. 4A). This finding indicated the protective effect of FAV against Cy-induced cell death and encouraged us to induce immunosuppressed mice by administering Cy (100 mg/kg). Expression of various immune modulators such as interleukin (IL)-6, IL-10, interferon (IFN)-γ, inducible nitric oxide synthase (iNOS), and tumor necrosis factor (TNF)-α by activated macrophages has been reported
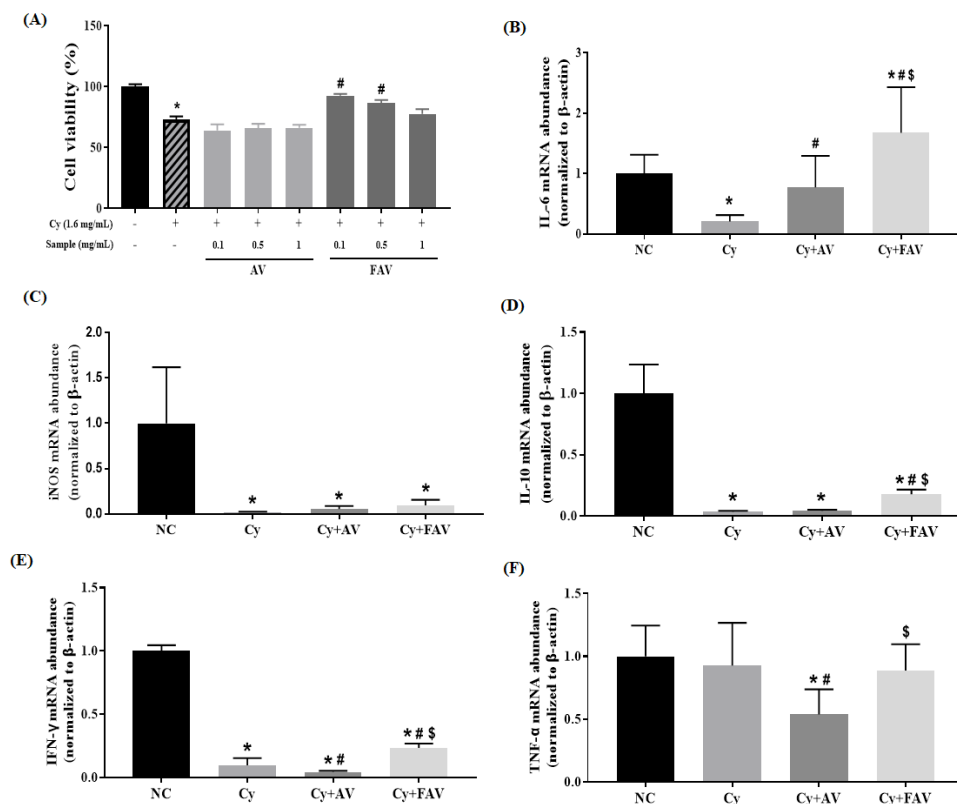
Fig. 4. Effects of FAV on the viability of splenocytes and on mRNA expression of immune promoting cytokine in CY-treated mice. (A) Splenocytes were treated with AV, FAV (0, 0.1, 0.5 and 1 mg/mL) and/or Cy (1.6 mg/mL). All results were expressed compared to untreated and shown as the means ± SD (n = 3). (B–F) As an immune promotion indicator, the expression of mRNA in cytokines as follows was expressed as a fold change to untreated (NC). (B) IL-6, (C) iNOS, (D) IL-10, (E) IFN-γ, and (F) TNF-α. Values are presented as mean ± SD (n = 4). *$p \leq 0.05$, compared with NC group, #$p \leq 0.05$, compared with Cy group; $$p \leq 0.05$, compared with Cy + AV group. FAV, fermented antler velvet; AV, non-fermented antler velvet; IL, interleukin; iNOS, inducible nitric oxide synthase; IFN, interferon; TNF, tumor necrosis factor.

to play an important role in host defense against harmful factors [40]. Cytokines such as IL-6 and TNF-α exhibit essential immune functions during early stages of infection [41,42]. IFN-γ, which is a type-1 T-helper (Th1)-derived cytokine, also plays important roles in cell-mediated immune response [43]. IL-6 and IL-10 are type-2 T-helper (Th2)-derived cytokines that promote humoral immunity [44]. NO is synthesized by iNOS, an important molecule that activates macrophages to neutralize pathogenic microorganisms and tumor cells [45]. We analyzed mRNA expression of immune modulators such as IL-6, IL-10, iNOS, IFN-γ, and TNF-α in splenocytes isolated from immunosuppressed mice. We observed that FAV promoted the production of immunomodulators in spleen tissue-derived cells while AV did not (Fig. 4). In addition to primary splenocytes, it is necessary to investigate the effect of FAV on the single type of isolated immune cells, such as natural killer cells, T/B cells, neutrophils, eosinophils, and dendritic cells, to describe the biological mechanisms for the immune-enhancing activity of FAV.

The health-promoting effects of antler velvet in terms of chemical composition, bioactive components, and pharmacological effects have been reported [46]; however, there is not adequate evidence for its immune-enhancing efficacy. Zhoa et al. have suggested that peptides (3.2 kDa) extracted from antler velvet can modulate the immune system via regulation of Th1- and Th2-related cytokines [47]. Consistently, in our study, antler velvet fermented with two bacterial strains

stimulated the immune system while AV did not.

The global demand for health- and immune-boosting functional foods has increased in the COVID-19 era, and studies on the development of immune-promoting substances and food processing are also increasing [48]. In this study, we demonstrated that FAV increased resistance to pathogenic bacteria in *C. elegans* and promoted splenocyte activation in mouse models by stimulating immune modulators. As deer antler velvet is fermented with LFR20-004 and LFR20-007, the bioactive molecules (GABA and sialic acid) as well as the immune function of deer antler velvet is expected to improve. In the future, an in-depth analysis of the metabolites from the LAB, *L. rhamnosus* LFR20-004 and *L. sakei* LFR20-007 that exhibit immune-promoting functions will be required. These LAB can be used as probiotic starters for health functional foods fermented for deer antler velvet as LAB.

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