Fermentation of Korean Red Ginseng by *Lactobacillus plantarum* M-2 and Its Immunological Activities

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Abstract We investigated ginsenoside transformation by fermentation of red ginseng with *Lactobacillus plantarum* M-2. We also examined the anti-metastasis and immune-stimulating activities of EtOH extracts of fermented red ginseng (FRG-E) in animal and human subjects. Total sugar decreased from 85.5 mg mL^{-1} to 44.1 mg mL^{-1} with increasing culture time during the fermentation with *L. plantarum* M-2. Uronic acid content reached a maximum level (534.3 µg mL^{-1}) at 3 days of fermentation and decreased thereafter. Ginsenoside metabolites increased from 4,637.0 to 7,581.1 µg mL^{-1} after 4 days. The prophylactic intraperitoneal injection of FRG-E (500 µg mouse^{-1}) inhibited lung metastasis about 81.1%, while the inhibitory effect against tumor metastasis by treatment of EtOH extract from non-fermented red ginseng (NFRG-E) was 66.9%. Immunoglobulin A (IgA)

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and G (IgG) levels in the serum of healthy subjects were higher after FRG-E administration than at baseline, whereas NFRG-E induced reductions of these variables related to immunity. At 1 week, the change in IgA level by FRG-E (5.14 mg mL^{-1}) was significantly higher than that by NFRG-E (-14.50 mg mL^{-1}; $p<0.05$). It was concluded that the immunological activities of FRG-E were higher than those of NFRG-E, indicating that fermentation helped enhance the immunological activities of red ginseng.

Keywords Korean red ginseng · Fermentation · *Lactobacillus plantarum* · Immunological activity

Introduction

Ginseng is 1 of 11 distinct species of slow-growing perennial plants with fleshy roots, belonging to the *Panax* genus in the family Araliaceae. In Asia, it is widely believed that ginseng is a miraculous medicine or mysterious tonic, improving one's physical condition and prolonging life with long-term administration [1]. Numerous studies have contributed to the accumulation of evidence that ginsenosides are responsible for the pharmacological effects of ginseng. Indeed, ginsenosides themselves can exert various pharmacological activities, by directly being added to cell cultures in vitro or by being intraperitoneally (i.p.) or intravenously (i.v.) injected into experimental animals. These results have led to the misunderstanding that intact ginsenosides might be the real active principles in the body [2].

However, orally ingested ginsenosides pass through the stomach and small intestine without decomposition by either gastric juices or liver enzymes into the large intestine, where ginsenosides are deglycosylated by colonic bacteria followed by transit into the circulation. The colonic bacteria cleave the oligosaccharide connected to the aglycone stepwise from the terminal sugar to generate major metabolites, namely 20S-protopanaxadiol 20-O-β-D-glucopyranoside (M1) and 20S-protopanaxatriol (M4) [3, 4]. Accumulating evidence strongly suggests that the metabolites are the active molecules in the body. Kim et al. [5] proposed the concept that plant glycosides act as pro-drugs that are metabolized to active forms by intestinal bacterial deglycosylation. Wakabayashi et al. [6] revealed that the anti-tumor activities of ginsenosides after oral administration are based on metabolites formed by intestinal bacterial deglycosylation. However, the metabolite-producing potential of intestinal bacteria differs among individuals. Therefore, it is easily considered that such individual differences in bacterial ginsenoside-hydrolyzing potentials may affect ginseng efficacy.

Fermented red ginseng (FRG) containing ginsenoside metabolites was developed utilizing specific microorganisms for fermentation to increase the absorption rate of ginsenosides, and may have merits for standardizing red ginseng efficacy [3, 7]. By fermentation, glycoside ginsenosides in red ginseng are converted into non-glycoside ginsenosides. In other words, glucose, attached to saponins is metabolized by bacteria and converted into small sized ginsenosides. FRG contains high concentrations of compound K (CK) as well as ginsenosides with enhanced biological activities such as Rb1, Rb2, Rg5, and Rk1. Therefore, their absorption rates by the body are high and these compounds are expected to maximize FRG's efficiency in pharmacology [8].

In a previous study [9], we screened edible *Lactobacillus* species for their potential in metabolizing ginsenosides from red ginseng. A recently isolated strain, *L. plantarum* M-2, that is food-grade, was used for the microbial conversion of ginsenosides in red ginseng powder. Thus, to utilize the beneficial properties of ginsenoside metabolites using food-compatible microorganisms, we investigated changes in total sugars, uronic acid,

polyphenols, and ginsenoside metabolites during fermentation by *L. plantarum* M-2. We also investigated the anti-metastasis and immunological activities of FRG in animal and human subjects.

