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Anti-oxidant and immune enhancement effects of *Artemisia argyi* H. fermented with lactic acid bacteria

Ji Yun Lee[1] · Ji Hyun Kim[2] · Ji Myung Choi[3] · Hyemee Kim[1] · Weon Taek Seo[4] · Eun Ju Cho[1] · Hyun Young Kim[2]

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Abstract This study investigated the antioxidant and immune enhancement activities of *Artemisia argyi* H. fermented by *Lactobacillus plantarum*. The fermented *A. argyi* H. ethanol extract increased scavenging activities of 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$), hydroxyl (·OH), and superoxide (O_2^-) radicals. Particularly, the ethanol extract of fermented *A. argyi* H. exhibited higher ·OH and O_2^- radical scavenging activities, compared with DPPH and $ABTS^+$ radical scavenging activities. To evaluate the immune enhancement effects of the fermented *A. argyi* H., mice were fed a normal diet supplemented the fermented *A. argyi* H. at concentrations of 1%, 2%, and 5%, respectively. The supplementation of fermented *A. argyi* H. dose-dependently increased splenocyte proliferation. In addition, mice fed with 5% fermented *A. argyi* H. showed enhanced proliferation of T-cells and B-cells, along with increased levels of interferon-γ, interleukin-10, and tumor necrosis factor-α, compared to the normal group. Furthermore, mice fed with fermented *A. argyi* H. exhibited an increase in prominent probiotics such as *Akkermansia muciniphila* and *Lactobacillus* in gut microbiota, compared to the normal group. This study suggests that fermented *A. argyi* H. with *Lactobacillus plantarum* could be used as a dietary antioxidant and immune enhancement agent.

Keywords Antioxidant · *Artemisia agryi* H. · Fermentation · Immune Improvement · Lactic acid

Introduction

The immune system is a defense mechanism against both internal and external threats to the body, comprising innate immunity and adaptive immunity [1,2]. The innate immunity is mediated by the phagocytes such as nature killer (NK) cells and macrophages, while the adaptive immunity involves the defense mechanisms of T-cells and B-cells [3]. The innate and adaptive immunity complementarily protect the body and these immune systems have the role of maintenance of the body function [4,5]. In addition, immune systems are maintained by the proliferation and activation of different immune cells and are closely related to oxidative stress [6]. There are two types of cellular metabolites that cause oxidative stress in the body such as reactive oxygen species (ROS) and reactive nitrogen species [7]. Excessive production of ROS such as peroxyl radical, hydrogen peroxide, hydroxyl radical (·OH), and super oxide (O_2^-) in the body is known to lead to dysfunction of immune cells, such as death of T-cell and suppression of NK cells [8-10]. Therefore, oxidative stress by the excessive production of ROS is closely associated with impaired immune activities, leading to inflammation, aging, and autoimmune diseases [11].

Ji Yun Lee and Ji Hyun Kim are contributed equally to this work.

Eun Ju Cho (✉)
E-mail: ejcho@pusan.ac.kr

Hyun Young Kim (✉)
E-mail: hyunyoung.kim@gnu.ac.kr

[1]Department of Food Science and Nutrition, Pusan National University, Busan 46241, Republic of Korea

[2]Department of Food Science and Nutrition, Gyeongsang National University, Jinju 52725, Republic of Korea

[3]Department of Food and Nutrition, Kyungsung University, Busan 48434, Republic of Korea

[4]Department of Food Science and Technology, Gyeongsang National University, Jinju 52725, Republic of Korea

In a previous study, microbial fermentation was found to enhance anti-oxidant effects, attributed to the increased production of various antioxidant compounds through microbial hydrolysis [12]. In particular, the role of lactic acid bacteria in supporting probiotics and antioxidants has been a subject of interest for many years [13]. The fermented kimchi is known for a high levels of lactic acid bacteria, has investigated the many health beneficial effects such as antioxidant, anticancer, and antiobesity [14]. A study has also reported that the fermentation of avocado leaves by lactic acid bacteria exhibited higher antioxidant activities [15]. Therefore, many studies focus on the immune improving effects of natural products to develop functional materials from food sources using lactic acid bacteria fermentation.

Artemisia argyi H. is a variation of Hwanghae mugwort, a unique resource in Namhae-gun, Gyeongsangnam-do, and it has abundant active components with antioxidant activity, such as jaceosidin, eupatilin, kaempferol, quercetin, etc. [16,17]. The *A. argyi* H. has been reported the physiological activities, including antioxidant and anti-inflammatory effects, as well as its potential for improving cognitive abilities [18-20]. In addition, *A. argyi* H. fermented by *Monascus purpureus* exhibited the neuroprotective effects and the enhanced memory and learning activities through improvement of oxidative stress [21]. Furthermore, the water extract of *A. argyi* H. fermented by *Lactobacillus brevis* and *Lactobacillus plantarum* has been shown to increase antioxidant activities by radical scavenging activities [22]. However, the immunity enhancement effects of the *A. argyi* H. fermented with lactic acid bacteria have not been investigated yet. Therefore, our study demonstrated the antioxidant activity and immune cell proliferation of *A. argyi* H. fermented by *Lactobacillus plantarum*, suggesting its potential as an antioxidant and immunostimulatory agents. In addition, we investigated the changes in intestinal microorganisms to evaluate the effects of *A. argyi* H. fermented by *Lactobacillus plantarum* on intestinal immune improvement.

Materials and Methods

Reagents

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2-deoxyribose were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). The $FeSO_4 \cdot H_2O$-EDTA was obtained from Daejung Chemicals & Mateals Co., Ltd. (Gyenggido, Korea), and H_2O_2, sodium hydroxide (NaOH), and acetic acid were purchased from Junsei Chemical Co. (Tokyo, Japan). Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Acros Organics (Morris Plains, NJ, USA) and Kanto Chemical Co., Inc. (Tokyo, Japan), respectively. The 2,2'-azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid ammonium salt) (ABTS) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and potassium persulfate was obtained from Samchun Chemical Co. (Gyenggido, Korea). Phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH) disodium salt, and nitrotetrazolium blue chloride (NBT) were obtained Bio Basic Inc. (Toronto, Canada). The Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Welgene Inc. (Daegu, Korea). The EZ-Cytox was obtained from DoGenBio Co., Ltd. (Seoul, Korea). Mouse tumor necrosis factor (TNF)-α, interferon-gamma (IFN-γ), and interleukin (IL)-10 enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D systems (Minneapolis, MN, USA). PowerFecal Pro DNA kit was purchased from QIAGEN (Hilden, Germany). All primers (Table 1) used for bacterial profiling were purchased from Macrogen (Seoul, Korea).

