

# Immunomodulatory Potential of *Weissella cibaria* in Aged C57BL/6J Mice

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Aging is associated with distinct changes in immune cells and a decline in immune function, leading to increased susceptibility to infection and reduced responses to vaccination. Certain strains of lactic acid bacteria exert beneficial effects on the immune system. Previously, we reported that *Weissella cibaria* JW15 isolated from kimchi possesses immune stimulatory activity in vitro. In the present study, we further investigated whether oral administration of JW15 improves immune function in aged mice. Eighteen-month-old female mice were administered JW15 daily at low (JW15-L;  $1 \times 10^8$  CFU/mouse) or high dosage (JW15-H;  $1 \times 10^9$  CFU/mouse), or with *Lactobacillus rhamnosus* GG (LGG) using oral gavage. Two-month-old female mice were included as healthy young mice. After 4 weeks, the mice were euthanized and immune profiles were analyzed using whole blood and spleen. In complete blood count analysis, the numbers of white and red blood cells were significantly increased in the JW15-L group compared with those in the old mouse (OM) control group. In addition, administration of either JW15 or LGG resulted in higher numbers of splenocytes in comparison with the OM group. Furthermore, proliferative potentials were higher in all probiotic groups than OM. Cytokines such as IFN- $\gamma$  and IL-6 were secreted at higher levels in splenocytes isolated from JW15-fed mice than in OM control mice. Similarly, mRNA expression of various cytokines was altered in the JW15 groups. Collectively, these results suggest that JW15 supplementation induces immunomodulatory effects in aged mice and indicate JW15 as a potential probiotic strain to improve immune function in aged animals.

**Keywords:** *Weissella cibaria* JW15, probiotics, aged mice, immunomodulatory effects

## Introduction

Aging is a complex process that negatively affects immunological function. The process of aging leads to gradual decline of the immune response, resulting in increased susceptibility to infectious diseases, impaired responses to vaccination, and increased chronic inflammatory diseases and cancer [1, 2]. These biological phenomena mainly occur in the adaptive immune system, which regulates humoral (B cell) and cellular (T cell) immune responses. In particular, a decrease in T cell number and function is an important factor in immunosenescence [3]. These changes in immune cells increase the susceptibility

to autoimmune, chronic, and infectious diseases. To improve health in elderly and aging animals, developments in research and materials for immune enhancement or immunomodulation in the elderly have actively been made in recent years [4–6].

Probiotics are defined as living microbial additives that show beneficial health effects on the host animal by improving the balance of the intestinal microflora [7]. Lactic acid bacteria are the most frequently used probiotics and are widely known for their beneficial effects, such as anti-inflammatory, anticarcinogenic, antimutagenic, immunostimulatory, and cholesterol-lowering properties [8]. Probiotics have been shown to improve mechanisms of the

host immune defense and to increase the immune response in the intestine by acting on immunomodulators [9]. Probiotics promote the activity of phagocytes and improve mucosal and systemic immune functions reduced by aging [10, 11]. In addition, research on the functional properties of novel lactic acid bacteria is actively under way.

*Weissella* species are gram-positive, non-spore-forming, catalase-negative, obligate heterofermentative bacteria, and ferment glucose to lactic acid and carbon dioxide through the heterolactic fermentation pathway. Among *Weissella* species, *Weissella cibaria* was first classified in a taxonomic study in 2002, and has been isolated from fermented food such as kimchi (a traditional Korean fermented vegetable) and by dominant species [12]. In addition, *W. cibaria* has shown immune effects involving the production of inflammatory mediators [13]. *W. cibaria* JW15 isolated from kimchi is reported to have properties of a probiotic, such as acid, bile, and heat tolerance, antimicrobial activities, and increasing nitric oxide (NO), nuclear factor (NF)- $\kappa$ B, IL-1 $\beta$ , and TNF- $\alpha$  in RAW 264.7 macrophage cells [14].

Although the immune response of lactic acid bacteria has been demonstrated in many studies, the immune-enhancing effect in aged mice was only partly investigated until now. In the present study, we evaluated the immune profiles of aged mice administered *W. cibaria* JW15 by analysis of complete blood count, splenocyte proliferation, and the concentration of cytokines secreted by splenocytes and cytokine production in 18-month-old mice.

## Materials and Methods

### Probiotics Preparation

*W. cibaria* JW15 isolated from kimchi and *Lactobacillus rhamnosus* GG (LGG, ATCC 53103), a well-known immune enhancing commercial strain, were cultured in de Man, Rogosa, and Sharpe (MRS) broth (BD, USA) at 37°C for 18 h and viable bacterial cells were counted on MRS plates. Then, the bacterial cells were collected by centrifugation at 14,000  $\times$ g for 10 min and washed twice with sterilized PBS (pH 7.2) [15]. The bacterial cells were diluted to a final dose of  $1.0 \times 10^8$  (low concentration) and  $1.0 \times 10^9$  (high concentration) CFU/mouse.

### Animals and Experimental Design

Female young (2-month-old) and old (18-month-old) C57BL/6J mice were purchased from Korea Basic Science Institute (Korea). The mice were housed at  $23 \pm 2^\circ\text{C}$  and  $50 \pm 10\%$  humidity under a 12-h light/dark cycle. Feed and water were provided ad libitum. The mice were divided into five groups ( $n = 6$ ): (i) young mice gavaged with PBS (YM; no probiotic-feeding); (ii) old mice gavaged with PBS (OM; no probiotic-feeding); (iii) old mice gavaged with *W. cibaria* JW15-low (JW15-L;  $1.0 \times 10^8$  CFU/mouse/day); (iv)

old mice gavaged with *W. cibaria* JW15-high (JW15-H;  $1.0 \times 10^9$  CFU/mouse/day); and (v) old mice gavaged with *L. rhamnosus* GG (LGG;  $1.0 \times 10^9$  CFU/mouse/day). After 4 weeks of feeding, animals were sacrificed by diethyl ether overdose. All animal experiments were approved by the Institutional Animal Care and Use Committee of Chungbuk National University (Approval No. CBNUA-978-16-01).