Materials and Methods

Materials

Six-year-old red ginseng powder was gifted from Sejong Korea Ginseng Co. (Incheon, Korea). Standard ginsenoside materials including CK, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg2, Rg3, Rg5, Rh1, Rh2, and Rk1 were purchased from Embo Laboratory (Daejeon, Korea). All other chemicals were of reagent grade and were obtained from local suppliers.

Laboratory-scale Fermentation Using Red Ginseng

Laboratory-scale fermentation using red ginseng was carried out in a 5 L vertical glass fermenter (Fermentec, Seoul, Korea) at 37°C with a 3 L working volume of medium (pH 6.0) including 150 g of red ginseng powder. A 4% (*v/v*) culture of *L. plantarum* M-2 grown in MRS broth was used as an inoculum. The cultures were stirred at 20 rpm throughout the fermentation. Samples (25 mL) were withdrawn through the sampling port every 24 h and extracted with 70 mL of 95% ethanol. The red ginseng was extract twice, with the extract solution under reflux in a water bath at 70°C, for 3 h each time. The combined extract was evaporated using a rotary evaporator under vacuum at 45°C. This extract was used in assays as an EtOH extract from fermented red ginseng (FRG-E) and compared to EtOH extract from non-fermented red ginseng (NFRG-E).

Analytical Methods

Total polyphenol content was determined using the Folin–Ciocalteu method [10] adapted to a microscale using gallic acid as a standard (50–800 $\mu g\ L^{-1}$). Total sugar and uronic acid levels were determined using the phenol-sulfuric acid [11] and *m*-hydroxydiphenyl methods [12], respectively, using glucose and galacturonic acid as the respective standards. In all cases, the analyses were performed in triplicate unless otherwise specified.

The levels of 14 major ginsenosides were analyzed using an HPLC-based technique developed by Lee et al. [13]. A Varian Prostar 200 HPLC system (Varian Inc., Palo Alto, CA) equipped with a quaternary solvent delivery system, an autosampler, and UV detector was used. The column configuration consisted of an IMtakt Cadenza CD-C18 (4.6×75 mm, Imtakt Co., Kyoto, Japan). UV absorption was measured at 203 nm. Gradient elution was employed using solvent A (10% acetonitrile) and solvent B (90% acetonitrile) at 40 °C; the gradient program was as follows: 0→11 min, 11% B (isocratic); 11→15 min, 11→16% B; 15→16 min, 16→20% B; 16→18 min, 20→21%; 18→24 min, 21% B (isocratic); 24→25 min, 21→22% B; 25→35 min, 22% B (isocratic); 35→36 min, 22→23% B; 36→40 min, 23% B (isocratic); 40→41 min, 23→24%; 41→45 min, 24% B (isocratic); 45→53 min, 24→37% B; 53→61 min, 37→45% B; 61→66 min, 45→46%; 66→73 min, 46→48% B; 73→75 min, 48% B (isocratic); 75→77 min, 48→11%; 77→85 min, 11% B (isocratic). The flow rate was kept at 1.3 mL min^{-1} and the sample injection volume was 5 μL. The level of total ginsenosides was determined by the sum of the 14 ginsenosides.

Experimental Lung Metastasis in Mice

Specific pathogen-free female BALB/c mice (6 weeks old) were purchased from NARA Biotech (Gyeonggi-do, Korea). The mice were housed in a specific pathogen-free environment. The experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of Yuhan University (2009E-1). Water and a diet of pellets were supplied ad libitum. A lung metastatic subline of a highly metastatic cell line of colon 26-M3.1 carcinoma was maintained as monolayer cultures in Eagle's medium supplemented with 7.5% fetal bovine serum, sodium pyruvate, non-essential amino acids, and L-glutamine, which were purchased from Gibco BRL (Grand Island, NY, USA).

Experimental lung metastasis was induced by i.v. inoculation of the colon 26-M3.1 carcinoma cells (3×10^4 mL^{-1} well^{-1}) into BALB/c mice. To study the anti-tumor metastasis activity, groups of 5 BALB/c mice were given i.p. FRG-E (50–5,000 μg mouse^{-1}) 2 days before tumor cell inoculation. The mice were also given oral administration of FRG-E (0.4-10 mg mouse^{-1}) seven times for everyday before and five times for 3 days interval after i.v. inoculation colon 26-M3.1 cells. The mice were sacrificed 14 days after tumor inoculation and their lungs were fixed in Bouin's solution. Lung tumor colonies were counted microscopically.