Preparation of ethanol extract from *A. argyi* H. fermented with lactic acid bacteria

The *A. argyi* H. were collected from Namhae-gun, Republic of Korea, in 2021 (Namhae, Korea). The leaves of *A. argyi* H. were steamed at 100 ºC for 5 min, then added to malt extract broth and crushed. After, these extract were sterilized at 100 ºC for 20 min, and it was used as a fermentation substrate. The *Lactobacillus plantarum* was inoculated into sterilized MRS agar media and allowed to grow for 2 days at 30 ºC with shaking at 150 rpm. And then, it was inoculated into fermentation substrate containing *A. argyi* H. at a 5% volume ratio and left to ferment at 30 ºC for 4 days to produce *A. argyi* H. fermented by *Lactobacillus plantarum*. The fermented *A. argyi* H. were freeze-dried, and then extracted three times in ethanol of an amount 20 times weight of *A. argyi* H. at room temperature for 24 h. After, this extract was dried and concentrated using a rotary vacuum evaporator.

DPPH radical scavenging activity

The DPPH radical scavenging activity was determined following the modified DPPH radical assay [23]. The ethanol extract of fermented *A. argyi* H. at concentrations of 100, 250, 500, and 1,000 μg/mL were reacted with 100 μL of 60 M DPPH solution in a 96 well plate. The mixture was then incubated at the room temperature in the dark for 30 min, and the absorbance was measured at 540 nm using microplate reader. DPPH radical scavenging activity was calculated by the following formula:

$$\text{DPPH radical scavenging activity (\%)} = 100 - [(A_s - A_b) \times 100 / A_c]$$

A_s is the experimental group to which samples of each concentration were added, A_b is the control group to which a blank was added, and A_c is the absorbance of the control group to which no sample was added.

$ABTS^+$ radical scavenging activity

ABTS radical solution ($ABTS^+$) was prepared by gently mixing 7 mM ABTS and 2.45 mM potassium persulfate in a 1:1 ratio. It was allowed to incubate at room temperature in the dark overnight. The mixed $ABTS^+$ was diluted 26 times with distilled water (DW) to achieve an absorbance value of 0.7±0.02. The 40

μL of fermented *A. argyi* H. ethanol extract diluted in DW at concentration of 100, 250, 500, and 1,000 μg/mL or blank and 160 μL of the $ABTS^+$ solution were mixed in a 96 well plate. The mixture was then incubated in the dark for 10 min, and measured at 734 nm using microplate reader [24]. $ABTS^+$ radical scavenging activity was calculated by the following formula:

$$ABTS^+ \text{ radical scavenging activity } (\%)=(1-A_s/A_b)\times 100$$

A_s is the experimental group to which samples of each concentration were added, and A_b is the control group to which a blank was added.

·OH radical scavenging activity

The 200 μL of 10 mM $FeSO_4 \cdot H_2O$-EDTA, 200 μL of 10 mM 2-deoxyribose, 200 μL of 10 mM H_2O_2, and 1,400 μL of the fermented *A. argyi* H. ethanol extract at concentrations of 100, 250, 500, and 1,000 μg/mL were mixed, and then incubated at 37 ºC for 4 h. Following incubation, 1 mL of 1.0% TBA and 1 mL of 2.8% TCA were added to the mixture. And then, it was reacted at 100 ºC for 20 min, and after cooling, the absorbance was measured at 490 nm using microplate reader [25]. ·OH radical scavenging activity was calculated by the following formula:

$$\cdot OH \text{ radical scavenging activity } (\%)=100-[(A_s-A_b)\times 100/A_c]$$

A_s is the experimental group to which samples of each concentration were added, A_b is the control group to which a blank was added, and A_c is the absorbance of the control group to which no sample was added.

O_2^- radical scavenging activity

The 100 μL of 0.1 M Tris-HCl (pH 7.4), 200 μL of 100 μM PMS, 200 μL of 500 μM NBT, 400 μL of 500 μM NADH, and 500 μL of the fermented *A. argyi* H. ethanol extract at concentration of 100, 250, 500, and 1,000 μg/mL were mixed, and then these mixtures incubated at room temperature for 10 min. Subsequently, the absorbance of the mixture was measured at 560 nm using microplate reader [26], and the O_2^- radical scavenging activity was calculated using the following formula:

$$O_2^- \text{ radical scavenging activity } (\%)=100-[(A_s-A_b)\times 100/A_c]$$

A_s is the experimental group to which samples of each concentration were added, A_b is the control group to which a blank was added, and A_c is the absorbance of the control group to which no sample was added.

Animals and experimental schedule

Five-weeks-old female Balb/C mice (13-16 g) were purchased from Orient Bio Inc. (Seongnam, Republic of Korea). The mice were kept in an animal laboratory and provided with free access to water, and the laboratory environment was maintained at a temperature of 20±2 ºC) and humidity of 55±5% in a 12-h light-dark cycle. The mice were allowed one week acclimatization period and randomly divided into four groups, each consisting of six mice (n =6). The mice were supplied with different treatment in the form of chow for 8 weeks experimental period. The normal group consumed the standard diet, while three experimental groups received a diet consisting of 1, 2, and 5% (*w/w*) fermented *A. argyi* H. The body weights of mice were measured once a week, and food intake was monitored daily. All experimental procedures were strictly followed animal experimental guidelines and were approved by the Animal Experimental Ethics Committee of Pusan National University (PNU-2022-0048).

