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Effect of fermented soybean products intake on the overall immune safety and function in mice

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Various functional activities have been reported for the fermented soybean products *doenjang* (DJ) and *cheonggukjang* (CGJ), although no systemic investigations of their immune functions have been conducted to date. We examined the effects of an experimental diet of DJ, CGJ, or a mixture of unfermented raw material for 4 weeks on overall immunity and immune safety in mice. No significant alterations were observed in peripheral or splenic immune cells among groups. Enhanced splenic natural killer cell activity was observed in the DJ and CGJ groups compared with the plain diet group. T helper type-1 (Th1)-mediated immune responses were enhanced in the DJ and CGJ groups with an upregulated production ratio of IFN-γ *vs*. IL-4 and IgG2a *vs*. IgG1 in stimulated splenic T and B cells, respectively. Resistance to *Listeria monocytogenes* infection was observed in the DJ and CGJ groups. Overall, the results of this study suggest that DJ and CGJ intake consolidates humoral and cellular immunity to Th1 responses.

Keywords: *cheonggukjang*, *doenjang*, immunity, mice, soybean products

Introduction

Since introduction of the concept of functional food in the 1980s, the role of food ingredients in health promotion or disease prevention has been extensively reported worldwide [9,27,35]. The functionality of food or food ingredients has primarily been investigated in phytochemicals from various plant sources including soy, flaxseed, garlic, tomatoes, broccoli, and citrus fruits [9]. A broad spectrum of diseases have been targeted for evaluation of the preventive and therapeutic effects of functional foods, such as metabolic syndrome, cardiovascular disease, cancer, and inflammatory diseases [8,23,24,28].

Among the various functional food ingredients, soybeans are known to contain functional compounds that contribute to health benefits. Two major bioactive compounds from soybean, isoflavones (*e.g*., genistein) and soybean peptides (*e.g*., lunasin), have frequently been reported to exhibit anti-carcinogenic, anti-diabetic, anti-oxidative, or anti-hypertensive effects [10,21,26]. Two Korean traditional fermented soybean products, *doenjang* (DJ) and *cheonggukjang* (CGJ), have been reported to exert anti-carcinogenic, anti-mutagenic, anti-inflammatory, or anti-diabetic effects [15,18,21,31]. CGJ is produced during fermentation with *Bacillus spp*. for a short period of 2 to 3 days, while DJ is produced via fermentation with *Bacillus spp*. and fungi for a period of 3 to 6 months [34]. DJ-mediated suppression of the inflammatory response has been demonstrated in experimentally induced colitis in mice [18]. Furthermore, an increase in the number of $CD4^+$ or $CD8^+$ T cells in the gastrointestinal tract of mice that were administered DJ has been reported [22]. Serum levels of proinflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor alpha were downregulated in ovariectomized mice administered CGJ [30]. Although several studies using mouse models have reported the immunomodulatory functions of DJ and CGJ, particularly inflammatory responses, no studies have investigated the systemic effects (*in vivo*) of DJ or CGJ intake on the immune system in humans or an animal model.

Therefore, in this study, we evaluated the effects of DJ and CGJ intake on the immune response in mice. Hematological parameters, histopathological findings regarding the major immune organs, natural killer (NK) cell function, serum immunoglobulin levels, cytokine production from stimulated

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splenic T cells, enumeration of splenic lymphoid cells, and host defense against *Listeria monocytogenes* infection were also examined.

Materials and Methods

Animals

Five-week-old male BALB/cByJ mice obtained from Orient Bio (Korea) were acclimated for at least 5 days before experimentation. The animals were housed in an animal room maintained at 22 ± 3°C and a relative humidity of 30 to 70%. The room was lit by artificial light for 12 h per day. During the experiment, animals were allowed free access to a standard laboratory solid diet and sterilized drinking water. The experimental mouse chow was prepared by mixing a standard rodent chow (Cargill Agri Purina, Korea) with freeze-dried DJ, CGJ, or raw material mixture (5% w/w). The raw material mixture was used as an unfermented control diet. Mice were randomly allocated into test groups (eight or four mice per group) and fed the experimental diet for 4 weeks. All of our animal maintenance and experimental procedures were approved by the Institutional Animal Care and Use Committee of the Catholic University of Daegu (approval No. CUD IACUC-2014-14).

