

Fermented Red Ginseng Alleviates Ovalbumin-Induced Inflammation in Mice by Suppressing Interleukin-4 and Immunoglobulin E Expression

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ABSTRACT Ginseng (the root of *Panax ginseng* Meyer) has been reported to have many biologic therapeutic effects, including anti-inflammatory properties, and ginsenosides are considered as one of the factors responsible for these therapeutic effects. To improve their therapeutic action, probiotic bacteria are used to ferment and chemically transform ginsenosides in red ginseng (RG). In this study, we aimed to investigate the beneficial effects of RG fermented by probiotic bacteria (FRG) against ovalbumin (OVA)-induced allergic rhinitis in a mouse model. We induced the mouse model via OVA inhalation; experimental results revealed increased immunoglobulin E (IgE) and interleukin (IL)-4 levels, leading to Th2-type cytokine response. The mice with induced allergy were then orally administered RG and FRG over 2 weeks, as a result of which, IL-4 and IgE levels in bronchoalveolar lavage fluid, nasal fluid, and serum were found to be ameliorated more effectively by FRG than by RG, suggesting that FRG has better immune regulatory effects than RG. FRG also downregulated immune cell levels, such as those of eosinophils and basophils, and significantly decreased the thickness of OVA-induced respiratory epithelium compared to RG. Collectively, the results showed that FRG treatment alleviates inflammation, thereby extending a protective effect to mice with OVA-induced inflammatory allergic rhinitis.

KEYWORDS: • *allergic rhinitis* • *fermented red ginseng* • *OVA* • *probiotics*

INTRODUCTION

ALLERGIES OR ALLERGIC DISEASES are immediate hypersensitivity immune reactions mediated by immunoglobulin E (IgE).^{1,2} Nearly 20% of the world's population suffer from allergies, and the number is increasing every year.³ Allergic reactions result from inflammatory mediators, such as histamines and cytokines.⁴ These have been recognized as critical mediators of allergic response, as they interact with each other to trigger acute inflammatory reactions against allergens.⁵ When an allergic reaction begins, IgE combines with mast cells or basophils to initiate the production of allergic mediators, such as histamine or cytokines.⁶ These steps induce inflammatory cell recruitment and eosinophil migration to the affected tissues.⁷

Various treatments are available for the treatment of this disorder, including calcium influx blockers, phosphodiesterase inhibitors, cyclic adenosine monophosphate stimulators, and Th2-type cytokine inhibitors.^{8,9} However, these treatments only serve as temporary measures for these allergic symptoms as most of them cannot completely alleviate the symptoms. Therefore, alternative methods with improved safety are necessary. Recently, natural medicines have been reported to be capable of regulating inflammatory responses

during allergic reactions.¹⁰ Hence, several studies are currently being conducted to find appropriate treatments for allergic diseases, with interest in natural therapeutic plants with less side effects.¹¹

Red ginseng (RG; the steamed ginseng) has been used as a beneficial therapeutic medicinal plant.¹² RG has been used as a functional food over several centuries, owing to its therapeutic actions, such as anti-inflammatory, antidiabetic, and antitumor activities.^{13–15} These effects have been attributed to the therapeutic active compounds known as ginsenosides. Ginsenosides may be divided into two types, namely Protoganaxadiol and Protoganaxatriol, and both groups have some clinical effects.¹⁶

Recently, various studies have shown red ginseng, fermented by gut microbiome (FRG), to have more potent biological activities than RG.^{17,18} During microbial fermentation, microorganisms or their enzymes change the structure of RG by hydrolyzing the glucose attached to ginsenosides, thereby improving the bioavailability of RG.^{19,20} Many kinds of probiotics have been developed to enforce the pharmacological effects of FRG.^{21,22} However, the difference between the antiallergic and anti-inflammatory effects of RG and fermented RG is not yet clear.

Based on the existing knowledge, we aimed to investigate the antiallergic effect of FRG in an ovalbumin (OVA)-induced mouse model of allergic rhinitis and understand its beneficial effects against OVA-induced inflammation.

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MATERIALS AND METHODS

Reagents and equipment

OVA, dexamethasone, A23187 (Sigma Aldrich), mouse interleukin (IL)-4, IL-6, mouse IgE Quantikine ELISA kit (R&D Systems), Easy-spin™ RNA kit (17221; Intron), lysing matrix D (6913-500; MP Bio), Fast-prep®-24 (MP bio), Omniscript® RT kit (205113; Qiagen), Gene Expression Master mix (4639016; Applied Biosystems), and QuantStudio™ 6 flex Real-Time instrument (Thermo Fisher Scientific) were used in the study. RG was prepared according to the method of Kim *et al.*²³ It was incubated with 1% *Lactobacillus paracasei* HY7016 and *Bifidobacterium longum* HY8004 at 37°C for 72 h and evaporated to obtain the concentrate (67.0 Brix).