Spleen index

The spleen was dissected and weighed, and then transported in the media (RPMI-1640+2% FBS+1% penicillin/streptomycin) for maintaining 4 ºC. Spleen index was calculated as following formula: Spleen index (mg/g)=spleen weight (mg)/body weight (g).

Isolation of splenocytes

The splenocytes were isolated by collected spleens and dissociated through a 70-μm cell strainer. After that, the splenocytes were washed with RPMI medium (RPMI-1640+2% FBS+1% penicillin/streptomycin) and centrifuged at 1,500 rpm for 3 min. The ACK lysis buffer was added to the pellets to removing the red blood cells. Finally, the splenocytes were washed with RPMI medium and were maintained in complete medium (RPMI-1640+ 10% FBS+1% penicillin/streptomycin).

T-cell and B-cell proliferation assay

Splenic lymphocyte proliferation was assessed by water-soluble tetrazolium (WST) assay. The splenocytes were seeded in a 96-well plate at a density of 2×10^4 cells/well, and then it is divided into two groups, such as untreated group and treated groups. The untreated group received complete medium in each well, while the treated groups had 10 μg/mL of LPS or 5 μg/mL of ConA added to each well. After incubation for 48 h at 37 ºC in a humidified 5% CO_2 incubator, a total of 20 μL EZ-cytox solution was added to each well and incubated for an additional 4 h. The absorbance was measured at 450 nm using a microplate reader. The cell proliferation was calculated as a percentage of 100% relative to the splenocytes isolated from the normal group.

Th1- and Th2-type cytokines production

The Th1-type cytokines (TNF-α, IFN-γ) and Th2-type cytokine (IL-10) were determined using an ELISA kit. Splenocytes were seeded in a U-bottomed 96 well plate at a density of 2×10^4 cells/well. To assess TNF-α and IL-10, 10 μg/mL of LPS was added to the plate, while for IFN-γ, 5 μg/mL of ConA was added to the plate. The plates were incubated for 24 h (for TNF-α and IL-10) or 72 h (for IFN-γ) at 37 ºC in a humidified 5% CO_2 incubator [27]. After centrifuged at 300 xg for 10 min, the concentration of the secreted cytokines in the supernatants was quantitated by

Table 1 The 16S rRNA gene-targeted group of the specific primers used for bacterial profiling

Target group	Sequences		Annealing temperature (°C)
	Forward	Reverse	
Uni (F341/R518)	CCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGG	59
Firmicutes (Phylum)	TGAAACTYAAAGGAATTGACG	ACCATGCACCACCTGTC	60
Bacteroidetes (Phylum)	GGARCATGTGGTTTAATTCGATGAT	AGCTGACGACAACCATGCAG	60
Akkermansia muciniphila (spp.)	CTGAACCAGCCAAGTAGCG	CCGCAAACTTTCACAACTGACTTA	55
Lactobacillus (genus)	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	55

measuring at 450 nm.

Quantitative real-time PCR

The fecal samples were collected within 30 min on the day before dissection, and they were stored −80 °C until DNA extraction. Bacterial DNA extraction was performed on fecal samples (250 mg) using the DNA extraction kit following the manufacturer's instructions. DNA purity was adjusted for 16S rRNA sequencing, and the DNA samples were evaluated using the iSeq platform (Illumina, San Diego, CA, USA). To confirm the abundance of gut microbiota, the DNA was quantified and amplified using qPCR (CFX Connect Real-Time PCR Detection system, Bio-Rad, Hercules, CA, USA). The PCR conditions were an initial denaturation at 95 °C for 5 min, and then 40 cycles at 95 °C for 5 s at the optimized annealing temperature (Table 1). The melt curve was analyzed to verify the specificity of the primers as follows: 5 min at 65 °C, 5 min at 95 °C, and 0.5 °C increments. The raw PCR data, which are the relative abundances of bacterial groups in feces, were normalized to the qPCR as the percentage of the total bacteria (universal primers F341/R518) [28].

Statistical analysis

All of the experimental results were expressed as mean ± standard deviation (SD). Statistical significance was analyzed using the Statistical Package for the Social Sciences 26.0 (SPSS, IBM Corporation, NY, USA). One-way analysis of variance (ANOVA) by Duncan's multiple range test was used to assess significant differences among groups. The significance level was $p < 0.05$ for all statistics.

Results

Effects of fermented *A. argyi* H. ethanol extract on radical scavenging activity

The results of the DPPH, $ABTS^{+}$, ·OH, and O_2^{-} radical

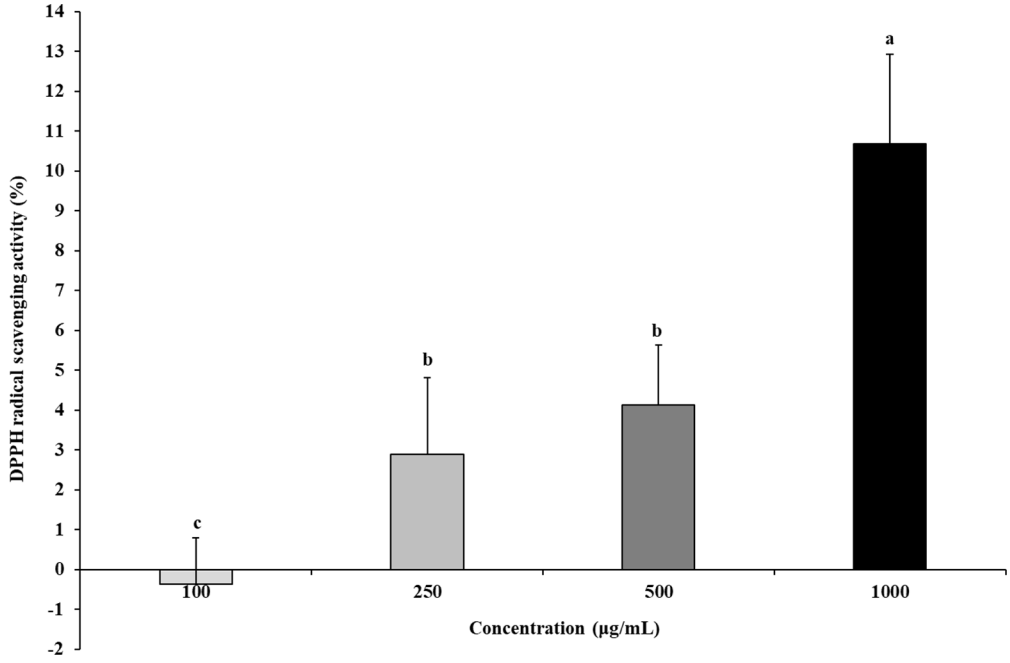

Fig. 1 DPPH radical scavenging activity of the ethanol extract from fermented *Artemisia argyi* H. Values are mean ± SD. [a-c]The different letters among groups represent significant differences ($p < 0.05$) by Duncan's multiple range test