Preparation of DJ and CGJ

The manufacture of fermented soybean products has been thoroughly investigated [34]. Briefly, three bacterial strains, *Bacillus amyloliquefaciens CJ3-27* (KCCM11317P, Korean Culture Center of Microorganisms, Korea), *Bacillus amyloliquefaciens CJ1526*, and *Bacillus subtilis CJ1553*, were inoculated onto three 500 mL flasks containing 200 mL of nutrient broth (pH 7.0; BD Biosciences, USA), then incubated in a shaking incubator at 37°C for 24 h. Next, equal amounts of the three cultured broths were mixed together. Meanwhile, soybeans (5 kg) were immersed in 15 L of water for 6 h, after which the water was removed. The wet soybeans were sterilized using an autoclave for 15 min at 1 atm pressure, then cooled to 35°C (steamed soybeans). The mixture of cultured broths was added to the steamed soybeans at 1% (v/w) and then incubated for 40 h in a chamber at 40°C and 99% relative humidity to give the final *cheonggukjang* product (CJ-CGJ-01). Meju (soybean block made of steamed soybeans) was fermented for 15 days in a chamber at 37°C and 35% relative humidity, then soaked in brine (22% w/w salt) for 15 days at 15°C. After decanting the supernatant liquid, the remaining soy blocks were aged for 6 months at 25°C to give the final DJ product (CJ-DJ-M01). The raw material mixture was made of 87% steamed soybeans and 13% salt. DJ, CGJ, and the raw material mixture were freeze-dried before use in the experiments.

Hematological and histopathological analysis

Blood samples were collected into Vacutainer tubes containing K_2EDTA (BD Biosciences) and analyzed for peripheral blood counts using the ADVIA 2120 automatic analyzer (Siemens, Germany). For examination of histopathological changes in immune organs, tissue samples were collected from the thymus, spleen, and mesenteric lymph nodes from one mouse per group since these organs from seven mice per group were used for immune cell phenotyping or *ex vivo* production of cytokines or immunoglobulins. Tissue samples were processed for hematoxylin and eosin staining by a CRO company (Croen Research, Korea), then subjected to microscopic examination by a histopathologist [33].

NK cell functional assay

Mitotracker Green FM dye (Molecular Probes, USA), which passively diffuses across the plasma membrane and accumulates in active mitochondria, was used for the NK cell functional assay [39]. K562 human leukemia cells (Korean Cell Line Bank, Korea) were used as target cells. The K562 cells were incubated with Mitotracker Green FM dye (300 nM) for 30 min at 37°C under 5% CO_2. The cells (10^5) were washed three times, then mixed with effector splenocytes (5×10^6) from mice administered the experimental diets for 4 weeks, after which they were incubated for 3 h at 37°C under 5% CO_2. Propidium iodide (2 μg; Sigma, USA) was added into each tube prior to flow cytometric analysis (FACSCalibur; Becton, Dickinson and Company, USA). The percentage of specific killing was calculated by subtraction of the percentage of dead cells in the control tube from the percentage of dead cells in the sample, where only target cells are present without effector cells.

Ex vivo production of cytokines or immunoglobulins from splenic lymphocytes

Mice were sacrificed and the spleens were collected following 29 days on the experimental diet. Blood was collected by cardiac puncture for serum separation. Single cell suspensions of spleen cells were prepared as described elsewhere [11]. Splenic T cells were stimulated *in vitro* with immobilized anti-CD3 monoclonal antibody (mAb; 5 μg/5 × 10^5 cells; BD Biosciences) for 48 h at 37°C in a 5% CO_2 incubator [12]. Splenic B cells (10^6 cells) were stimulated *in vitro* with a mixture of lipopolysaccharide (1 μg; Sigma), recombinant mouse IL-4 (50 ng; R&D Systems, USA), and recombinant human APRIL (10 ng; R&D Systems) for 96 h at 37°C in a 5% CO_2 incubator [29]. The culture medium was BioWhittaker RPMI1640 (Lonza Cologne, USA) supplemented with nonessential amino acid (1 mM), sodium pyruvate (1 mM), sodium bicarbonate (1%), glutamine (2 mM), 2-mercaptoethanol (50 μM), and 10% heat-inactivated fetal bovine serum (HyClone, USA). Culture supernatants were collected and stored at −80°C until use.