### Complete Blood Count

Four weeks after the administration of probiotics, blood of the mice was collected to assess the complete blood count (CBC). The white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), platelet (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH) levels were measured using an automated hematology analyzer (Advia2120, USA).

### Splenocyte Isolation and Cell Count

The experimental animals were sacrificed by diethyl ether overdose and spleens were weighed. Splenocytes were obtained by passing through a cell strainer (SPL, Korea) and centrifuged at 200  $\times$ g for 10 min. Lysis of erythrocytes was conducted using  $1 \times$  RBC lysis buffer (0.154 M  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$ , 0.1 mM ethylenediaminetetraacetic acid). After centrifugation at 200  $\times$ g for 10 min, the splenocytes were counted using the trypan blue exclusion method [16].

### Cell Surface Staining for Flow Cytometry

Single-cell suspensions obtained from spleen tissue were stained with CD4-PECy5.5, CD8-PE, NK1.1-FITC, and B220-PE antibodies for 30 min on ice and then washed with fluorescence-activated cell sorting buffer (2% FBS in PBS). The cell surface expression of CD4, CD8, NK1.1, and B220 was evaluated by flow cytometry analysis performed with the Accuri analyzer (BD Biosciences). All antibodies used were purchased from eBioscience (USA).

### Splenocyte Proliferation

Fresh splenocytes ( $1 \times 10^5$  cells/well) in 96-well plates (Costar; Corning, USA) were cultured and stimulated with the B cell mitogen lipopolysaccharide (LPS; Sigma-Aldrich, USA) at 5  $\mu\text{g}/\text{ml}$  and T cell mitogen concanavalin A (ConA; Sigma-Aldrich) at 10  $\mu\text{g}/\text{ml}$  in Dulbecco's modified Eagle's medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) with 100 U/ml penicillin-streptomycin (Gibco) for 72 h. Then, sodium 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (Sigma-Aldrich) at 1 mg/ml and phenazine methosulfate (Sigma-Aldrich) at 10 mM were added to the culture medium for 2 h. Plates were read at 450 nm with an ELISA reader (Tecan, Austria) [17].

### Cytokine Measurement

The cytokines interleukin (IL)-6, IL-10, tumor necrosis factor

(TNF)- $\alpha$ , and interferon (IFN)- $\gamma$  were analyzed in culture supernatants or serum using ELISA kits (eBioscience) according to the manufacturer instructions. Briefly, 96-well plates (SPL) were coated with 100  $\mu$ l/well of capture antibodies to IL-6, TNF- $\alpha$ , and IFN- $\gamma$  in coating buffer overnight at 4°C. The plates were washed five times with PBS containing 0.05% (v/v) Tween 20 (PBST; BioShop, Canada), and then each well was incubated with 200  $\mu$ l of 10% (v/v) FBS in PBS at 37°C for 1 h to block nonspecific protein binding. After five washes with PBST, standards of cytokine and supernatant samples of 100  $\mu$ l were incubated at room temperature for 2 h. After five washes with PBST, 100  $\mu$ l of antibodies was reacted and incubated at RT for 1 h. After five washes with PBST, the plate was reacted with avidin-HRP at room temperature for 30 min and washed seven times with PBST. Then, wells were incubated with 100  $\mu$ l of tetramethylbenzidine in the dark for 15 min and the reaction was stopped with 50  $\mu$ l of 2 N H<sub>2</sub>SO<sub>4</sub>. The absorbance was read at 450 nm using a microplate reader. The production levels of cytokines were calculated from the standard curves of each of the cytokine standards (0–1,000 pg/ml for IL-6, IL-10, TNF- $\alpha$ , and IFN- $\gamma$ ) [15].

#### RNA Extraction and Real-Time PCR

Total RNA was isolated using RNAiso plus (Takara Bio, Japan). The RNA concentration was measured on a BioSpec-nano (Shimadzu, Japan). One microgram of RNA was heated to 65°C for 5 min prior to transcription to cDNA using a PrimeScript First Strand cDNA Synthesis Kit (Takara Bio). The relative expression of IFN- $\gamma$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-10, IL-12, IL-17, T-bet, and Nkp46 was detected by quantitative PCR (Rotor-Gene 6200; Corbett Research, Australia) with SYBR Green PCR Master Mix reagent (Takara Bio) and normalized to the level of  $\beta$ -actin as a reference gene using the following primers: IFN- $\gamma$  (forward 5'-GGACAA TCTCTTCCCCACCC-3' and reverse 5'-CATGAAAATCCTGCA GAGCC-3'), IL-1 $\beta$  (forward 5'-GCCTTGGGCTCAAAGGAAAG AATC-3' and reverse 5'-GGAAGACACAGATTCCATGGTGAAG-

3'), IL-6 (forward 5'-TCCATCCAGTTGCCTTCTTG-3' and reverse 5'-GGTCTGTTGGGAGTGGTATC-3'), TNF- $\alpha$  (forward 5'-GAA CTGGCAGAAGAGGCACT-3' and reverse 5'-AGGGTCTGGGCC ATAGA-3'), IL-10 (forward 5'-GCGCTGCATCGATTCT CC-3' and reverse 5'-GCTCTGTCTAGGCTCTGGAGT-3'), IL-12 (forward 5'-AACCTCACCTGTGACACGCC-3' and reverse 5'-CAAGTCCATGTTTCTTTGCACC-3'), IL-17 (forward 5'-ACTCTT CATCCACCTCACACGA-3' and reverse 5'-GCCATGATATAG ACGTGTGGC-3'), IL-17 (forward 5'-ACTCTTATCCACCTCAC ACGA-3' and reverse 5'-GCCATGATATAGACGTTGTGGC-3'), T-bet (forward 5'-CAACAACCCCTTGGCAAAG-3' and reverse 5'-TCCCCCAAGCAGTTGACAGT-3'), Nkp46 (forward 5'-CCC CCTGACACCAGATGTTC-3' and reverse 5'-CTGTGCCTTGGG CTATGCT-3'), and  $\beta$ -actin (forward 5'-GAAATCGTGCCTGAC ATCAAAG-3' and reverse 5'-TGTAGTTTCATGGATGCCACAG-3') [18].