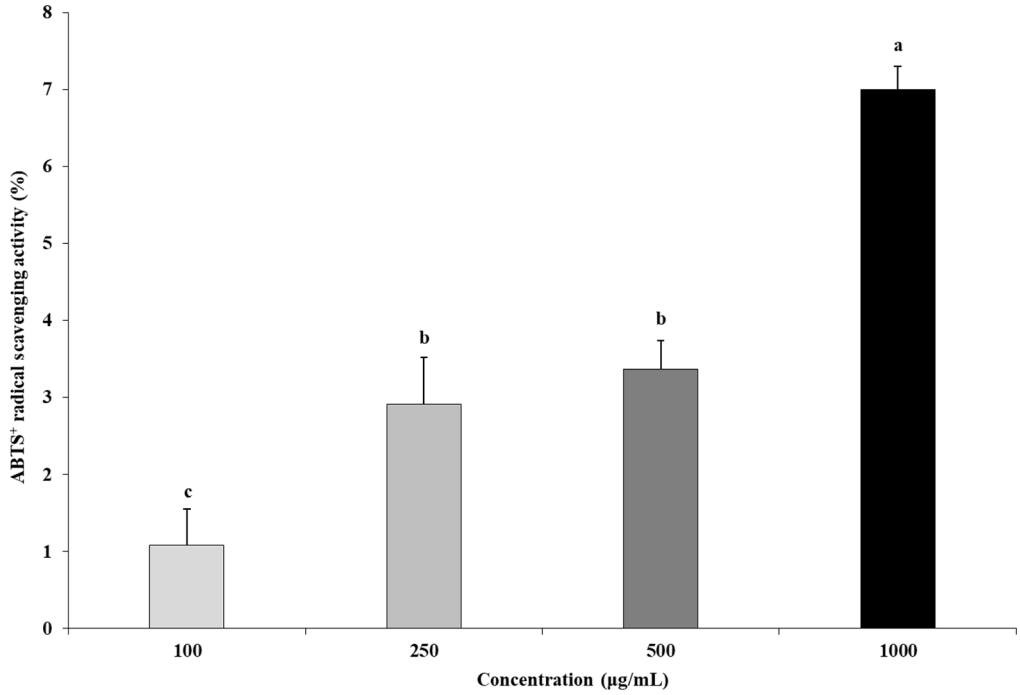

Fig. 2 $ABTS^+$ radical scavenging activity of the ethanol extract from fermented *Artemisia argyi* H. Values are mean ± SD. [a-c]The different letters among groups represent significant differences (p <0.05) by Duncan's multiple range test

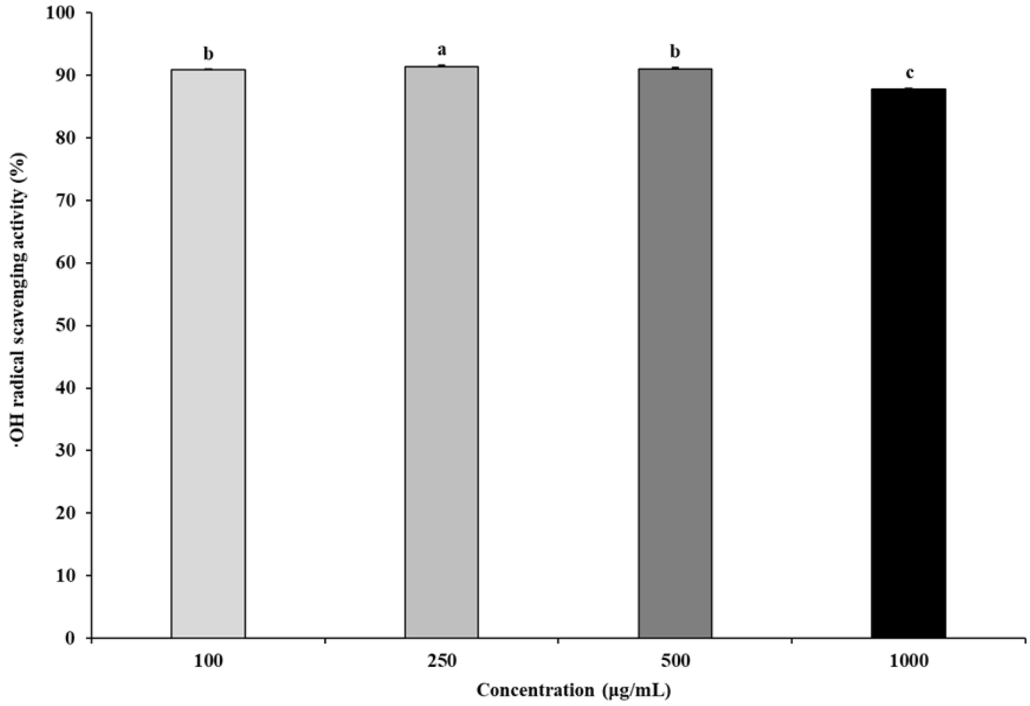

Fig. 3 ·OH radical scavenging activity of the ethanol extract from fermented *Artemisia argyi* H. Values are mean ± SD. [a-c]The different letters among groups represent significant differences (p <0.05) by Duncan's multiple range test

scavenging activity of fermented *A. argyi* H. ethanol extract at concentrations ranged from 100 to 1,000 μg/mL were measured. The fermented *A. argyi* H. ethanol extract at concentration of 250, 500, and 1,000 μg/mL were observed DPPH radical scavenging activity in a dose-dependent manner, showing 2.90, 4.13, and 10.68%, respectively (Fig. 1). The $ABTS^+$ radical scavenging