Animals

Balb/c mice (male, 6 weeks old, 20–25 g) were purchased from DooYeol Biotech (Seoul, South Korea) for this study. The mice were housed in a room in the animal laboratory (20–23°C, 12-h light/dark cycle, nonspecific pathogen free), fed an autoclaved standard laboratory diet, and allowed free access to water. All experimental procedures were approved by the Ethics Review Committee of the Korean Yakult Company Limited R&D Center, Korea (AEC-2020-0002-Y(2)).

Mouse model of OVA-induced allergic rhinitis

The protocol for sensitization and inhalation challenge was performed according to the method of Kim *et al.*, with slight modification.²³

In brief, male Balb/c mice were randomly divided into five groups, each with nine mice: (1) control group (vehicle), (2) OVA-induced group (OVA), (3) dexamethasone-treated group (Dex), (4) red ginseng-administered group (RG), and (5) fermented red ginseng-administered group (FRG). Mice were intraperitoneally (i.p.) sensitized with 45 µg of OVA emulsified with 0.1–0.2 mL of 1% aluminum hydroxide [% (w/w)] in phosphate-buffered saline (PBS, pH 7.4). Injections were performed thrice, on days 1, 8, and 15. Challenges were continued with repeated exposure to an aerosol of 1% OVA, using a nebulizer (NE-C28; Omron, South Korea). Challenge procedure was conducted for 30 min, once a day, for 5 consecutive days (days 25–29). The vehicle-treated group was subjected to i.p. injection of 0.2 mL of PBS, and then challenged with PBS. For Dex, RG, and FRG groups, mice sensitized and challenged with OVA, as described, were orally administered RG, FRG, and Dex for 2 weeks (days 16–29). PBS was used as vehicle. RG and FRG were used as feed at 6 mg/mouse once a day, and dexamethasone was administered at 500 µg/kg.

After 29 days, mice were sacrificed by CO₂ inhalation, and bronchoalveolar lavage fluid (BALF), nasal fluid, and blood were collected, and nasal cavity and lung were extracted. The obtained organs, samples, and blood were stored at –70°C (deep freezer) for subsequent cytokine analysis and gene expression analysis.

Cell culture

RAW264.7 and RBL-2H3 cells were cultured in 5% CO₂ and 37°C in Dulbecco's modified Eagle's medium containing 1% antibiotic-antimycotic solution (Gibco) and 10% heat-inactivated fetal bovine serum. For *in vitro* assay, cells (3×10^5 cells/mL) were incubated with liposaccharides (LPS) (1 µg/mL) or A23187 (1 µM) in the presence or absence of RG or FRG (5 µg/mL) for 24 h.

Preparation of BALF and nasal fluid samples

The bronchial sections of sacrificed mice were vertically divided into half. The mouse lungs were lavaged with 1 mL of PBS and a specimen is collected from there. The collected BALF was centrifuged at 2000 g for 3 min, and the supernatant used for cytokine analysis. The remaining pellet was resuspended in 100 µL PBS and used for cell analysis.

Analysis of immunocytes in BALF and blood

The BALF was analyzed using Mindray, BC-5000Vet. The number of cells was indicated as 1×10^3 cells/µL.

Analysis of cytokines in blood, BALF, and nasal fluid

Cytokines in blood, nasal fluid, and BALF were analyzed using ELISA kits. For the separation of serum, blood from mice was left at 25°C for 30 min. The blood samples were then centrifuged at 2000 g for 10 min. Serum IL-4 and IgE were determined using ELISA kits (R&D System), according to the manufacturer's manual. BALF and nasal fluid were centrifuged at 3000 g for 10 min. The cytokine content in the supernatant of BALF and nasal fluid was analyzed using IL-4 and IgE ELISA kits, according to the manufacturer's instruction.

Gene expression analysis in the liver and lung tissues

Total RNA was extracted using the protocol of Easy-spin RNA kit. Liver and lung tissue samples were homogenized in Matrix D tubes with lysis buffer using Fast-prep-24. After grinding the tissue, the remaining protocol of Easy-spin RNA kit was followed. Total RNA was stored at –70°C for subsequent gene expression analysis. Total RNA was reverse transcribed into cDNA using Omniscript RT kit. The cDNA was amplified with Gene Expression Master Mix and TaqMan Probe on QuantStudio 6 Flex Real-Time Instrument. Quantification was performed by using TaqMan primer, *IL-4* (Mm_00445260_m1), inducible nitric oxide synthase (*iNOS*) (Mm_00440502_m1), cyclooxygenase-2 (*COX-2*) (Mm_00478374_m1), and *GAPDH* (Mm99999915_g1).