#### Statistical Analysis

All data were analyzed using SPSS 12.0 for Windows (SPSS, USA). Significant differences between groups were tested using ANOVA and compared using Duncan's test. Statistical significance was considered at  $p < 0.05$ .

## Results

#### Effects of *W. cibaria* JW15 on CBC in Aged Mice

In the OM group, the WBC, RBC, platelets, and hemoglobin levels were significantly reduced compared with those in the YM group, indicating the detrimental effects of aging on hematology (Table 1). When the OM group was administered JW15 daily, CBC parameters such as WBC and RBC counts were recovered to levels similar to those of the YM group. The WBC number in the JW15-L group ( $3.0 \pm 0.63 \times 10^3/\text{mm}^3$ ) was significantly higher ( $p < 0.05$ ) than

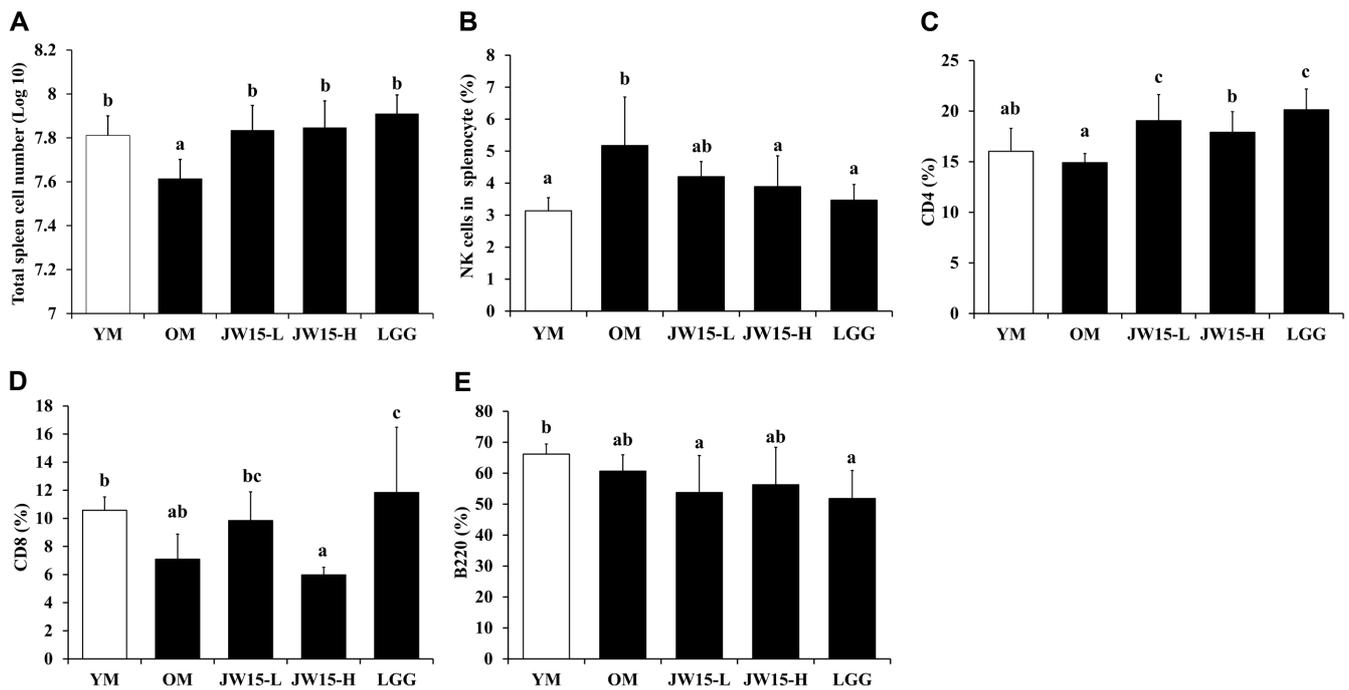
**Table 1.** Effect of *Weissella cibaria* JW15 on the hematological indices in 18-month-old C57BL/6J mice.

Hematological index	Group				
	YM	OM	JW15-L	JW15-H	LGG
WBC ( $10^3/\text{mm}^3$ )	$3.6 \pm 0.99^c$	$1.5 \pm 0.32^a$	$3.0 \pm 0.63^{bc}$	$2.2 \pm 0.98^{ab}$	$1.9 \pm 0.33^a$
RBC ( $10^6/\text{mm}^3$ )	$8.6 \pm 0.35^b$	$7.2 \pm 0.15^a$	$8.1 \pm 0.93^b$	$6.9 \pm 0.59^a$	$6.9 \pm 0.50^a$
Platelet ( $10^3/\text{mm}^3$ )	$975.6 \pm 81.80^{ab}$	$638.2 \pm 323.18^a$	$1,055.8 \pm 251.15^b$	$921.5 \pm 134.44^a$	$851.6 \pm 294.37^a$
Hgb (g/dl)	$12.5 \pm 0.58^c$	$10.4 \pm 0.25^{ab}$	$11.2 \pm 0.49^b$	$10.2 \pm 0.88^a$	$9.8 \pm 0.79^a$
MCHC (g/dl)	$24.5 \pm 0.36^a$	$19.7 \pm 9.78^a$	$24.3 \pm 1.00^a$	$24.9 \pm 0.47^a$	$24.4 \pm 0.88^a$
MCV ( $\mu\text{m}^3$ )	$60.3 \pm 0.71^a$	$58.5 \pm 1.24^a$	$60.8 \pm 2.29^a$	$59.4 \pm 2.57^a$	$61.0 \pm 1.79^a$
MCH (pg)	$14.7 \pm 0.28^a$	$14.4 \pm 0.13^a$	$14.5 \pm 0.19^a$	$14.8 \pm 0.36^a$	$14.8 \pm 0.56^a$

WBC: white blood cell; RBC: red blood cell; Hgb: hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin.