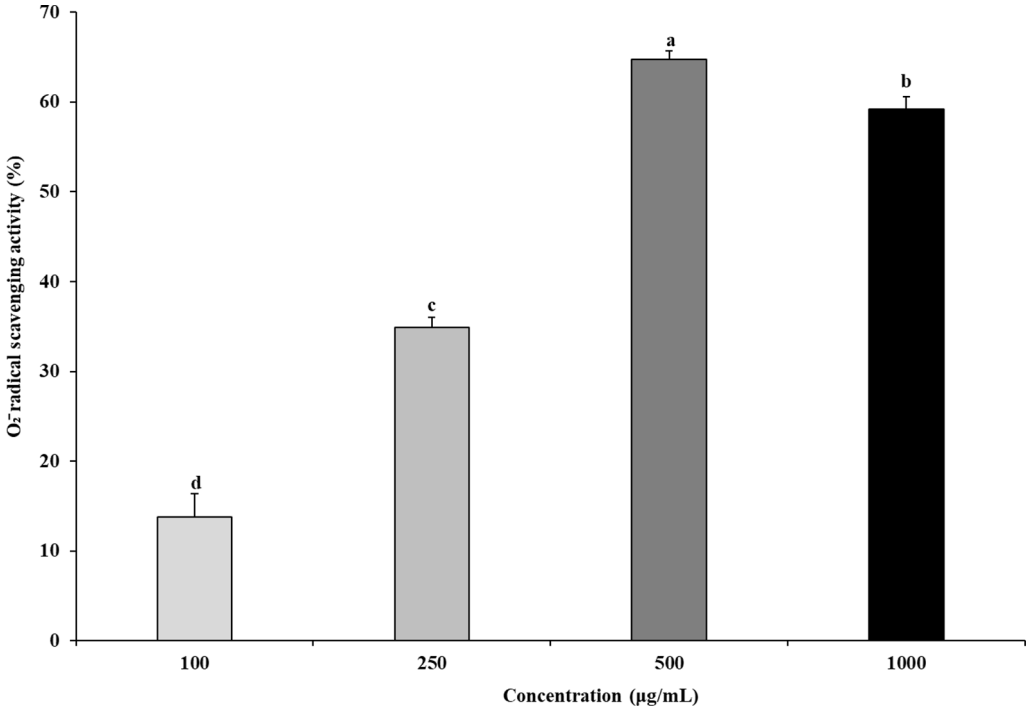

Fig. 4 O_2^- radical scavenging activity of the ethanol extract from fermented *Artemisia argyi* H. Values are mean ± SD. [a-d]The different letters among groups represent significant differences (p <0.05) by Duncan's multiple range test

activity of fermented *A. argyi* H. ethanol extract at concentrations of 100, 250, 500, and 1,000 μg/mL were 1.08, 2.91, 3.37, and 7%, respectively (Fig. 2). The fermented *A. argyi* H. ethanol extract dose-dependently increased $ABTS^+$ radical scavenging activity. In particular, 1,000 μg/mL of fermented *A. argyi* H. ethanol extract showed the significantly highest DPPH and $ABTS^+$ radical scavenging activities. The fermented *A. argyi* H. ethanol extract at concentration of 250, 500, and 1,000 μg/mL were 90.91, 91.37, 90.97, and 87.86% of ·OH radical scavenging activity, respectively (Fig. 3). In particular, all of the concentrations of fermented *A. argyi* H. ethanol extract showed more than 80% of ·OH radical scavenging effects. The fermented *A. argyi* H. ethanol extract at 500 μg/mL was the highest O_2^- radical scavenging activity among other concentrations (Fig. 4). The treatment of fermented *A. argyi* H. ethanol extract at 250, 500, and 1,000 μg/mL exhibited for more than 30% on the O_2^- radical scavenging effects, which are 34.89%, 64.74%, and 59.24%, respectively.

Effects of fermented *A. argyi* H. on the spleen weights and spleen indices of Balb/c mice

For measuring the immune-enhancement effects for fermented *A. argyi* H. extract, the spleen weight was measured on the day of dissection, and the value of the spleen indices was derived using the body weight measured the day before mice were sacrificed. The principle on the spleen index is that as the spleen weight decreases or the body weight increases, the spleen index is increased [29]. In this study, although the immune-enhancement effects of fermented *A. argyi* H. were demonstrated, there were no significant differences in the spleen weight and the spleen indices (Table 2).

Table 2 Effect of fermented *Artemisia argyi* H. on the spleen weight and index in Balb/c mice

Group	Spleen weight (mg)	Spleen index (mg/g)
Normal	73.17±2.04^{NS}	3.62±0.16^{NS}
Fermented *Artemisia argyi* H. 1%	73.33±4.76	3.65±0.14
Fermented *Artemisia argyi* H. 2%	74.50±5.32	3.81±0.24
Fermented *Artemisia argyi* H. 5%	71.67±2.66	3.62±0.22

Spleen index (mg/g) = (weight of spleen / body weight). Values are mean ± SD. Means are determined by Duncan's multiple range test. NS means no significant differences among groups

Effects of fermented *A. argyi* H. on the proliferation of splenocytes of Balb/c mice

To investigate the immune enhancing effects of fermented *A. argyi* H., the cell proliferations of splenocytes were measured by WST assay (Fig. 5). The results of the splenocyte proliferation for fermented *A. argyi* H., without any mitogen treatment, showed that it was significantly increased at all concentrations, and in particular, all of the concentrations for fermented *A. argyi* H. were increased compared to the normal group (Fig. 5A). B-cell and T-cell proliferation showed significant increases in the treatment of