Cytokine and immunoglobulin measurements in the culture supernatants and serum

IL-4 and interferon-gamma (IFN-γ) were measured in the culture supernatants using the OptEIA ELISA sets (BD Biosciences) as a paired capture and detection antibody as described elsewhere [12]. Serum IgE levels were determined by sandwich ELISA using anti-mouse IgE mAb as a capture Ab and biotinylated anti-mouse IgE mAb as a detection Ab (BD Biosciences). IgG1 and IgG2a were measured in the culture supernatants and serum with a sandwich ELISA using goat anti-mouse IgG1 or IgG2a (Serotec, UK) as a capture Ab and goat-anti-mouse IgG conjugated with horseradish peroxidase as a detection Ab (Sigma) [13,19].

Flow cytometric analysis

Single cell suspensions of splenocytes were analyzed for $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, and $B220^+$ B cell phenotyping as described elsewhere [13]. Anti-CD4-FITC Ab and anti-CD8-PE Ab were used to identify $CD4^+$ T cell and $CD8^+$ T cell populations. Anti-CD3-PE Ab and anti-B220-FITC Ab were used for sorting B cell populations. All fluorescent Abs were purchased from BD Biosciences. Cells were incubated with FcBlock (1 μg/10^6 cells; BD Biosciences) to avoid nonspecific binding of fluorescent Abs. FITC-conjugated or PE-conjugated isotype controls were used to determine nonspecific binding of fluorescent Abs.

Assessment of host resistance against Listeria infection

To evaluate host resistance against *Listeria monocytogenes* infection, various groups of mice were composed. Following 4 weeks of consumption of the experimental diet, mice (n = 4 per group) were infected with 1.5×10^5 colony-forming units (CFU) of *Listeria monocytogenes* through the tail vein on day 29 [20]. Mice were observed twice daily and mortality was noted. Mice that survived 68 h after infection were sacrificed under CO_2 anesthesia. Livers and spleens were collected and homogenized in 5 mL of saline. The homogenates were serially diluted (10^{-2} to 10^{-4}) in saline and plated on blood agar plates. Numbers of viable bacteria were determined following incubation for 48 h in a 37°C microbiological incubator.

Statistical analyses

Statistical analyses were performed using SigmaStat (ver. 3.5; Systat Software, USA). Data were expressed as the means ± standard error of the mean (SEM). One-way analysis of variance was used to examine significant differences among groups. Student's *t*-tests or the Mann-Whitney rank sum test were used depending on the normal distribution of the data. A p value of less than 0.05 was considered significant.

Results

Effects of the experimental diet on body weight gain and histopathology

Gross adverse effects of the experimental diet were evaluated via comparison of body weight gain before and after the 4 week diet intake among groups. No significant differences in body weight gain were found among groups (Table 1), indicating that the experimental diets containing 5% DJ, CGJ, or the mixture of unfermented raw materials did not influence the normal diet intake in mice. Histopathological analysis of the thymus, spleen, and mesenteric lymph node did not reveal any abnormal changes among mice examined.

Quantitative analysis of the number of peripheral blood cells and proportion of splenic lymphocyte subpopulation

Quantitative changes in peripheral blood cells were monitored in the mice (Table 2). No significant differences were observed in the DJ, CGJ, and unfermented diet groups when compared with mice in the plain diet group for all hematological parameters measured. The proportion of major immune cells in the spleen was evaluated. There were no significant differences in the percentage of $CD4^+$ T cells among groups (DJ, 35.6% ± 1.2%; CGJ, 34.2% ± 1.5%; unfermented diet, 37.1% ± 0.8%; and plain diet, 40.4% ± 3.0%). There were also no significant differences in the percentage of $CD8^+$ T cells (DJ, 14.8% ± 0.8%; CGJ, 17.1% ± 1.2%; unfermented diet, 17.0% ± 1.7%; and plain diet, 17.9% ± 1.2%) or $B220^+$ B cells (DJ, 22.3% ± 2.6%; CGJ, 19.0% ± 1.5%; unfermented diet, 20.2% ± 3.7%; and plain diet, 16.7% ± 1.9%) among groups.