YM, young mice group (no probiotic feeding); OM, old mice group (no probiotic feeding); JW15-L, *Weissella cibaria* JW15-low group ( $1.0 \times 10^8$  CFU/mouse/day); JW15-H, *Weissella cibaria* JW15-high group ( $1.0 \times 10^9$  CFU/mouse/day); LGG, *Lactobacillus rhamnosus* GG group ( $1.0 \times 10^9$  CFU/mouse/day).

<sup>abc</sup>Different superscript letters indicate the statistical differences determined by ANOVA ( $p < 0.05$ ).



**Fig. 1.** Analysis of immune cells in the spleen after treatment with *Weissella cibaria* JW15.

(A) Total spleen cell number (Log 10); (B) NK cells (%); (C) CD4<sup>+</sup> T cell; (D) CD8<sup>+</sup> T cell; (E) B220 cell. Mice were orally administered *W. cibaria* JW15 for 4 weeks. Data represent the mean  $\pm$  SD of six mice in each group. Different superscript letters (a, b, and c) indicate the statistical differences determined by ANOVA ( $p < 0.05$ ). YM, young mice group (no probiotic feeding); OM, old mice group (no probiotic feeding); JW15-L, *W. cibaria* JW15-low group ( $1.0 \times 10^8$  CFU/mouse/day); JW15-H, *W. cibaria* JW15-high group ( $1.0 \times 10^9$  CFU/mouse/day); LGG, *Lactobacillus rhamnosus* GG group ( $1.0 \times 10^9$  CFU/mouse/day).

that in the OM group ( $1.5 \pm 0.32 \times 10^3/\text{mm}^3$ ) and comparable to that of the YM group ( $1.5 \pm 0.32 \times 10^3/\text{mm}^3$ ). In addition, the RBC count was significantly higher in the JW15-L group ( $8.1 \pm 0.93 \times 10^6/\text{mm}^3$ ) than in the OM group ( $7.2 \pm 0.15 \times 10^6/\text{mm}^3$ ). A similar trend was also observed in platelet counts among the groups. Interestingly, the CBC results were not different between the LGG and OM groups.

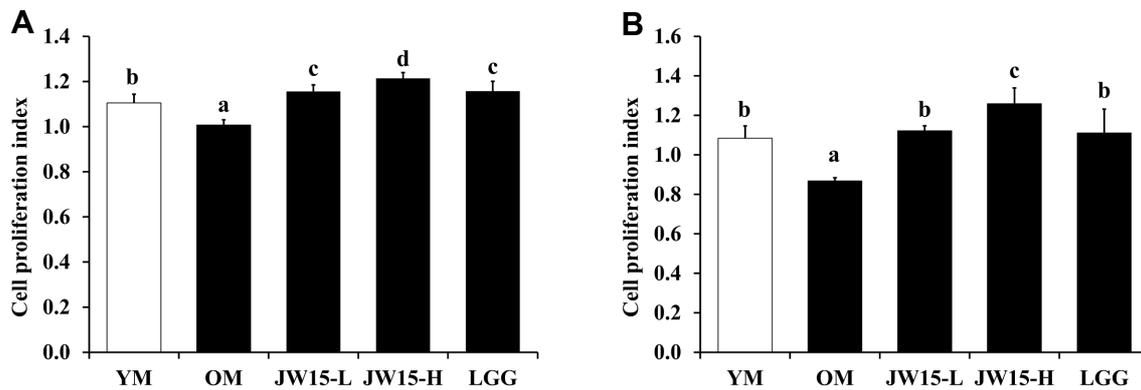
#### Effects of JW15 on Spleen Cell Count and Splenocyte Proliferation

After 4 weeks of oral administration of JW15 to aged mice, the splenocytes were isolated and counted (Fig. 1). As expected, the total splenocyte count was statistically lower in the OM group than in the YM group, reflecting the WBC count in whole blood as shown above (Table 1). When mice were fed with probiotics for 4 weeks, the spleen cell numbers in all three probiotic groups were significantly higher than that in the OM group, and were similar to that of the YM group ( $p < 0.05$ ). This result implies that JW15 as well as LGG administration may enhance hematopoiesis in bone marrow, followed by increased immune cell numbers

in peripheral tissues.

The distribution of NK cell, T cell (CD4<sup>+</sup>, CD8<sup>+</sup>), and B cell (B220) in mouse spleen was analyzed by flow cytometry. With JW15 treatment or LGG, the proportion of CD4<sup>+</sup>T cells was increased compared with that in the OM group, whereas the levels of NK cells and B cells were not significantly different among the groups. In the aspect of CD8<sup>+</sup>T cells, only the LGG group showed statistically significant difference compared with OM group (Fig. 1).

In order to determine whether splenocytes efficiently respond to stimuli, cells were treated with TLR4 ligand (LPS) or mitogen (ConA) for 72 h, and the level of cell proliferation was compared among the mouse groups. The proliferative potential of splenocytes was slightly reduced with statistical significance in the OM group as shown in Fig. 2, suggesting that aging affected both immune cell development and function. Regardless of the stimulant, splenocytes derived from the JW15 or LGG group showed higher levels of proliferation than those from the OM group. Oral administration of JW15 either at low or high dose significantly enhanced TLR4-mediated splenocyte



**Fig. 2.** Effects of *Weissella cibaria* JW15 on proliferation of splenocytes in 18-month-old C57BL/6J mice.

*W. cibaria* JW15 promotes LPS and ConA-induced splenocyte proliferation. Splenocytes were cultured in the presence of LPS (5 µg/ml) or ConA (10 µl/ml) for 72 h. (A) LPS-induced splenocyte proliferation. (B) ConA-induced splenocyte proliferation. Mice were orally administered *W. cibaria* JW15 for 4 weeks. Data represent the mean ± SD of six mice in each group. Different superscript letters (a, b, c, and d) indicate the statistical differences determined by ANOVA ( $p < 0.05$ ). YM, young mice group (no probiotic feeding); OM, old mice group (no probiotic feeding); JW15-L, *W. cibaria* JW15-low group ( $1.0 \times 10^8$  CFU/mouse/day); JW15-H, *W. cibaria* JW15-high group ( $1.0 \times 10^9$  CFU/mouse/day); LGG, *Lactobacillus rhamnosus* GG group ( $1.0 \times 10^9$  CFU/mouse/day).

proliferation to a level similar to that of cells from the YM group ( $p < 0.05$ ). Increased proliferation of splenocytes was also observed when cells for JW15-H were stimulated with ConA. In the case of the LGG group, splenocyte proliferation was significantly higher than that in the OM group only when cells were stimulated with ConA ( $p < 0.05$ ).