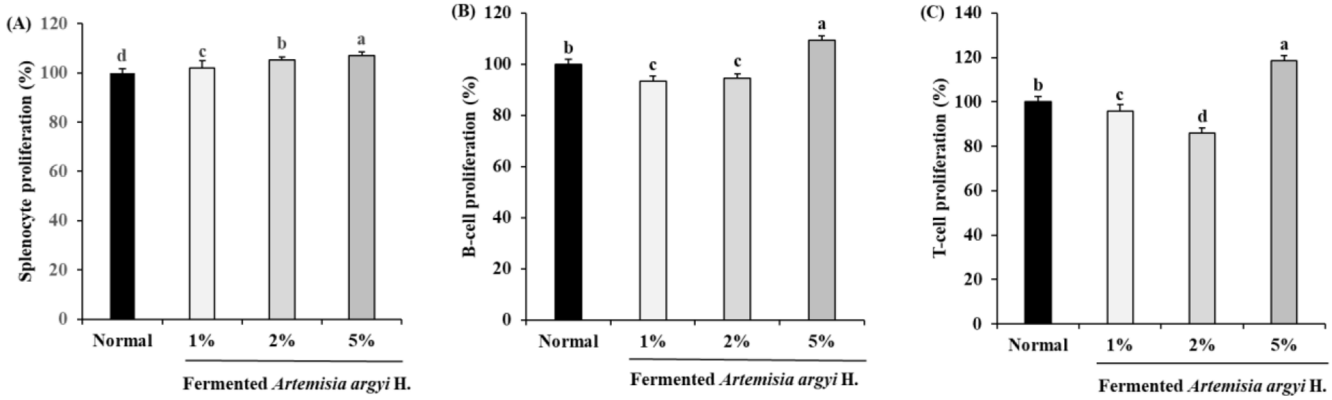

Fig. 5 Effect of fermented *Artemisia argyi* H. on the proliferations of splenocyte (A), B-cells (B), and T-cells (C) in Balb/c mice. Values are mean ± SD. [a-d]The different letters among groups represent significant differences (p <0.05) by Duncan's multiple range test

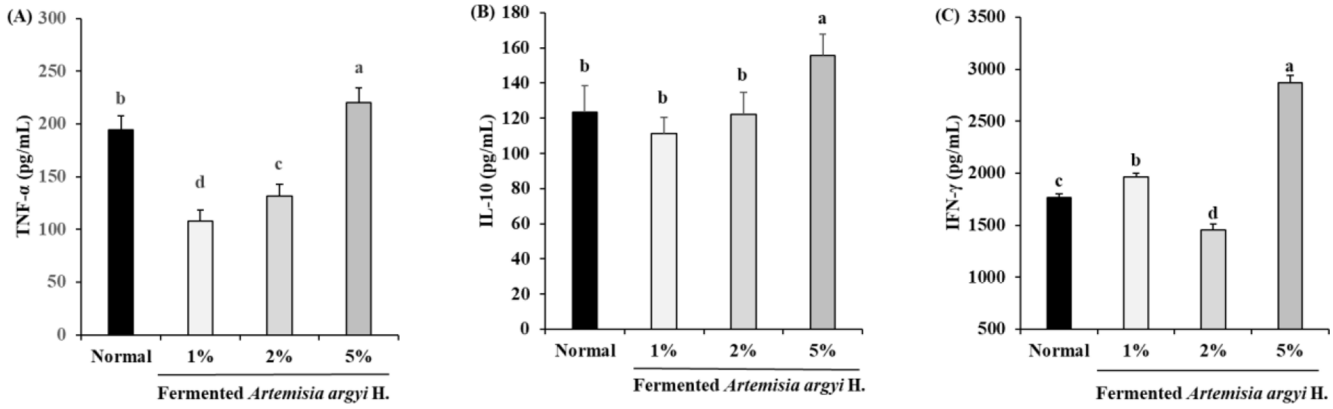

Fig. 6 Effect of fermented *Artemisia argyi* H. on production of TNF-α (A), IL-10 (B), and IFN-γ (C) in splenocytes of Balb/c mice. Values are mean ± SD (n =6). [a-d]The different letters among groups represent significant differences (p <0.05) by Duncan's multiple range test

fermented *A. argyi* H. at 5%, compared to the normal group, which are 109.48 and 118.39%, respectively (Fig. 5B, C). Additionally, it was observed that T-cell proliferation on 5% fermented *A. argyi* H.-treated group was higher than B-cell proliferation. These results suggest that the immune-enhancement effects of fermented *A. argyi* H. may be significantly increased on the splenocyte proliferations.

Effects of fermented *A. argyi* H. on production of Th1- and Th2-type cytokines in splenocytes of Balb/c mice

The higher production of TNF-α, which corresponds to the Th1-type cytokine, was showed only in the group treated with 5% fermented *A. argyi* H. (219.92 pg/mL), than the normal group, which are 25.79 pg/mL higher (Fig. 6A). And the significant production of IL-10 belongs to Th2-type cytokines, was exhibited only in the 5% fermented *A. argyi* H.-treated group (155.54 pg/mL), and there were no significant differences in the 1% and 2%, compared to the normal group, which are 32.11 pg/mL higher (Fig. 6B). Furthermore, in the production of IFN-γ, which is one of the Th1-type cytokines, it showed remarkable increase in the fermented *A. argyi* H.-treated group at 1 and 5%, compared to the normal group (Fig. 6C). In particular, the group treated with 5% fermented *A. argyi* H. showed the highest production of IFN-γ, which are 2,871.92 pg/mL. Among these results of cytokines production, IFN-γ was prominently stimulated in the treatment of fermented *A. argyi* H. at 5%, compared to the other cytokines.