Nasal tissue slides and measurement of nasal epithelial thickness

H&E slides of nasal tissue, fixed with 10% formalin solution, were prepared by KPC (Gwangju-si, South Korea). The thickness of the epithelium was measured under an Olympus CK2 microscope (Tokyo, Japan) with 20× magnification. The epithelial thickness was analyzed using ImageJ (National Institutes of Health [NIH]) software.

High-performance liquid chromatography analysis

The ginsenosides were measured using an Agilent Technologies 1260 Infinity high-performance liquid chromatography (HPLC) system connected with the C18 column (250×4.5mm, 6.0 μ m, Supelco). The mobile phase consisted of water (solvent A, 85%) and acetonitrile (solvent B, 15%) at a flow rate of 1.6 mL/min at 45°C.

Statistical analysis

All data are expressed as mean $\pm$ standard error. Significant differences were statistically analyzed using one-way analysis of variance, followed by Duncan's multiple range test ($P < .05$). The obtained data were analyzed using GraphPad Prism 5 program (GraphPad Software, Inc.).

RESULTS

Preparation of microorganisms for fermented RG

We selected two microorganisms from our own library that showed the highest beta-glucosidase enzyme activity (data not shown). Fermentation of RG with *L. paracasei*

HY7016 and *B. longum* HY8004 hydrolyzed it into minor ginsenosides, which elevated the amount of Rg3, F2, Rh1, Rh2, and Rg2 (Supplementary Table S1). Ginsenoside content was measured by HPLC.

Fermented RG inhibited IgE and IL-4 expression in the mouse model of OVA-induced allergic rhinitis

We examined the antiallergic effect of RG and FRG in mice with OVA-induced allergic rhinitis. A variety of allergic symptoms was observed in the OVA-induced group; typically, the animals were seen scratching their noses or sneezing. When allergic rhinitis was induced by OVA, the levels of IL-4 were significantly increased in the serum, BALF, and nasal fluid. However, the levels were significantly reduced in all the samples after RG and FRG administration ($P < .05$). FRG-gavaged mice showed much greater reduction of IL-4 levels in serum than the mice in the RG-administered group (Fig. 1).

IgE secretion is known to be associated with IL-4 responses and Th2-type cytokine activation. We found IgE secretion to be increased in BALF and nasal fluid of the OVA-induced group, and remarkably in serum ($P < .05$).