Macrophage Stimulation of FRG-E by Oral Administration in Mice

Male ICR mice (6-weeks old, NARA Biotech) were randomly divided into five groups with eight mice per group. These consisted of a control group (saline administration without sample), NFRG-E (1.0 g kg^{-1} body weight (BW) day^{-1}), two groups of FRG-E (0.5 and 1.0 g kg^{-1} BW day^{-1}), and a LPS group (0.1 g of lipopolysaccharide kg^{-1} BW day^{-1}), and each was orally administered aqueous solution using intragastric tubes daily for 7 days. After 1 week, the macrophage stimulating activity of FRG-E by oral administration was determined according to the following method [14]. After the ICR mice administered FRG-E were injected i.p. with thioglycollate medium, macrophage suspensions were prepared from the peritoneal cavities of the mice after 3 days. Two hundred microliters of the cell suspension (1×10^6 cells mL^{-1}) was seeded in a 96-well microplate for 2 h to prepare the macrophage monolayer, and non-adherent cells were removed by washing with RPMI 1640 medium [15]. After the secreted phosphatase from macrophages was solubilized by 0.1% Triton X-100 and reacted with p-nitrophenyl phosphate, the absorbance at 405 nm was photometrically measured using a microplate reader (Sunrise, Tecan, Grödinger, Austria).

Cytokine Production Assay

The determination of tumor necrosis factor alpha (TNF-α) for conditioned medium recovered from the macrophage cultures was performed by enzyme-linked immunosorbent assay (ELISA) kits (Pharmingen, San Jose, CA, USA) according to the manufacturer's recommendations. The solution of purified anti-mouse TNF-α monoclonal antibody (mAb) was added to microtiter plates for ELISA overnight at 4°C. The cultured medium of macrophages was added to the wells, and then the plates were incubated at room temperature for 2 h. After biotinylated anti-mouse TNF-α mAb and alkaline phosphatase labeled streptoavidin were added to each well, the wells were incubated with 150 μL of chromogenic substrate solution (p-nitrophenylphosphate disodium salt in diethanolamine

buffer pH 9.8), and subsequently the absorbance at 405 nm was measured using a microplate reader.

Immunoglobulin Level in Human Serum

Approval for the experiment was obtained from the Ethical Committee for Human Experimentation at Korea University (KU-IRB-09-09-A-3). The study protocol was explained, and written informed consent was obtained. Volunteers were excluded if they were smokers or taking prescription medications. Thirty-three participants were selected for the study and three subjects failed to complete the study. Thus, the final subjects consisted of 30 healthy adults (17 male and 13 female). The general characteristics of the subjects are provided in Table 1.

This single-blind, random-allocation and parallel study was completed over a 6-week period using a two-phase crossover design with a 2-week washout period. The subjects, randomly allocated to one of two groups, received either 2 g of FRG-E or NFRG-E before breakfast each day for 2 weeks. Prior to the FRG-E or NFRG-E consumption period, fasting blood samples were collected at baseline. The subjects were instructed to maintain their regular diet and exercise patterns as usual. To help ensure compliance, they were asked to record the number of red ginseng samples taken at the end of each week. The subjects were questioned about any symptoms or medication changes at weekly visit.

Blood samples were collected from a superficial vein in the forearm into dry non-heparinized centrifuge tubes. For biochemical analysis, the blood was centrifuged at $1,500 \times g$ for 10 min to obtain the serum. The immunoglobulin G (IgG) and immunoglobulin A (IgA) in the serum were quantitated using a BN II Nephelometer (Dade Behring, Marburg, Germany) by immunonephelometric methods calibrated against the certified reference material (CRM) 470 [11].

Statistical Analysis

All data were reported as means±standard deviations (SD). The statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS Inc.,

Table 1 General characteristics of human subjects

Characteristics	Subjects	
	Male (n=17)	Female (n=13)
Age (year)	40.3±8.2	30.5±7.0
Height (cm)	170.4±5.2	162.5±4.6
Weight (kg)	72.7±12.2	60.1±8.0
BMI (kg m^{-2})	25.0±3.6	22.8±2.8
Fat mass (kg)	17.3±6.0	19.9±5.8
% Body fat (%)	23.3±5.4	32.7±5.9
Lean body mass (kg)	55.5±7.8	40.2±4.1
Systolic blood pressure (mmHg)	124.3±17.8	108.6±12.1
Diastolic blood pressure (mmHg)	82.5±12.0	75.1±7.8

Values are means±SD

BMI Body mass index, body weight (kg) [height (m)]$^{-2}$

Chicago, IL, USA). The values between extracts (FRG-E vs. NFRG-E) were compared using an independent two-tailed t test assuming equal variance. Control and extract (FRG-E or NFRG-E) values were also evaluated by independent two-tailed t tests. In the human experiment, values between treatments (FRG-E vs. NFRG-E) were compared using paired two-tailed t tests. p values less than 0.05 were considered significant.