Effect of the experimental diet on NK cell activity

The effects of the experimental diet on NK cell functional activity in the spleen were evaluated. Enhanced killing of K562 target cells was observed for the effector splenocytes from mice

Table 1. Average body weight gain (g, mean ± SEM) of mice fed experimental diets over 4 weeks, and histopathological alterations

Group	Body weight at day 0 (g)*	Body weight gain	Histopathological alterations†
DJ	19.8 ± 0.2	4.3 ± 0.4	ND
CGJ	19.5 ± 0.3	4.9 ± 0.4	ND
Unfermented	19.2 ± 0.3	4.4 ± 0.2	ND
Plain	20.2 ± 0.3	3.9 ± 0.3	ND

DJ, *doenjang*; CGJ, *cheonggukjang*; Unfermented, unfermented raw material mixture; Plain, standard rodent chow; ND, not detected. *Body weight measured 1 day prior to commencement of the experimental diet (eight mice per group). †Histopathological alterations in the thymus, spleen, and mesenteric lymph node were not observed in any of the mice. One mouse per group was used for histopathologic examination.

Table 2. Average number of peripheral blood cells (mean ± SEM) in the groups of mice (n = 8)

Parameters	DJ	CGJ	Unfermented	Plain
WBCs (10^6/mL)	4.48 ± 0.92	4.17 ± 1.18	4.42 ± 0.74	5.37 ± 0.70
RBCs (10^6/μL)	11.04 ± 0.44	10.19 ± 0.43	10.13 ± 0.54	10.15 ± 0.10
Platelets (10^3/μL)	804.7 ± 136.4	672.6 ± 139.0	759.6 ± 126.2	743.2 ± 88.1
Neutrophils (10^6/mL)	1.29 ± 0.48	0.71 ± 0.18	0.63 ± 0.11	1.33 ± 0.22
Lymphocytes (10^6/mL)	2.76 ± 0.59	3.12 ± 0.91	3.51 ± 0.64	3.71 ± 0.76
Monocytes (10^6/mL)	0.06 ± 0.01	0.05 ± 0.02	0.07 ± 0.02	0.07 ± 0.01
Eosinophils (10^6/mL)	0.10 ± 0.03	0.11 ± 0.05	0.09 ± 0.02	0.09 ± 0.02
Basophils (10^6/mL)	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01

WBCs, white blood cells; RBCs, red blood cells; DJ, *doenjang*; CGJ, *cheonggukjang*; Unfermented, unfermented raw material mixture; Plain, standard rodent chow.

Table 3. Distribution of IgG isotypes (mean ± SEM) in serum or B cell culture supernatants of mice

Specimen	Group	IgE (ng/mL)	IgG2a (mg/mL)	IgG1 (mg/mL)	IgG2a/IgG1
Serum*	DJ	316.5 ± 36.1	0.899 ± 0.069[a]	0.391 ± 0.063[a,b]	2.57 ± 0.31
	CGJ	349.6 ± 59.7	0.795 ± 0.043[a]	0.225 ± 0.013	3.61 ± 0.28[c]
	Unfermented	357.2 ± 51.9	0.753 ± 0.067	0.298 ± 0.026[a,b]	2.53 ± 0.10
	Plain	320.0 ± 55.3	0.562 ± 0.072	0.183 ± 0.021	3.10 ± 0.28
Culture supernatants[†,‡]	DJ	NT	227.8 ± 27.9	105.0 ± 6.7	2.14 ± 0.17[a]
	CGJ	NT	212.2 ± 30.4	103.1 ± 13.9	2.07 ± 0.10[a]
	Unfermented	NT	316.5 ± 46.8	118.1 ± 14.5	2.66 ± 0.22[a,b]
	Plain	NT	146.4 ± 31.5	112.8 ± 8.5	1.22 ± 0.18

DJ, *doenjang*; CGJ, *cheonggukjang*; Unfermented, unfermented raw material mixture; Plain, standard rodent chow; NT, not tested. *Serum samples from eight mice per group were analyzed. †B cell culture supernatants from seven mice per group were analyzed. ‡IgG2a or IgG1 production in the culture supernatants is reported in ng/mL. [a]$p < 0.05$ compared with the plain diet group. [b]$p < 0.05$ compared with the CGJ diet group. [c]$p < 0.05$ compared with all other groups.

in the DJ (32.4% ± 6.8%; $p = 0.163$), CGJ (26.4% ± 2.2%; $p = 0.089$) and unfermented diet (26.0% ± 1.4%; $p = 0.211$) groups relative to those in the plain diet group (20.5% ± 1.4%).