#### Splenocyte Cytokines (IL-6, IL-10, TNF- $\alpha$ , and IFN- $\gamma$ )

When immune cells are activated, they produce various types of cytokines and modulate immune responses. To estimate whether oral administration of JW15 affected cytokine production by splenocytes, levels of major immune-stimulatory cytokines such as IL-6, TNF- $\alpha$ , and IFN- $\gamma$  were measured using supernatants harvested from splenocyte cultures. As shown in Fig. 3A, TNF- $\alpha$  levels in splenocyte culture of the JW15-L and JW15-H groups ( $39.2 \pm 10.60$  pg/ml and  $44.7 \pm 4.36$  pg/ml, respectively) were significantly higher than that in the OM group ( $28.6 \pm 3.72$  pg/ml,  $p < 0.05$ ). The splenocyte levels of IFN- $\gamma$  in JW15-H ( $55.5 \pm 6.58$  pg/ml) group mice were shown to be more secreted than those in the OM group ( $41.7 \pm 3.74$  pg/ml) (Fig. 3C). JW15 showed increased production of some proinflammatory cytokines (TNF- $\alpha$  and IFN- $\gamma$ ) in aged splenocytes.

IL-10 is a well-known anti-inflammatory cytokine, which inhibits various types of immune cell function, and its dysregulation is accompanied by aging. In the OM group, the serum level of IL-10 was slightly higher than that in the YM group, and this difference was statistically significant

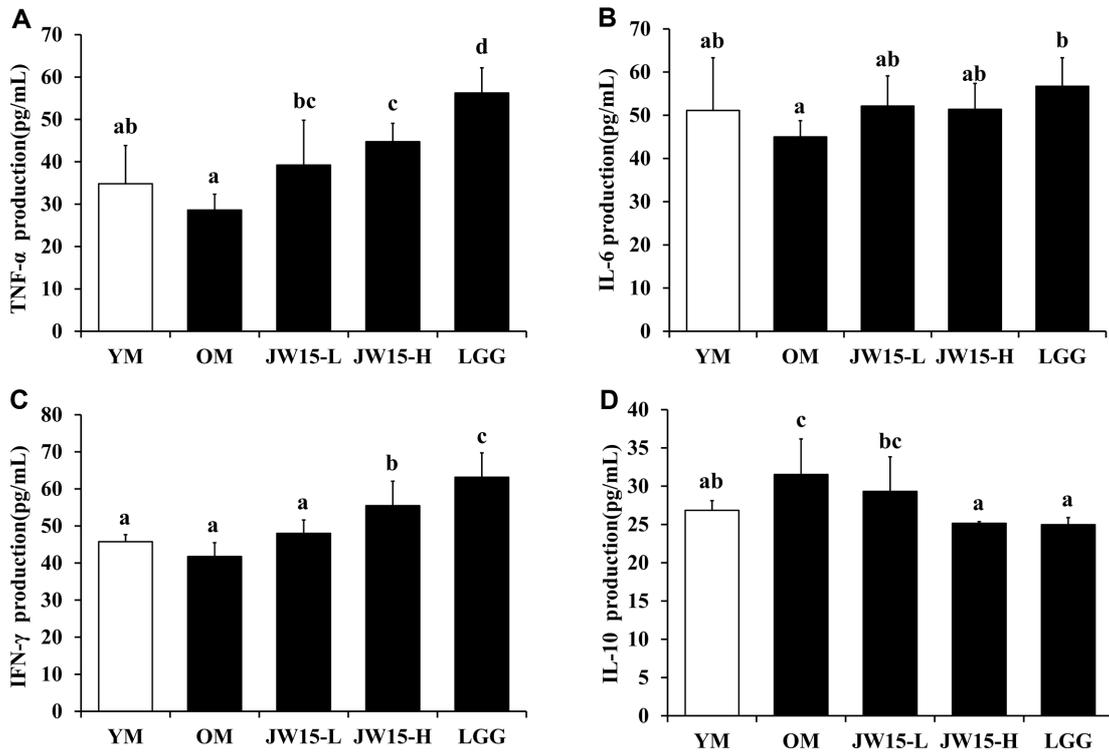
( $p < 0.05$ ). Administration of JW15-H or LGG for 4 weeks downregulated serum IL-10 to the levels of the YM group (Fig. 3D). However, the physiological significance of reduced serum IL-10 was not apparent in the present study ( $p < 0.05$ ).

#### Serum IFN- $\gamma$ and TNF- $\alpha$

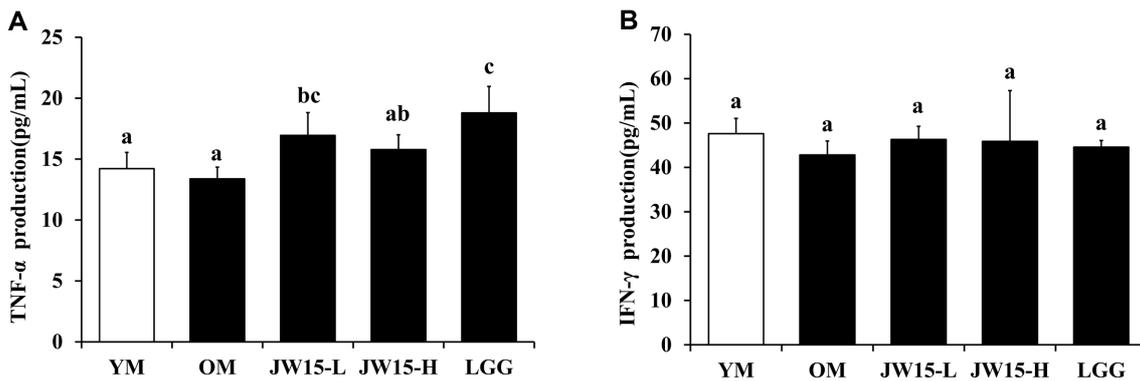
At 4 weeks after oral administration of JW15 to aged mice, cytokines IFN- $\gamma$  and TNF- $\alpha$  were measured in mouse serum. The production of IFN- $\gamma$  and TNF- $\alpha$  in aged mouse serum is represented in Fig. 4. TNF- $\alpha$  production in the JW15-L group ( $16.9 \pm 1.88$  pg/ml) showed a significant elevation compared with that in the OM group ( $13.4 \pm 0.96$  pg/ml,  $p < 0.05$ ). There was no significant difference in IFN- $\gamma$  production from aged mouse serum between any groups ( $p < 0.05$ ).