Results

Changes in Chemical Composition During Fermentation of Red Ginseng by *L. plantarum* M-2

Changes in the chemical composition of red ginseng fermented by *L. plantarum* M-2 were monitored during fermentation (Fig. 1). The control (0 day, non-fermented red ginseng) had the highest total sugar content (85.5 mg mL^{-1}), which decreased to 44.1 mg mL^{-1} with increasing fermentation time, indicative of the use of sugars as a carbon source for growth throughout fermentation. Uronic acid content reached its maximum (534.3 µg mL^{-1}) at 3 days of fermentation and decreased from 4 days. The polyphenol content of the FRG showed a maximum value (35.6 µg mL^{-1}) at 2 days of fermentation and then decreased slightly with increasing culture time. However, the polyphenol content of FRG changed slightly throughout fermentation, and there were no significant differences ($p > 0.05$). Cell growth (cell number) showed a maximum value at 3 days of fermentation and then decreased slightly with increasing culture time.

Changes in Ginsenosides During Fermentation of Red Ginseng by *L. plantarum* M-2

The total ginsenoside and ginsenoside metabolite contents of the red ginseng fermented by *L. plantarum* M-2 increased with fermentation time (Table 2). The total contents of major ginsenosides, such as Rb1, Rb2, Rc, and Rd (protopanaxadiol type), changed slightly during fermentation, and the total contents of protopanaxatriol type ginsenosides (Re, Rg1, and Rf) were not significantly different throughout fermentation. However, ginsenoside
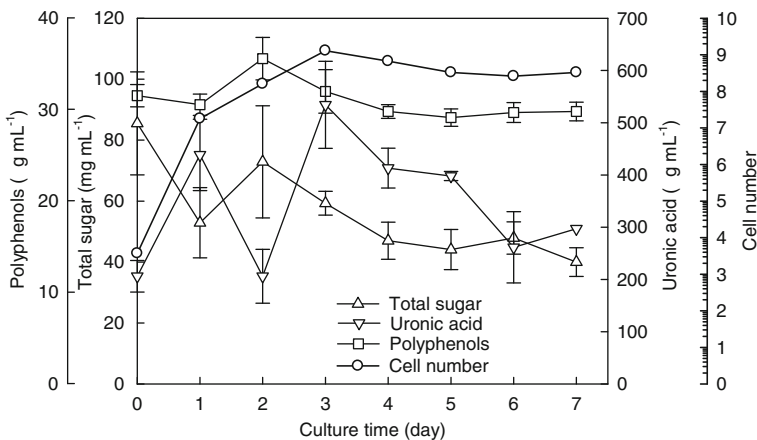
Fig. 1 Changes in total sugar, uronic acid, polyphenols contents, and cell number during fermentation of red ginseng powder by *Lactobacillus plantarum* M-2. Values are means±SD

Table 2 Changes in ginsenoside contents during fermentation of red ginseng powder by *Lactobacillus plantarum* M-2