Effect of experimental diet on immunoglobulin levels in serum and B cell culture supernatants

Among mouse IgG isotypes, IL-4 induces isotype switching to IgG1 and IFN-γ induces IgG2a. Therefore, analysis of the relative level of these IgG isotypes has been used to evaluate the skewedness toward T helper type-1 (Th1) cell or T helper type-2 (Th2) cell predominance responses. Furthermore, IL-4 causes isotype switching to IgE, the primary mediator of various allergic diseases. No significant difference was found in the levels of serum IgE among groups (Table 3). The ratio of IgG2a:IgG1 in the serum samples was significantly higher in the CGJ group than all other groups, indicating a predominant Th1-mediated immune response in the CGJ group. The ratio of IgG2a:IgG1 in the splenic B cells was observed to be higher in the CGJ diet group than the plain diet group. Compared with the plain diet group, the IgG2a:IgG1 ratio was higher in the DJ diet group and the raw materials mixture diet group. This upregulation in serum and B cell culture supernatants may be attributable to enhancement of the IgG2a synthesis rather than downregulation of IgG1 synthesis, as IgG1 levels in the DJ and CGJ diet groups did not differ significantly from the plain diet group.

Effect of experimental diet on cytokine production by splenic T cells

To further investigate the possibility of a bias toward a predominant Th1 response in mice that were administered a DJ or CGJ diet that was observed from the IgG isotype production described above, IFN-γ and IL-4 levels in splenic T cell culture supernatants were determined because IFN-γ is known to favor a Th1 response and IL-4 favors a Th2 response. The level of IFN-γ was significantly higher in the DJ (7,602.3 ± 402.1 pg/mL) and CGJ (7,215.5 ± 511.4 pg/mL) diet groups than the plain diet group (4,338.3 ± 957.7 pg/mL) (Fig. 1). Furthermore, IFN-γ levels were significantly higher in the DJ intake group

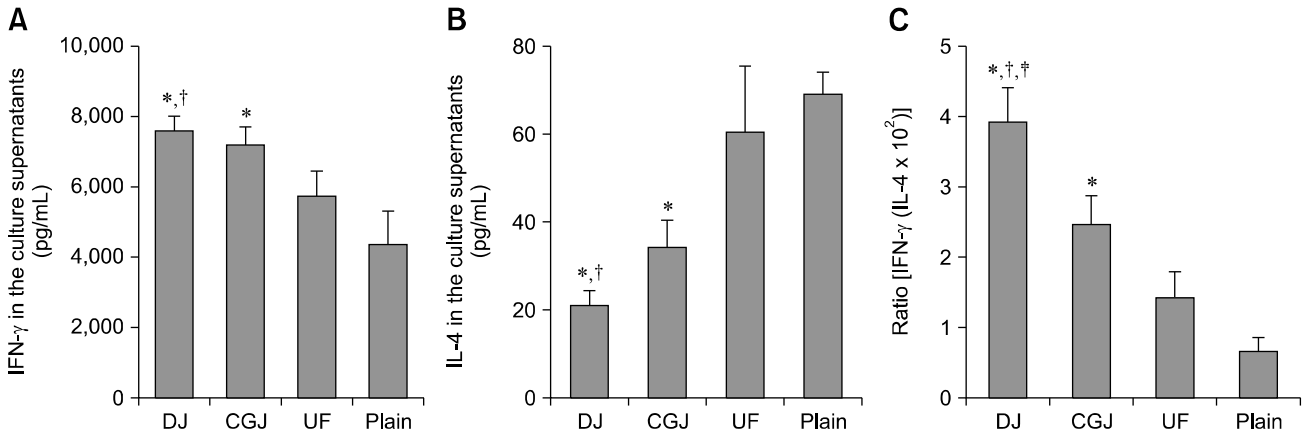

Fig. 1. Effect of a 4 week experimental diet of *doenjang* (DJ), *cheonggukjang* (CGJ), or unfermented raw material mixture (UF) on interferon-γ (IFN-γ) and interleukin (IL)-4 production from splenic T cells stimulated *ex vivo* compared with standard rodent chow intake (Plain). The IFN-γ:IL-4 ratio was calculated by dividing the IFN-γ level with the IL-4 level and then multiplying the result by 100 for each individual culture supernatant. Values are expressed as the mean ± SEM for seven mice per group. *$p < 0.05$ *vs*. the plain group. †$p < 0.05$ *vs*. the UF group. ‡$p < 0.05$ *vs*. the CGJ group.