#### Cytokine and T Cell-Mediated Transcription Factors mRNA Expression in Mouse Colon and Mesenteric Lymph Nodes

JW15 has been shown to induce the production of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in macrophages [14]. In this study, the effect of JW15 on expression of IFN- $\gamma$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-10, IL-12, and IL-17 in colon and mesenteric lymph nodes (MLN) was determined. In colon tissue of the JW15-H group, a significant increase ( $p < 0.05$ ) in IFN- $\gamma$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-10, IL-12, and IL-17 was observed as compared with that in the OM group (Fig. 5). We analyzed mRNA levels of T-bet and NKp46 (NK cell marker) and found that those of T-bet displayed a tendency to be increased in JW15-fed groups but there was no significant difference



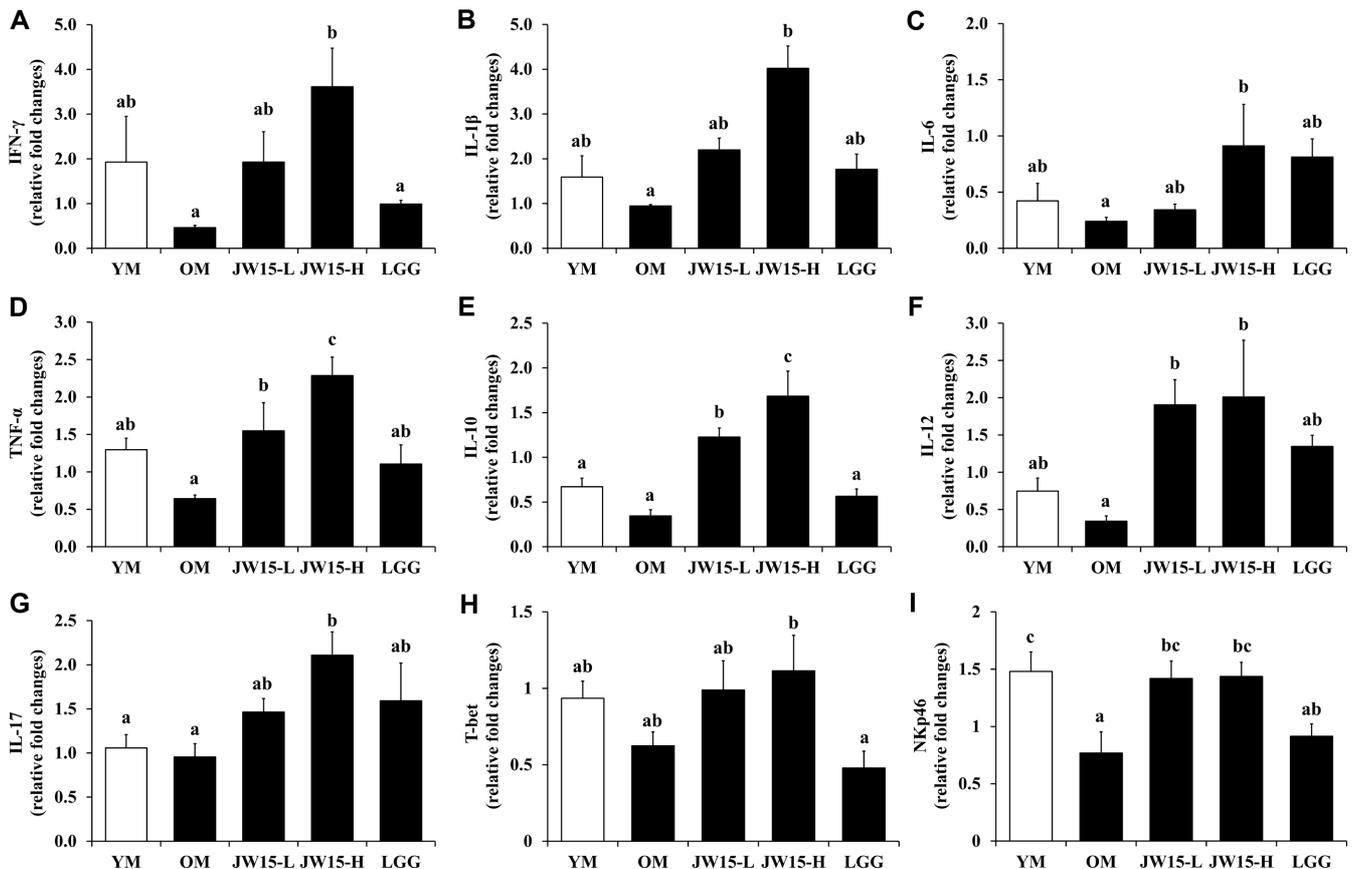
**Fig. 3.** Splenocyte levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ, and IL-10 production in 18-month-old C57BL/6J mice. Productions of (A) TNF-α, (B) IL-6, (C) IFN-γ, and (D) IL-10 in mouse splenocytes. Mice were orally administered *Weissella cibaria* JW15 for 4 weeks. Data represent the mean ± SD of six mice in each group. Different superscript letters (a, b, c, and d) indicate the statistical differences determined by ANOVA ( $p < 0.05$ ). YM, young mice group (no probiotic feeding); OM, old mice group (no probiotic feeding); JW15-L, *W. cibaria* JW15-low group ( $1.0 \times 10^8$  CFU/mouse/day); JW15-H, *W. cibaria* JW15-high group ( $1.0 \times 10^9$  CFU/mouse/day); LGG, *Lactobacillus rhamnosus* GG group ( $1.0 \times 10^9$  CFU/mouse/day).