Ginsenoside ($\mu g\ mL^{-1}$)	Fermentation time (day)							
	0	1	2	3	4	5	6	7
CK	0.0±0.0	0.0±0.0	0.0±0.0	7.5±0.6	29.8±2.1	52.1±2.3	84.7±3.0	11.2±4.1
Rh2	100.3±12.4	110.8±23.3	92.2±24.0	154.3±19.5	174.8±20.6	166.2±10.6	156.0±10.4	175.3±8.7
Rh1	112.0±11.6	106.9±24.6	120.1±24.9	150.1±18.5	177.9±21.6	161.5±20.9	164.7±19.9	184.0±16.7
Rg5+Rk1	3362.2±151.4	3206.3±188.9	3476.8±174.1	4287.6±127.2	5486.4±185.0	5058.8±151.3	5273.0±258.4	5551.4±262.1
Rg2	266.8±33.3	260.7±65.3	287.1±62.3	314.3±52.3	379.4±59.3	359.7±28.5	373.6±29.1	406.3±22.3
Rg3	896.0±79.9	925.0±69.3	1015.2±172.0	1242.8±135.8	1507.6±147.8	1430.8±93.0	1486.5±81.9	1615.5±72.3
Rg1	212.9±22.5	187.6±40.7	218.6±41.6	176.7±31.9	213.3±35.4	205.9±31.8	216.1±30.4	216.6±27.4
Rf	313.4±14.3	262.1±31.0	332.2±27.3	338.9±24.1	390.8±26.9	390.2±17.3	403.4±16.1	531.6±13.0
Re	823.3±40.7	769.9±95.3	881.2±91.1	735.5±96.0	868.9±74.6	836.8±54.0	867.6±46.3	886.6±38.0
Rd	832.1±60.3	804.6±121.3	920.9±82.5	863.3±71.1	1031.8±92.8	991.2±72.5	1033.7±89.8	1076.8±68.0
Rb2+Rc	2223.0±74.1	2096.0±127.4	2400.4±109.2	2211.1±61.4	2616.1±132.6	2517.5±93.6	2601.7±107.2	2693.9±101.9
Rb1	2018.1±142.2	1842.4±71.9	2030.3±79.8	1827.4±68.0	2286.9±75.1	2097.1±165.0	2249.8±166.5	2435.1±125.3
Total	11160.1±842.8	10572.3±1658.9	11775.0±1618.8	12309.5±1326.4	15163.7±1483.8	14267.8±1140.8	14910.8±1127.4	15784.3±889.8
Metabolite	4637.0±276.1	4498.9±348.1	4899.2±433.3	6002.3±334.4	7581.1±415.9	7062.9±297.0	7382.5±392.4	7768.4±377.6

Ginsenoside metabolites are sum of CK, Rh2, Rh1, Rg5, Rk1, Rg2, and Rg3

metabolites (CK, Rh2, Rh1, Rg5, Rk1, Rg2, and Rg3) easily absorbed by the small intestine showed significantly higher levels at 4 days of fermentation compared with 0 day, increasing from 4,637.0 to 7,581.1 $\mu g\ mL^{-1}$. New peaks were also evident in the chromatogram after 4 days of fermentation. Since these unknown peaks were considered to be minor ginsenosides between Rh1 and Rg5+Rk1 (data not shown), we assumed that the new peaks might be the easily absorbed metabolites of ginsenosides.

Inhibitory Effect Against Lung Metastasis in Mice

Tables 3 and 4 show that the systemic administration of FRG inhibited tumor metastasis induced by colon 26-M3.1 carcinoma cells. To investigate whether the fermented red ginseng could enhance natural immunity against tumors, we examined the effect of FRG-E in an experimental lung tumor metastasis model of colon 26-M3.1 cells. The prophylactic i.p. administration (50–5,000 $\mu g\ mouse^{-1}$) of FRG-E inhibited tumor metastasis (Table 3). In particular, FRG-E inhibited lung metastasis about 81.1% at 500 $\mu g\ mouse^{-1}$ whereas the inhibitory effect against tumor metastasis by EtOH extract from NFRG-E was 66.9% at the same dose. High dose (>500 $\mu g\ mouse^{-1}$) administration of both FRG-E and NFRG-E showed some piloerection (data not shown). In addition, we confirmed whether the FRG could augment anti-metastasis by oral administration in experimental lung metastasis of colon 26-M3.1 carcinoma cells (Table 4). The results indicated that the oral administration of FRG-E dramatically inhibited lung metastasis in a dose-dependent manner. Consequently, these results show that FRG-E may enhance systemic natural immunity against inoculated tumors in the mucosal immune system administered via the oral route.

Macrophage Stimulating Effect of Orally Administered FRG-E in Mice

The effect of FRG-E on the stimulation of macrophages was investigated ex vivo. After FRG-E had been orally administered for 7 days at different doses, the stimulatory response of the macrophages was investigated by using a cellular lysosomal enzyme. The administration of 0.5 and 1.0 $g\ kg^{-1}\ BW\ day^{-1}$ of FRG-E for 7 days revealed a dose-dependent increase in the relative activity of the macrophage lysosomal enzyme. A

Table 3 Inhibition of tumor metastasis for experimental lung metastasis in mice that were given intraperitoneal injections 2 days before tumor cell inoculation at the indicated doses of EtOH extracts from fermented ginseng (FRG-E) and non-fermented red ginseng (NFRG-E)