Effects of fermented *A. argyi* H. on the gut microbiota composition of the feces of Balb/c mice

In the gut bacteria of phylum and genus levels from feces, the ratio of *Firmicutes* to *Bacteroidetes* showed no significant differences at all of the concentrations for fermented *A. argyi* H., compared to the normal group (Fig. 7A). On the other hand, *Lactobacillus* and *Akkermansia muciniphila*, which are the probiotics, were significantly increased in the groups treated with fermented *A. argyi* H. (Fig. 7B, C). In particular, the expression level of *Akkermansia muciniphila* showed a more prominent increase in the that 5% fermented *A. argyi* H.-administered group than in normal group. These findings suggest that fermented *A. argyi* H. may enhance the gut immunity through the increases of

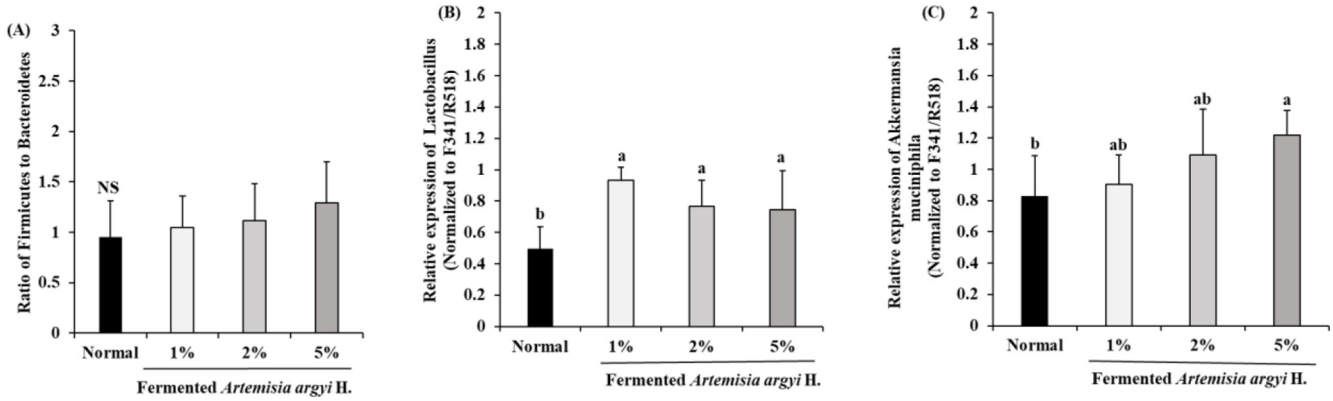

Fig. 7 Effects of fermented *Artemisia argyi* H. on the ratio of *Firmicutes* to *Bacteroidetes* (A), *Lactobacillus* (B), and *Akkermansia muciniphila* (C) in the feces of Balb/c mice. Values are mean ± SD. [a-b]The different letters among groups represent significant differences (p <0.05) by Duncan's multiple range test. NS means no significant differences among groups

the beneficial microbiota, in addition to immune-enhancement effect.

Discussion

Among the free radicals used for measuring the anti-oxidant activity, DPPH and $ABTS^+$ radical are known to be relatively stable [30]. In the assay of DPPH radical scavenging activity, its principle is that as diphenyl picrylhydrazyl, which has the colour of purple, has radicals in the molecular, it receives electrons or hydrogen from antioxidants (sulfur-containing amino acids such as cysteine and glutathione or aromatic amines such as L-ascorbic acid, tocopherol, hydroquinone, and phenylenediamine). And then, it is reduced to from a stable form of diphenyl picryl-hydrazine, and the colour of purple is decolorized [31]. This assay is known as the method widely used, as it has the characteristics of being relatively stable, simple, highly reproducible, and reliable [32]. In the assay of $ABTS^+$ radical scavenging activity, it was developed based on the mechanism that $ABTS^+$ cation radicals in plasma was inhibited by antioxidants. The principle of this assay is that $ABTS^+$ radicals generated from the reaction with potassium persulfate are removed by antioxidants and decolorized to blue-green, which is a color of radical. This assay has the characteristics that it could measure hydrogen-donating antioxidants and chain breaking antioxidants [24]. In this study, we confirmed the DPPH and $ABTS^+$ radical scavenging activities of fermented *A. argyi* H. ethanol extract at concentrations of 100, 250, 500, and 1,000 μg/mL. Our findings indicate that both DPPH and $ABTS^+$ radical scavenging activity were higher at the highest concentration tested. Jaceosidin, a major active compounds found in *Artemisia* species, is particularly abundant in *A. argyi* H., containing twice as much as other *Artemisia* species [17]. However, a previous study has reported a negative correlation between fermentation time and the amount of jaceosidin [33]. Moreover, both the total polyphenol and flavonoid contents were observed to decrease with prolonged fermentation time [33]. Another study indicated a decrease in DPPH and $ABTS^+$ radical scavenging activities during the fermentation of *A. argyi* H. with *Acetobacter pasteurianus* A8. [34]. Therefore, it suggests that the fermentation time in the production of fermented *A. argyi* H. ethanol extract may be related to lower DPPH and $ABTS^+$ radical scavenging activities.

To further investigate antioxidant activity in comparison to previous studies, we also confirmed the ·OH and O_2^- radical scavenging activities. Among various types of ROS, ·OH radicals are highly toxic and reactive, leading to lipid oxidation, DNA damage, and biomolecules mutations, thereby inducing the various diseases. The ·OH radicals are generated from H_2O_2 through the fenton reaction or from the decomposition of $ONOO^-$ [35]. The O_2^- radicals are the initial ROS generated and decomposed by antioxidant enzymes such as peroxidase and catalase and converted into water or oxygen molecules [35]. However, O_2^- radicals can indeed inactivate antioxidant enzymes such as glutathione peroxidase and catalase in the presence of transition metals [36]. In addition, O_2^- radicals promoted the generation of ·OH radicals, leading to DNA modification [36]. In this study, the ·OH and O_2^- radical scavenging activities of fermented *A. argyi* H. ethanol extract was higher than DPPH and $ABTS^+$ radical scavenging activities. Taken together, these results suggest that the fermented *A. argyi* H. ethanol extract exhibited relatively high antioxidant activity through the ·OH and O_2^- radical scavenging.