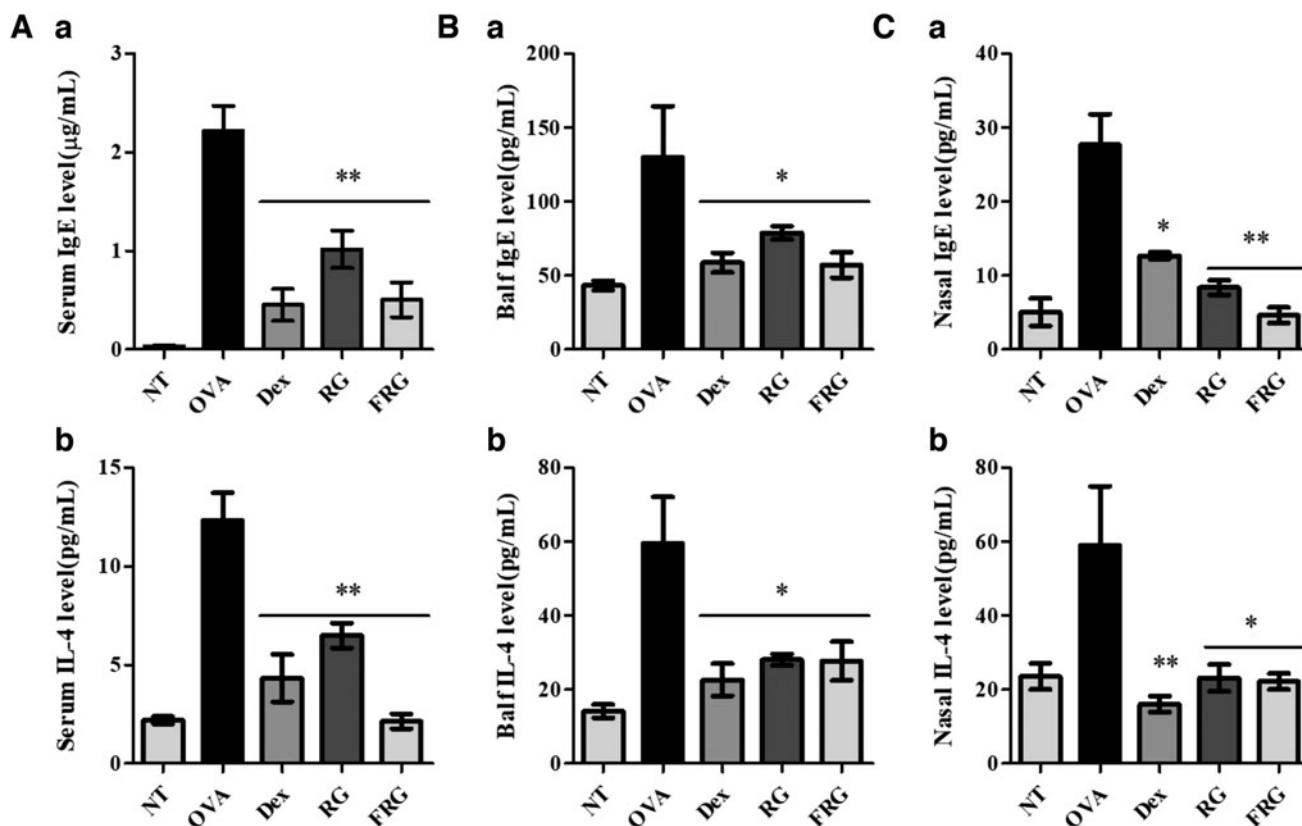


FIG. 1. Effect of red ginseng (RG) and fermented red ginseng (FRG) on blood, BALF, and nasal fluid of mice with OVA-induced allergic rhinitis. Effect on (a) IgE and (b) IL-4 levels in (A) blood, (B) BALF, and (C) nasal fluid samples. IgE and IL-4 levels were measured using ELISA kits. Data are shown as the mean $\pm$ SE. Significant differences are indicated as * $P < .05$ and ** $P < .01$, compared to the OVA group. NT, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group (1 mg/[kg·day]); RG, red ginseng-fed OVA group (6 mg/[mouse·day]); FRG, fermented red ginseng-fed OVA group (6 mg/[mouse·day]). BALF, bronchoalveolar lavage fluid; ELISA, enzyme linked immunosorbent assay; OVA, ovalbumin; IgE, immunoglobulin E; IL, interleukin; SE, standard error.

However, OVA-specific IgE levels in BALF and nasal fluid significantly decreased in the RG- and FRG-treated groups compared with levels in the nontreated OVA-induced group ($P < .05$) (Fig. 1).

Immune cell composition changed in OVA-induced mouse model

Mice with OVA-induced allergic response showed increased levels of eosinophil and basophil populations in both serum and BALF compared with levels in normal mice. Mouse serum from both RG- and FRG-administered groups showed decreased eosinophil and basophil cell population; especially, significant basophil cell inhibition was observed in FRG-treated mice ($P < .05$) (Fig. 2).

BALF from both RG- and FRG-administered groups also tended to exhibit decreased eosinophil and basophil cell population compared with that from nontreated OVA-induced group (Fig. 2).

Nasal respiratory epithelial thickness was alleviated by FRG

Representative histological and immunohistochemical images of mouse nasal respiratory epithelium from each group are shown in Figure 3.

Higher-magnification figures reveal the respiratory epithelial surface of mouse nasal mucosa. The normal group showed histologically normal respiratory epithelial thickness of $\sim 20 \mu\text{m}$. A significant increase was observed in the

thickness of nasal epithelium in the nontreated OVA group compared with that in the normal group. OVA inhalation induced notably increased nasal respiratory epithelial thickness of $\sim 45\text{--}50 \mu\text{m}$, compared to that in the normal group ($P < .05$) (Fig. 3). However, when RG and FRG were orally administered, the epithelial thickness was reduced; in fact, it was remarkably reduced by FRG administration ($\sim 30 \mu\text{m}$).

Lung inflammation in OVA-exposed mice was attenuated by FRG

We confirmed the occurrence of inflammatory response in the lung tissue of mice exposed to OVA. Mice that inhaled OVA exhibited a significant increase in the RNA expression level of *IL-4*, *COX-2*, and *iNOS* in lung tissue ($P < .05$) (Fig. 4). In contrast, FRG-treated mice showed downregulation of the OVA-induced increase in *IL-4*, *COX-2*, and *iNOS* ($P < .05$).