Sample	Conc. ($\mu g\ mouse^{-1}$)	Lung metastasis		
		No.	Range	Inhibition (%)
Control		64.7±8.1	59–80	–
NFRG-E	50	42.8±2.9	39–46	33.6
	500	21.4±7.3	15–29	66.9
	5000	52.6±6.5	45–60	–
FRG-E	50	13.8±4.1	9–19	78.7
	500	12.2±3.1	8–15	81.1
	5000	41.2±12.5	29–52	36.3

Values are mean±SD for five mice

Table 4 Inhibition of tumor metastasis for experimental lung metastasis in mice that were given oral administration 2 days before tumor cell inoculation at the indicated doses of EtOH extracts from fermented ginseng (FRG-E) and non-fermented red ginseng (NFRG-E)

Sample	Conc. (mg mouse^{-1})	Lung metastasis		
		No.	Range	Inhibition (%)
Control		102.2±8.6	91–110	–
NFRG-E	0.4	96.6±2.7	95–100	–
	2	100.8±9.9	90–117	–
	10	35.0±10.6	20–49	65.8
FRG-E	0.4	84.2±8.8	96–71	–
	2	46.2±5.5	39–53	54.8
	10	28.6±9.1	18–43	72.0

Values are mean±SD for five mice

significant increase and maximum stimulation of relative activity was observed at 1.0 g kg^{-1} BW day^{-1} (1.44-fold of the saline-administered group; control; Fig. 2a). These results suggest that the oral administration of FRG-E enhanced the stimulatory response of macrophages. Activated macrophages are able to recognize and lyse tumor cells including those that are resistant to cytostatic drugs. Therefore, macrophage activation can play a role in novel immunotherapeutic approaches in the treatment of cancer. TNF-α, produced by activated macrophages, is considered one of the most important mediators directly involved in tumor cell killing [16]. The ability of FRG-E to activate macrophages was investigated by observing TNF-α production. As presented in Fig. 2b, treatment of peritoneal macrophages with FRG-E in the ex vivo experiment induced TNF-α (3.22-fold of the control) at 1.0 g kg^{-1} BW day^{-1}. This result suggests that FRG-E can activate macrophages, and its ability to induce TNF-α production from macrophages may enhance macrophage-mediated cytotoxicity against tumor cells [17].

Immunoglobulin Levels in Serum from Humans Administered FRG-E

Figure 3 presents the changes in IgA and IgG levels in serum by FRG-E administration. IgA and IgG levels were higher after FRG-E administration than at baseline, whereas NFRG-E induced reductions of these variables related to immunity. At 1 week, the change in IgA level by FRG-E (5.14 mg mL^{-1}) was significantly higher compared to that by NFRG-E (−14.50 mg mL^{-1}; $p<0.05$). For IgG level, the change by FRG-E was also slightly higher than that by NFRG-E, although it did not reach statistical significance.

Discussion

Ginseng roots contain various pharmaceutical components such as ginsenosides (saponins), polyacetylenes, polyphenolic compounds, and acidic polysaccharides. Among these components, ginsenosides have been reported as the most pharmaceutically active ingredients. Until now, various ginsenosides, with five major ginsenosides (Rb1, Rb2, Rc, Re, and Rg1) constituting more than 80% of the total ginsenosides, have been isolated from ginseng roots [18].