The spleen is an immune system that plays a protective role against antigens [37]. Spleen contains spleen cells, which are mainly composed of various immune cells such as T-lymphocytes, B-lymphocytes, and macrophages [37]. Splenocyte proliferation is a crucial indicator of immunity enhancement. Splenocyte proliferation is influenced by substances such as probiotics, in particular, the increase of splenocyte proliferation is known to be important in immunosuppression models [38]. LPS is a mitogen that stimulates B-cells, inducing their proliferation and differentiation of B-cells

and stimulating the secretion of Th2-type cytokines such as IL-4, IL-6, and IL-10. On the other hand, ConA is a mitogen that stimulates T-cell proliferation, and it is a plant lectin combined with mannose/glucose isolated from Jack bean (*Canavalia ensiformis*) seeds [39,40]. In this study, the proliferation of splenocytes, which had no treatment with any mitogen, was dose-dependently increased in the fermented *A. argyi* H.-fed group. In addition, the proliferation of B-cells and T-cells was significantly increased only in the 5% fermented *A. argyi* H.-fed group. Although there was no significant difference in the spleen index, these results demonstrated the effectiveness of fermented *A. argyi* H. in enhancing immunity through the increase of splenocyte proliferation.

Th-cells play an important role in adaptive immunity and are composed of Th1-cells and Th2-cells [1]. Th1-cells play a role in stimulating cellular immune responses and stimulating the activation of macrophages and B-cells [1, 41]. Cytokines secreted by Th1-cells typically include IFN-γ, IL-2, IL-12, and TNF-α. Th2-cells are important in humoral immunity and play a role in promoting B-cell proliferation, inducing antibody production, and inducing proliferation and differentiation of mast cells and eosinophilic leukocytes [41]. Cytokines secreted by Th2-cells include IL-4, IL-10, IL-6, IL-13, and IL-5. It is considerably important if it is under normal body system, balanced differentiation of Th1-cells and Th2-cells can be occurred [41]. In this study, fermented *A. argyi* H.-fed group showed an increase in the production of Th1-type cytokines such as TNF-α and IFN-γ, as well as Th-2 type cytokines such as IL-10. These result indicated that regulation of the balance between Th1-cells and Th2-cells. Particularly, IFN-γ was significantly produced in 5% fermented *A. argyi* H.-fed group, compared to other cytokines, supporting the potential immune-enhancement effects of fermented *A. argyi* H.

Firmicutes and *Bacteroidetes* account for approximately 90% of the bacteria in the colon [42]. Typically, the *Firmicutes* phylum prefers the fiber and includes gram-negative bacteria belonging to the genera such as *Bacillus*, *Clostridium*, *Enterococcus*, and *Lactobacillus*, while the *Bacteroidetes* phylum prefers refined carbohydrates and added sugars and includes gram-negative bacteria belonging to the genera such as *Bacteroides*, *Alistipes*, and *Parabacteroides* [43,44]. The ratio of *Firmicutes* and *Bacteroidetes* plays a crucial role in determining intestinal health. Imbalance of gut microorganisms has pathological effects on the gastrointestinal tract, such as diarrhea and irritable bowel syndrome, and on the immune system, central nervous system, and energy metabolism system [45]. As a result of measuring the ratio of *Firmicutes* to *Bacteroidetes* in this study, it was confirmed that the concentration of fermented *A. argyi* H.-administered groups was numerically increased compared to the normal group, but it was not statistically significant. Therefore, we additionally confirmed the gut microorganism composition among other beneficial probiotics, as *Lactobacillus* and *Akkermansia muciniphila* is a gram-negative anaerobic bacterium that decomposes the mucus layer, and it colonizes the gastrointestinal tract early in life and accounts for 3% of the total microbiota in healthy adults [46,47]. As it decomposes mucin, beneficial probiotics generates acetate and propionate, which are short-chain fatty acids, through substrate interactions with other bacteria and the host, potentially regulating gut-immunity responses [48]. In all groups treated with fermented *A. argyi* H., the expression of *Akkermansia muciniphila* was relatively increased, compard with the normal group. In particular, 5% fermented *A. argyi* H.-fed group significantly increased the expression of *Akkermansia muciniphila*. Probiotics are known to be caused by an increase in innate and adaptive immune responses and play a role in regulating immune responses [49]. Among several probiotics, bacteria such as *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus plantarum*, which correspond to *Lactobacillus*, have been reported in research to improve systemic and mucosal immunity [50]. In our results, the expression level of *Lactobacillus* genera was significantly increased in fermented *A. argyi* H.-fed group. Taken together, these findings suggest that fermented *A. argyi* H. has an improving effect on intestinal immunity by up-regulation of *Lactobacillus* and *Akkermansia muciniphila.* In previous study reported the immune function of the polyphenol mixture of *A. argyi* H. on inflammation in lipopolysaccharide-induced Raw 264.7 cells [51]. However, immune enhancement effects of *A. argyi* H. extract has not been investigated in previous studies. The kaempferol, one of the bioactive compounds of *A. argyi* H., is known to exert immunostimulatory effects on immune responses mediated by immune cells such as splenocytes, macrophages, peripheral blood mononuclear cells, and NK cells [52]. In addition, quercetin showed an immunomodulatory effect by Th1/Th2 balance, and gut immunity by protecting the intestinal structure and integrity [53,54]. Furthermore, apigenin, which is one of the active compounds of *A. argyi* H., is known to have the immunoregulatory activities by regulation of the gut microbiota and up-regulation of microbial diversity [55, 56However, further study needs to comparison of *A. argyi* H. and fermented *A. argyi* H., and its active compounds on immune enhancement effects.

In conclusion, this study investigated the antioxidant activity and immune-enhancement effects of *A. argyi* H. fermented with *Lactobacillus plantarum*. The fermented *A. argyi* H. ethanol extract showed DPPH, $ABTS^{+}$, ·OH, O_2^{-} radical scavenging activities. In particular, ·OH and O_2^{-} radical scavenging activities of fermented *A. argyi* H. ethanol extract were higher than DPPH, $ABTS^{+}$ radical scavenging activities. In addition, administration of fermented *A. argyi* H. showed the immune enhancement effect through the proliferation of T-cells and B-cells belonging to the splenocytes, and the balanced production of Th1-and Th2-type cytokines. Furthermore, fermented *A. argyi* H.-fed mice increased production of beneficial gut microbial flora such as *Lactobacillus* and *Akkermansia muciniphila* in feces. Therefore, we suggests that *A. argyi* H. fermented with *Lactobacillus plantarum* could be

used as a dietary antioxidant and immunostimulatory agent.

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