Production of IL-4 and NO and expression of iNOS and COX2 mRNA were inhibited by FRG in vitro

We investigated the anti-inflammatory effect of RG and FRG in RAW264.7 and RBL-2H3 cells (Fig. 5). Treatment with the response inducer LPS significantly increased IL-4 and NO production. It also increased *iNOS* and *COX-2* mRNA expression levels in nontreated RAW264.7 cells. However, RG and FRG treatment significantly inhibited the IL-4 and NO levels induced by LPS. RG or FRG treatment inhibited both *iNOS* and *COX-2* mRNA expression in

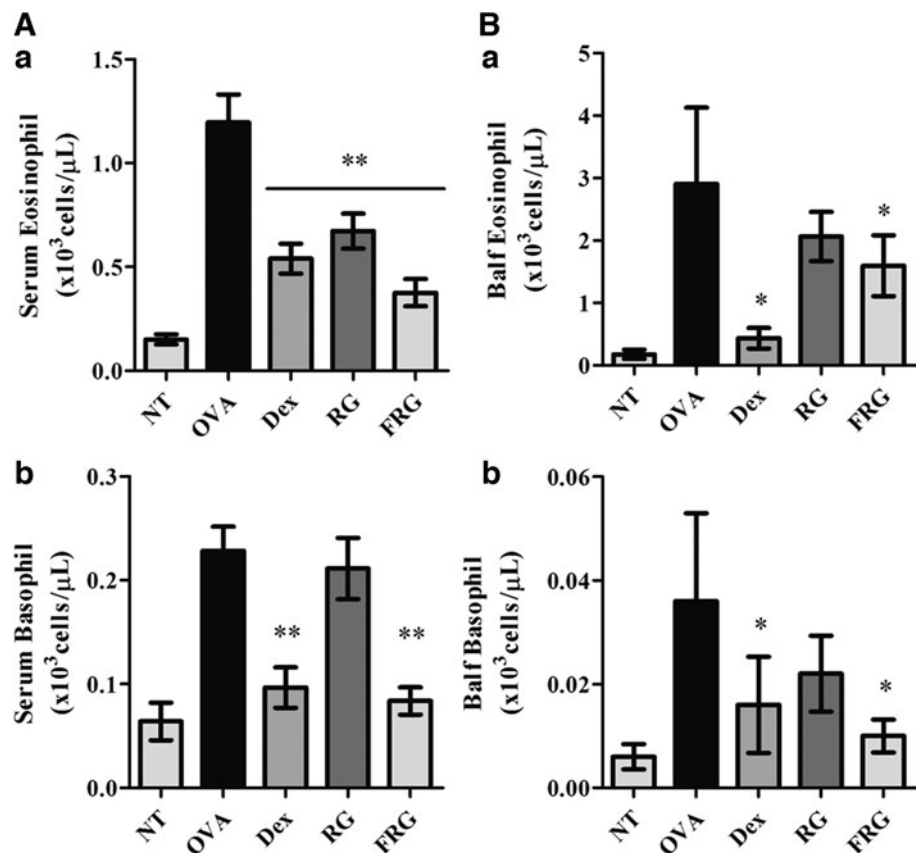


FIG. 2. Effects of RG and FRG on immune cell composition, namely eosinophil and basophil populations, in the blood and BALF of mice with OVA-induced allergic rhinitis. The immune cell count was increased upon OVA exposure and decreased in the experimental groups. (A) Effects on (a) blood eosinophil and (b) BALF eosinophil population. (B) Effects on (a) blood basophil and (b) BALF basophil population. Data are shown as the mean $\pm$ SE. Significant differences are indicated as * $P < .05$ and ** $P < .01$, compared to the OVA group. NT, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group (1 mg/[kg $\cdot$ day]); RG, red ginseng-fed OVA group (6 mg/[mouse $\cdot$ day]); FRG, fermented red ginseng-fed OVA group (6 mg/[mouse $\cdot$ day]).