**Fig. 4.** Tumor necrosis factor (TNF)-α and interferon (IFN)-γ production in 18-month-old C57BL/6J mouse serum. Production of (A) TNF-α and (B) IFN-γ in mouse serum. Mice were orally administered *Weissella cibaria* JW15 for 4 weeks. Data represent the mean ± SD of six mice in each group. Different superscript letters (a, b, and c) indicate the statistical differences determined by ANOVA ( $p < 0.05$ ). YM, young mice group (no probiotic feeding); OM, old mice group (no probiotic feeding); JW15-L, *W. cibaria* JW15-low group ( $1.0 \times 10^8$  CFU/mouse/day); JW15-H, *W. cibaria* JW15-high group ( $1.0 \times 10^9$  CFU/mouse/day); LGG, *Lactobacillus rhamnosus* GG group ( $1.0 \times 10^9$  CFU/mouse/day).

among the groups. The level of NKp46 transcript, a NK cell marker, was remarkably higher in JW15-fed groups than

in the OM group and similar to the level of the YM group. This result indicates that NK cells could be responsible for



**Fig. 5.** Effects of *Weissella cibaria* JW15 on production of cytokines and T cell-mediated transcriptional factors in 18-month-old C57BL/6J mouse colon.

mRNA expression of (A) IFN- $\gamma$ , (B) IL-1 $\beta$ , (C) IL-6, (D) TNF- $\alpha$ , (E) IL-10, (F) IL-12, (G) IL-17, (H) T-bet, and (I) NKp46 was assessed by quantitative PCR in colon samples from mice. Mice were orally administered *W. cibaria* JW15 for 4 weeks. Data represent the mean  $\pm$  SD of six mice in each group. Different superscript letters (a, b, and c) indicate the statistical differences determined by ANOVA ( $p < 0.05$ ). YM, young mice group (no probiotic feeding); OM, old mice group (no probiotic feeding); JW15-L, *W. cibaria* JW15-low group ( $1.0 \times 10^8$  CFU/mouse/day); JW15-H, *W. cibaria* JW15-high group ( $1.0 \times 10^9$  CFU/mouse/day); LGG, *Lactobacillus rhamnosus* GG group ( $1.0 \times 10^9$  CFU/mouse/day).

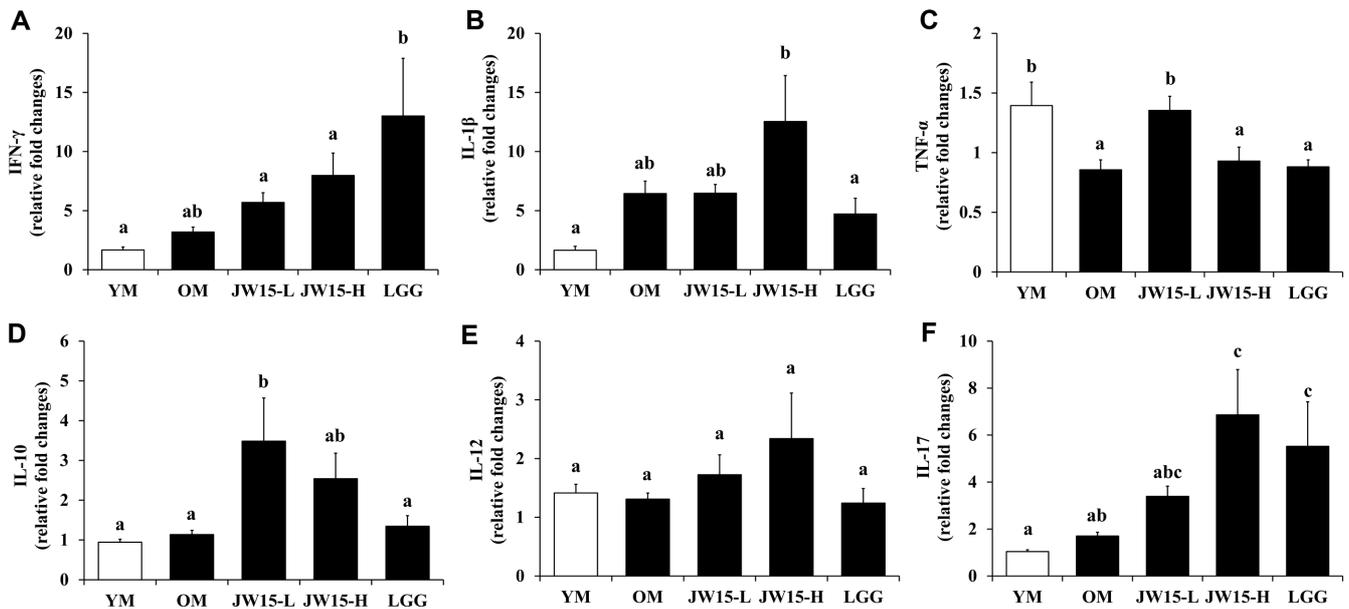
the increased mRNA expression of IFN- $\gamma$ , although we cannot exclude other cell types such as Th1 cell. For MLN, the relative expression of IL-1 $\beta$  and IL-17 was highest in the JW15-H group (Fig. 6). In mesenteric lymph nodes, there was no significant increase in mRNA level of T-bet, GATA-3, ROR- $\gamma$ , and Foxp3 (data not shown). Therefore, these results suggest that administration of JW15 could increase cytokines in the colon and MLN of aged mice.