than in the unfermented raw material diet group (5,746.7 ± 707.3 pg/mL). The level of IL-4 was significantly lower in the DJ (21.3 ± 3.1 pg/mL) and CGJ (34.3 ± 6.1 pg/mL) groups than in the plain diet group (69.1 ± 5.0 pg/mL). IL-4 levels were also significantly lower in the DJ diet group than in the unfermented raw material diet group (60.4 ± 15.0 pg/mL). The upregulated IFN-γ and downregulated IL-4 production in the DJ and CGJ groups resulted in a significantly higher IFN-γ:IL-4 ratio in both the DJ group (3.92 ± 0.49) and the CGJ group (2.47 ± 0.41) than in the plain diet group (0.67 ± 0.18). The IFN-γ:IL-4 ratio was significantly higher in the DJ group than in the CGJ group and the unfermented raw material intake group (1.42 ± 0.37).

Host resistance against *Listeria monocytogenes* infection

Cell-mediated immunity *in vivo* was evaluated via adoption of a *Listeria monocytogenes* infection model. As shown in Table 4, mortality was observed to be lowest until 68 h post-infection in the CGJ diet group, whereas all mice that were administered unfermented raw material died. Lower burdens of *Listeria monocytogenes* were recovered from the spleens and livers of mice from the DJ and CGJ diet groups compared with the plain diet group. Since protection from *Listeria monocytogenes* infection is mediated by the innate and Th1 immune response, mice from the CGJ diet group appear to have a stronger innate and Th1 immune response than those from other groups.

Table 4. *In vivo* assessment of the influence of the experimental diet on mortality and viable bacterial count following *Listeria monocytogenes* infection*

Group	Organ	% Mortality (number of surviving mice)	Colony-forming units (10^6)
DJ	Spleen	75 (1)	4.0
	Liver		2.9
CGJ	Spleen	25 (3)	0.67 ± 0.04
	Liver		10.3 ± 0.7
Unfermented	Spleen	100 (0)	NT
	Liver		NT
Plain	Spleen	75 (1)	310
	Liver		2,400

DJ, *doenjang*; CGJ, *cheonggukjang*; Unfermented, unfermented raw material mixture; Plain, standard rodent chow; NT, not tested as all mice deceased. *Following 4 weeks of the experimental diet, mice were infected with *Listeria monocytogenes* on day 29. Mice that survived until 68 h following infection were sacrificed, and the spleens and livers were collected (No. of mice per group = 4).

Discussion

In the present study, intake of the fermented soybean products DJ or CGJ did not induce any significant abnormalities in body weight gain, histopathology, peripheral blood cell numbers, or composition of the major immune cell types of the spleen. Furthermore, serum levels of IgE did not differ between groups administered the fermented soybean products and the control group that was fed standard rodent chow. Considering upregulation of serum IgE levels as an immunological marker of food allergy [14,32,38], our results may indicate that the intake of fermented soybean products had no effect on allergenic responses. Measurement of soy-specific IgE level might be necessary to support the above explanation. Furthermore, a higher Th1 cell predominance, indicated by a significantly higher IFN-γ:IL-4 ratio and IgG2a:IgG1 ratio in splenic lymphocyte culture supernatants [17,40], was observed in mice from the DJ and