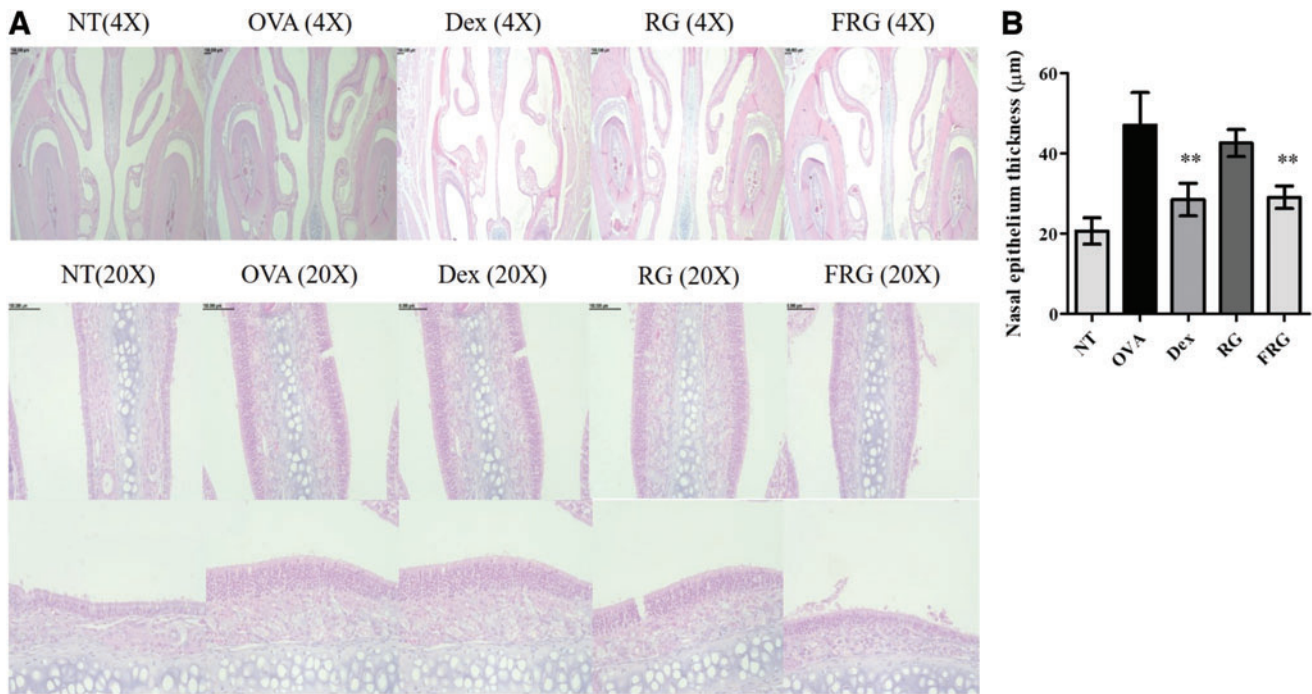


FIG. 3. Histopathological changes in the nasal epithelial tissues and thickness. **(A)** Representative images of hematoxylin and eosin-stained sections of the respiratory epithelium. **(B)** Thickness of nasal epithelium. Data are shown as the mean $\pm$ SE. Significant differences are indicated as $**P < .01$, compared to the OVA group. NT, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group (1 mg/[kg $\cdot$ day]); RG, red ginseng-fed OVA group (6 mg/[mouse $\cdot$ day]); FRG, fermented red ginseng-fed OVA group (6 mg/[mouse $\cdot$ day]). Color images are available online.

in vitro assays. Thus, RG and FRG treatment could decrease the expression of inflammatory signals in immune cells.

DISCUSSION

RG is one of the most widely used functional health foods and is an herbal medicine. Its major constituents include

ginsenosides, which have exhibited therapeutic effects in various human disorders, such as cancer, allergic inflammation, and immune disorder.^{13–15,22} Microorganisms, such as *Lactobacillus*, are used for ginseng fermentation, since they can change the composition and enhance minor ginsenosides,^{24,25} and deglycosylated ginsenosides are known to be absorbed in the intestine.²⁶ However, the effects of RG,