Fig. 2 Effects of EtOH extracts from fermented ginseng (FRG-E) and non-fermented red ginseng (NFRG-E) on: macrophage stimulating activity by oral administration (A) and TNF-α production from macrophages (B). Bars are means±SD for five samples. Asterisk means a significant difference ($p<0.05$) between the saline-administered group (control) and each EtOH extract-administered group by independent two-tailed t tests. The control group (saline administration without sample), NFRG-E (1.0 g kg^{-1} BW day^{-1}), two groups of FRG-E (0.5 and 1.0 g kg^{-1} BW day^{-1}) and LPS group (0.1 g of lipopolysaccharide kg^{-1} BW day^{-1}) were each orally administered aqueous solution using intragastric tubes daily for 7 days
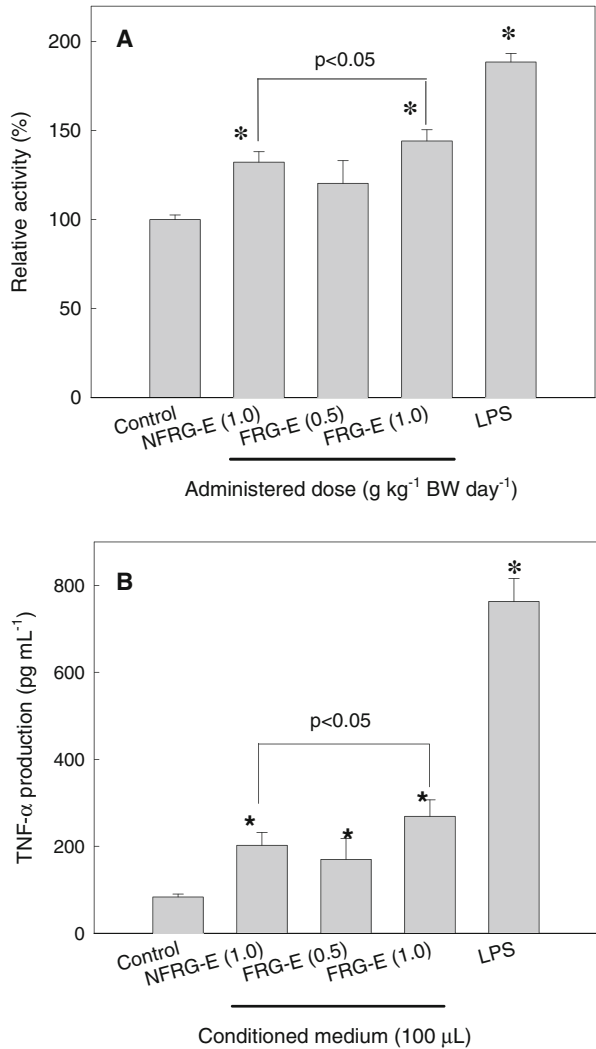

Several studies have shown that the transformation of ginsenosides into deglycosylated ginsenosides is required to achieve more effective physiological action by the compounds [19]. Recently, many researchers have also focused on the pharmaceutical activities of minor ginsenosides such as Rd, Rg3, Rh2, and CK, because their activities were found to be superior to those of major ginsenosides. Various transformation methods including mild acid hydrolysis [18], enzymatic conversion [20], and microbial conversion [3] have been used. However, chemical methods have been avoided because of side reactions such as epimerization, hydration, and hydroxylation, and most of the microorganisms used for ginsenoside transformation are not of a food-grade standard.

In this study, the fermentation of red ginseng by *L. plantarum* M-2 was carried out for the transformation of ginsenosides. This FRG had increased total ginsenoside and metabolite contents, especially for Rh1, Rh2, Rg5, Rk1, Rg2, and Rg3 ginsenosides with increasing culture time (Table 2). Red ginseng mainly contains ginsenosides such as Rg3,

Rb1, Rb2, and Rc. Rh1, Rh2, and Rg3, which are representative constituents in red ginseng, and are produced from protopanaxadiol ginsenosides by steaming raw ginseng [21]. When red ginseng is fermented by *Bifidobacterium* H-1, Rg3 is transformed to Rh2, which is a representative constituent in FRG [3, 22]. Compared to Rg3, Rh2 has exhibited potent cytotoxicity against tumor cells, antiallergic effects against mast cells, anti-inflammatory activity in microbial cells [23], secretion of insulin, and the reduction of plasma glucose in streptozotocin-diabetic rats [3]. Rh1 is produced from Re via Rg1 and Rg2 by *Bifidobacterium* sp. Int57, *Bifidobacterium* sp. SJ32, *Aspergillus niger*, and *Aspergillus usamii* [24]. Rg2 has been found to reduce the acetylcholine-evoked secretion of catecholamines from cultured bovine adrenal chromaffin cells [25]. Rh1 is known to possess antiallergic and anti-inflammatory activities [24]. Therefore, fermentation may be a means to obtain red ginseng components that have immunopotentiating activity and potent cytotoxicity against tumor cells, and that are easily absorbed in the human intestinal tract.

Both systemic injection and oral administration of FRG-E showed higher anti-tumor metastatic activity than NFRG-E in mice (Tables 3 and 4). Appropriate enhancement of anti-tumor activity by treatment of bioactive compounds can augment host defense responsiveness [26]. Oral administration of FRG-E also enhanced macrophage stimulating activity ex vivo. Significant maximum stimulation was observed at 1.0 g kg^{-1} BW day^{-1} (1.44-fold of the saline-administered control group; Fig. 2a). As presented in Fig. 2b, oral administration of FRG-E in mice also induced TNF-α (3.22-fold of the control) at the same dose. These results suggest that FRG-E has potent immunomodulatory properties by enhancing macrophage function, followed by the contribution of the potent anti-tumor activity of FRG in vivo. It is well documented that several ginsenosides from ginseng may contribute to the activation of systemic and mucosal immunity in rats by oral administration [27]. In addition, our previous data revealed that FRG-E seemed to increase anti-tumor activity compared to NFRG-E in a lung metastasis model of colon 26-M3.1 carcinoma cells [28]. Thus, the anti-metastatic activity of FRG-E indicates that several kinds of ginsenoside metabolites may contribute to the activation of the innate immune system including macrophages in mice [17, 29].