CGJ diet groups compared with the control diet group. Because host resistance to cancer occurrence or viral infection is primarily involved with Th1 cell-mediated immunity [1], the augmented Th1 response in response to the administration of DJ or CGJ fermented soybean products suggests a certain effector function of these fermented soybean products on T helper cell-mediated immune surveillance against infection or oncogenesis. Anti-cancer and anti-mutagenic effects have been reported in *in vitro* and *in vivo* studies on DJ, but the background mechanism has not yet been intensively investigated [15,31]. Enhanced production of IFN-γ from splenic T cells may have contributed to protection against *Listeria monocytogenes* infection in the CGJ diet group since mortality was found to be 25% in the CGJ group compared with 75% in the plain diet group and 100% in the unfermented soybean diet group. No definitive conclusion regarding the protective effects of fermented soybean products against *Listeria* infection can be made as the protective effect was not demonstrated in response to the intake of DJ; nevertheless, there was significantly higher production of IFN-γ in the DJ intake group. Since activated $CD4^+$ or $CD8^+$ T cells or NK 1.1^+ cells are known to be major sources of IFN-γ [37], a key mediator in the defense against *Listeria* infection, further investigation of the effects of DJ or CGJ intake on these cell populations is necessary because the results on IFN-γ production were obtained from stimulated splenic T helper cells. Regarding the enhanced splenic NK cell activity through the intake of DJ or CGJ, predominance of Th1 cell activity in the DJ and CGJ diet groups may have been due to the induction of NK cell activation, which has been reported to mediate differentiation of progenitor helper T cells to Th1 via secretion of IFN-γ [4]. An increase in NK cell activity has been reported in mice treated with DJ extracts dissolved in drinking water [15]. Since the predominance of Th1 cell activity was not observed in the unfermented diet group, despite enhanced NK cell activity in the unfermented diet group relative to the plain diet control, the fermented soy products could be responsible for the immune upregulation described above.

Soybeans contain many biologically functional components including isoflavones, unsaturated fatty acids, and bioactive peptides, and the fermentation process is known to improve nutritional and functional properties of soybeans [6,21,34]. Among these components, isoflavone, a well-known phytoestrogen, has been reported to possess various functional properties against carcinogenesis [24], cardiovascular diseases [3], virus infection [2], and inflammation [7]. Furthermore, suppressive effects against peanut-mediated or chicken egg ovalbumin-mediated allergenic sensitization were evoked via consumption of soybean isoflavones [25] or green soybean extracts [16], respectively. This inhibitory function has been speculated to be attributable to the dendritic cell-mediated downregulation of Th2 reactivity, resulting in decreased secretion of IL-5, IL-9, and IL-13 [25]. Therefore, the isoflavones in DJ and CGJ may be responsible for the skewed T helper cell response toward Th1 activity that culminated in both a significantly higher IFN-γ:IL-4 ratio and IgG2a:IgG1 ratio following *ex vivo* splenic lymphocyte stimulation. This predominance of Th1 reactivity was also reflected in a higher IgG2a:IgG1 ratio [36] in the sera of mice from the CGJ diet group than that of mice from the unfermented soybean diet group and the plain diet control group. Delineation of the type(s) of isoflavones that are major players in the augmented Th1 reactivity among various isoflavone forms including daidzin, genistin, glycitin, genstein, glycitein, and malonyl genistin in fermented soybean products is preferable [21,34]. Although there is very little data available regarding the effects of lunasin, a representative bioactive soybean peptide, on helper T cell-mediated immune regulation, based on the anti-cancer and anti-inflammatory functions of this peptide [5,6], luansin may also be involved in the predominance of Th1 reactivity that was demonstrated in mice from the DJ and CGJ diet groups. The effects of fermented soybean peptides including lunasin on T cell-mediated immune responses should be an avenue for further research. Moreover, it will be necessary to delineate the effects of bacterial products such as poly-γ-glutamic acid from the fermented CGJ on the skewed Th1 reactivity [18].

Although the number of mice used in this study may have been insufficient for investigating certain immunological parameters such as NK cell functional analysis and defense against *Listeria* infection, intake of DJ and CGJ, which are representative fermented soybean products in Korea, resulted in enhanced NK cell activity. This led to consolidation of innate immunity and upregulation of Th1 reactivity over the Th2 response in both humoral and cellular immunity with no apparent quantitative alteration in major immune cell populations in the spleen and peripheral blood that may exert resistance against *Listeria* infection. Further investigation of the particular components of fermented soybean products that contribute to the immune regulation observed in this study are recommended. In addition, the present observations should be further confirmed through sub-chronic or chronic administration of DJ or CGJ since those fermented soybean products are generally taken throughout one's lifetime in Korea.

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Conflict of Interest

The authors declare no conflicts of interest.

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