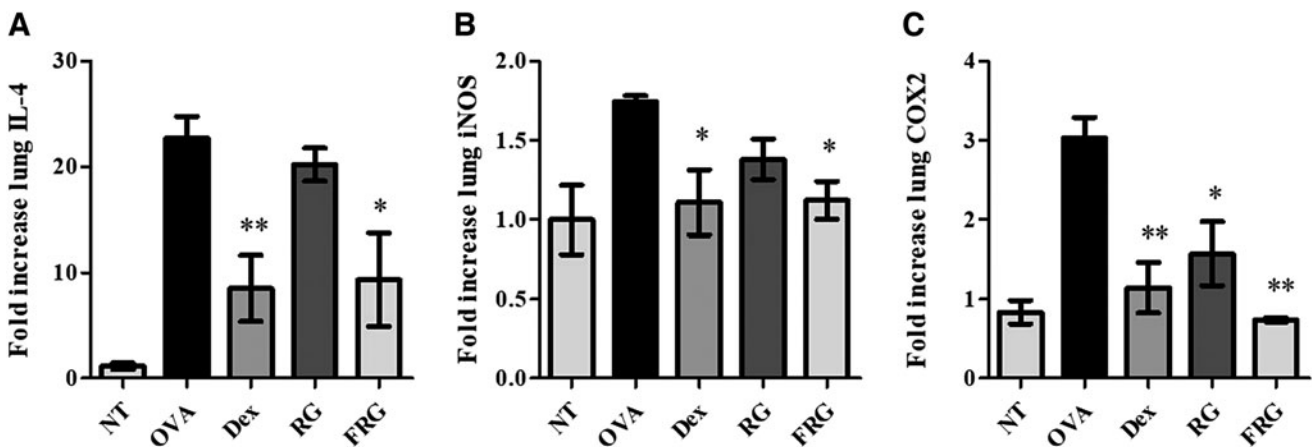


FIG. 4. Effect of RG and FRG on inflammation-related gene expression levels in lung. **(A)** Reduction of *IL-4* mRNA level in lung tissue. **(B)** *iNOS* mRNA expression. **(C)** OVA-induced increase in *COX-2* decreased in the experimental groups. Data are shown as the mean $\pm$ SE. Significant differences are indicated as $**P < .01$ and $*P < .05$, compared to the OVA group. NT, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group (1 mg/[kg $\cdot$ day]); RG, red ginseng-fed OVA group (6 mg/[mouse $\cdot$ day]); FRG, fermented red ginseng-fed OVA group (6 mg/[mouse $\cdot$ day]). *COX-2*, cyclooxygenase-2; *iNOS*, Inducible nitric oxide synthase.

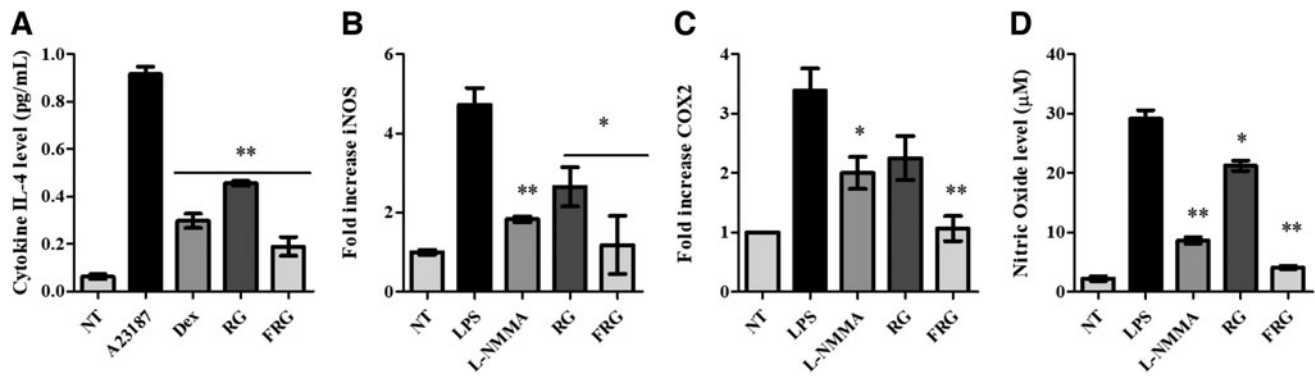


FIG. 5. Effects of red ginseng (RG) and fermented red ginseng (FRG) in LPS-induced RAW264.7 cells or A23187-induced RBL-2H3 cells. **(A)** Reduced cytokine IL-4 levels in RBL-2H3 cells. **(B, C)** Reduction of mRNA levels in LPS-induced RAW264.7 cells. **(D)** Nitric oxide expression level in RAW264.7 cells. Data are shown as the mean ± SE. Significant differences are indicated as ** $P < .01$ and * $P < .05$, compared to the LPS group. NT, normal cell; LPS, LPS-treated group (1 $\mu\text{g/mL}$); PC, positive group treated with A23187 or L-NMMA; LPS, liposaccharides.

fermented by a probiotic mixture, in a mouse model of rhinitis or in the lungs have not been sufficiently studied till date; therefore, in this study, we fermented RG with *L. paracasei* HY7016 and *B. longum* HY 8004.