The results of this study exhibit that FRG, a newly developed red ginseng fermented with *L. plantarum* M-2, is capable of increasing IgG and IgA in serum in a healthy human subject model (Fig. 3). The hyper-IgG and -IgA effects in serum may be attributable to the ability of immunoactive constituents from FRG [30]. IgA, which is secreted from
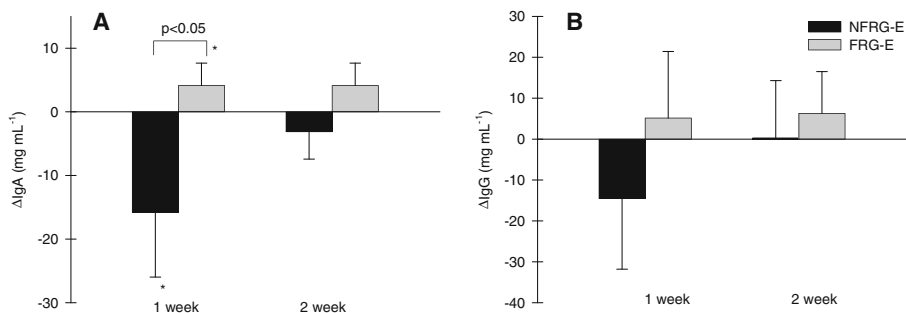
Fig. 3 Effects of EtOH extracts from fermented ginseng (FRG-E) and non-fermented red ginseng (NFRG-E) on immunoglobulin A (IgA) and immunoglobulin G (IgG) levels in serum of healthy subjects. Bars are mean±SD for 30 subjects. The values between groups (FRG-E vs. NFRG-E) at each week were compared using a paired two-tailed *t* test

conventional B cells or from B-1 cells, plays a prominent role in the gut mucosal immune system, which serves to protect the body from the continuous threat of infection from intestinal bacteria or exogenous pathogenic bacteria [31]. Although there are significant levels of IgA in human serum, it is generally accepted that the secretory form of the protein remove, in a functional sense, which is most important [32]. Secretory IgA is assembled during an active transport process as locally produced dimeric IgA passes across the mucosal epithelium in seromucous secretions such as saliva, colostrums, breast milk, tracheobronchial, and genitourinary secretions [33]. Our results demonstrate that the serum level change in IgA among the groups treated with FRG-E and NFRG-E was varied at the first week after continuous administration. The change in serum IgA level in humans administered FRG-E was higher than at baseline (5.14 mg mL^{-1}) whereas NFRG-E was lower (−14.50 mg mL^{-1}). Serum IgA antibodies are implicated in host defense against bacterial infections [33]. IgG is the most important class of immunoglobulin in secondary immune responses and is distributed evenly between the intravascular and extravascular pools [34]. The major effecter mechanisms of human IgG are the activation of the classical complement pathway, the elimination of pathogens, and the mediation of inflammation [35]. IgG also acts as an anti-toxin and as an opsonin by its ability to bind Fc receptors on macrophages and neutrophils, and IgG is important in conferring immunity to neonates and in the first months of life [36]. In determining IgG level, the change in IgG level by FRG-E was slightly higher than that by NFRG-E ($p>0.05$). Serum immunoglobulin levels represent a non-specific, relatively insensitive measure of immune function, however total serum immunoglobulin levels might moderately reflect actual humoral immunity in vivo. The data acquired from this experiment have proven that FRG-E can act as a biological response modifier by enhancing the magnitude of a specific immune response in humans.

There are some limitations to this human study; FRG-E administration induced a significant difference in IgA, but no significant difference in IgG was shown. If the duration and/or amount of red ginseng administration were greater in other studies than in our human study, the immunostimulating effect might be more effective than our results. The mechanism of increase in serum IgA and IgG concentrations by oral administration of FRG-E still remains obscure, but these findings encourage a more in-depth analysis of the mechanism involved.

In conclusion, our results suggest that fermented red ginseng may improve immunos-timulating activities and have beneficial properties as a useful functional herb. While experimental studies have demonstrated the pharmacological effects of fermented red ginseng, more studies are needed to support the role of ginseng in medical care.

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