## Discussion

Probiotics have been considered to possess beneficial effects on host health, including enhancing the immune system. Probiotics that regulate humoral (B cell) and cellular (T cell) immunity induce splenocyte proliferation and

cytokine production. The probiotic strain *W. cibaria* JW15 increased the levels of NO, NF- $\kappa$ B, IL-6, and TNF- $\alpha$  in RAW 264.7 macrophage cells [14]. In addition, oral administration of JW15 showed resistance to *Listeria monocytogenes* infection by increasing proinflammatory cytokine (IL-1 $\beta$ , TNF- $\alpha$ ) levels [19]. Therefore, we hypothesized that *W. cibaria* JW15 administration induces splenocyte cytokines (IL-6, TNF- $\alpha$ , and IFN- $\gamma$ ) and improves the immune response in 18-month aged C57BL/6J mice. In this study, we investigated the immune-enhancing effects of *W. cibaria* JW15 by analyzing the number of WBCs and RBCs in blood, ability for splenocyte proliferation, and the cytokine levels (IL-6, TNF- $\alpha$ , and IFN- $\gamma$ ) in splenocytes and serum of aged mice.

The immunological function of JW15 may be initiated by the activation of cells such as lymphocytes and macrophages.



**Fig. 6.** Effects of *Weissella cibaria* JW15 on production of cytokines in 18-month-old C57BL/6J mouse mesenteric lymph nodes. mRNA expression of (A) IFN- $\gamma$ , (B) IL-1 $\beta$ , (C) TNF- $\alpha$ , (D) IL-10, (E) IL-12, and (F) IL-17 was assessed by quantitative PCR in mesenteric lymph node samples from mice. Mice were orally administered *W. cibaria* JW15 for 4 weeks. Data represent the mean  $\pm$  SD of six mice in each group. Different superscript letters (a, and b) indicate the statistical differences determined by ANOVA ( $p < 0.05$ ). YM, young mice group (no probiotic feeding); OM, old mice group (no probiotic feeding); JW15-L, *W. cibaria* JW15-low group ( $1.0 \times 10^8$  CFU/mouse/day); JW15-H, *W. cibaria* JW15-high group ( $1.0 \times 10^9$  CFU/mouse/day); LGG, *Lactobacillus rhamnosus* GG group ( $1.0 \times 10^9$  CFU/mouse/day).

To examine the effect of JW15 on immune cells in aged mice, the number of spleen cells and the splenocyte proliferative capacity were determined. The spleen is the largest secondary immune organ in the body and controls immune responses. The proliferation of splenocytes is critical in the immune system, since lymphocyte differentiation occurs by maturation of B cells and T cells [20]. In particular, the size and number of spleen cells can be used as a direct indicator of lymphocyte differentiation and are utilized as an immunity indicator in many studies [21]. In the present study, the splenocyte proliferation ratio in the JW15-fed group was significantly activated compared with that in the OM group. In particular, LPS- and ConA-stimulated splenocytes of the JW15-H group showed a higher splenocyte proliferative ability. Similar activity of LPS and ConA has been demonstrated in *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, which increase the proliferation of aged mouse splenocytes [17]. The synthesized splenocytes are shown to induce inflammatory substances such as NO, IL-6, and TNF- $\alpha$ , which activate tissue regeneration and immune responses [22]. Therefore, these results suggest that JW15 may induce the production of inflammatory mediators (IL-6, TNF- $\alpha$ , and IFN- $\gamma$ ).

Immune cells such as B cells, T cells, and macrophages

were included in the spleen cell count, and these cells secreted many cytokines that regulate immune responses. The production of proinflammatory cytokine IL-6 activated B cells and T cells, which produced the cytokine TNF- $\alpha$  [22]. The concentration of cytokines plays an important role during aging, and some probiotics are shown to regulate the production of cytokines and modulate immune function in aged animals [10]. The probiotic dahi, containing *L. acidophilus* and *B. bifidum*, induced an increase in the level of IL-6 produced by macrophages in aging mice [23]. In this experiment, JW15 significantly increased TNF- $\alpha$  and IL-6 production, which induces T cell and B cell activity in aging animals, with reduced proinflammatory cytokine production. The present results show that JW15 induced immune activity by increasing the secretion of proinflammatory cytokines involved in T cell and B cell activity in aged mice.

IFN- $\gamma$  is produced by NK cells in nonspecific immune responses and has immunosuppressive and immunomodulation abilities [24]. In the innate immune system, T cells are particularly susceptible to the injurious effects of aging, and IFN- $\gamma$  and IL-2 are known to be representative indicators of T cell function [25]. Thus, the ability to produce IFN- $\gamma$  and IL-2 shows a decline in the aging process. Administration of *L. rhamnosus* increases the production of

IFN- $\gamma$  in splenocytes of aged mice [26], and it has been reported that *B. bifidum* increases the production of IFN- $\gamma$  in aged mouse serum [27]. In this study, it was confirmed that splenocyte IFN- $\gamma$  significantly increased in aged mice treated with JW15 compared with that in the OM group. These results suggest that JW15 may significantly delay immune aging and improve immune system function.

A number of studies have shown that age-associated changes in the immune system can lead to dysfunction in immune cells and accompanying imbalance in Th1/Th2 cytokine production [26, 28]. Similarly, the number of immune cells and immune cell proliferative capacity in aging animals decreased in the present study. Therefore, it has been confirmed that many responses of the immune system are diminished with aging. Several probiotics have been reported to restore impaired immune system function in aged animals through immune cell proliferation and regulation of cytokine production [4, 10, 23]. Similarly, the present results show that JW15 improved splenocyte proliferation and regulated the production of various cytokines.

In conclusion, the present study demonstrated that dietary supplementation with JW15 resulted in increased WBC and RBC counts in peripheral blood, and enhanced splenocyte proliferation and cytokine production in C57BL/6J aged mice. These findings imply that JW15 could modulate immune cell development and function and thus restore the impaired immune response in aged mice. Further studies are necessary to confirm our findings and expand the current knowledge on the specific molecular and immunological mechanisms by which JW15 modulates the immune system in elderly humans as well as other animals. In addition, we found that *W. cibaria* JW15 possessed stronger immunomodulatory activity than the most well-known commercial probiotic strain, *L. rhamnosus* GG. Therefore, we propose that JW15 is a potential candidate for use as a probiotic bacterium along with LGG or even in place of LGG.

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