Rhinitis, an allergic disorder, is a serious public health concern across all age-groups worldwide.²⁷ It is an inflammatory disease that occurs in the nose or nasal cavity due to an immoderate immune reaction to protein allergens.²⁸ Patients with rhinitis are estimated to account for ~10% of the world's population.^{2,29} Rhinitis is triggered in the nasal epithelial membrane due to allergen-protein interacting with IgE.³⁰ Although not a critical disease, its symptoms (including itching and sneezing) can affect the quality of life and overall health.³¹ Symptoms resulting from allergic and inflammatory reactions usually arise from eosinophils, basophils, and mast cells.^{8,32} Among the mediators, the expression of IL-4, IL-5, IL-6, and IgE is usually enhanced while that of IL-2, IL-10, and interferon gamma may be reduced.³³

In this study, we found mice with OVA-induced allergy show symptoms of allergic rhinitis and pathological changes, such as scratching and sneezing. Sensitization by OVA is associated with high levels of IgE and IL4 expression, as reported previously,³³ thus suggesting that the successful induction of OVA-induced allergic rhinitis. However, fermented RG, produced by a mixture of probiotics, including *B. longum* HY8004 and *L. paracasei* HY7016, could inhibit IL-4 and IgE secretion in blood, BALF, and nasal fluid in OVA-challenged mice. Sensitization by OVA increased eosinophil and basophil populations, whereas administration of FRG suppressed the same in both blood and BALF. Figure 3 depicts the induction of allergic rhinitis by OVA inhalation and the remarkable thickness of nasal epithelium, compared to that in the normal group. However, both RG- and FRG-treated groups had much reduced respiratory epithelial thickness; in fact, treatment with FRG seemed to have more therapeutic effects than that with RG.

Allergic rhinitis is associated with IgE production, and recent studies on the effects of anti-IgE treatment and IL-4 reduction have confirmed both mediators to play an im-

portant role in therapy.^{34,35} Balza *et al.* reported a connection between IgE expression and serum IgE levels.³⁶ Many studies have shown the initiation of IgE production to depend on the selective Th2-type immune response, and Th2 cells to produce cytokines, such as IL-4, which are required for the development of IgE responses.³³

IgE level was found to be upregulated in patients with allergic rhinitis, and high levels of inflammation mediators, such as iNOS, COX-2, and NO, were found in their blood.^{37,38} OVA inhalation and allergic rhinitis have been found to increase inducible NO synthase and COX-2 expression. Both *iNOS* and COX-2 gene expression levels have been reported to be involved in inflammation, and related to Th2-type immune response.³⁹ We confirmed both *iNOS* and COX-2 mRNA expression in OVA-challenged mouse lung tissue to be elevated; oral RG and FRG administration suppressed the expression of these inflammatory cytokines in the mouse model of allergic rhinitis. In addition, we confirmed the anti-inflammatory effects of FRG *in vitro* by investigating the reduction of IL-4 cytokines and production of NO in *in vitro* assay. Fermented RG suppressed *iNOS* and COX-2 mRNA expression level in LPS-induced RBL-2H3 cells. FRG may also suppress Th2 cytokines and proinflammatory cytokines and maintain the balance between Th1- and Th2-type cytokines.

In this study, we confirmed that FRG is more effective for alleviating inflammation in OVA inhalation-induced allergic rhinitis mouse model than RG. During fermentation, our two bacteria increased the amount of ginsenosides, such as Rg3, Rh1, Rh2, F2, and Rg2. According to previous studies, ginsenosides Rh1 and Rh2 have essential anti-inflammatory effect, and Rg2 is the major active component exhibiting antioxidant activity in neuronal PC12 cells.^{40,41} Minor ginsenosides, produced by fermentation with microorganisms, have potential therapeutic effects on allergic rhinitis. However, the mechanism underlying the activity or action of these ginsenosides is not clear and further studies are needed.

Collectively, our findings suggested probiotic-fermented RG to possibly suppress IL-4- and IgE-mediated allergic

rhinitis response, thereby decreasing the nasal respiratory epithelial thickness and alleviating lung inflammation in mouse models and mast cells.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

FUNDING INFORMATION

No funding was received for this article.

SUPPLEMENTARY MATERIAL

Supplementary Table S1

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Supplement Table 1. Ginsenoside content of RG and FRG.

(mg/g)	RG	FRG
Rg1	0.120±0.008	0.025±0.013
Re	0.489±0.021	0.068±0.016
Rb1	1.791±0.123	0.680±0.086
Rd	0.339±0.012	0.207±0.097
Rg3	0.404±0.045	0.786±0.033
Rh`	0.048±0.009	0.083±0.007
Rg2	0.202±0.054	0.310±0.048
F2	0	0.107±0.008
Rf	0.104±0.034	0.091±0.008
Rh2	0.860±0.071	1.927±0.108