

Dietary Supplementation with a Probiotic Fermented Four-Herb Combination Enhances Immune Activity in Broiler Chicks and Increases Survivability against *Salmonella Gallinarum* in Experimentally Infected Broiler Chicks

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ABSTRACT. Herbs including *Curcuma longa*, *Houttuynia cordata*, *Prunus mume* and *Rubus coreanus* have potential immune enhancing and antimicrobial effects. Probiotics also have antibacterial effects, and some are important in regulating the immune system. The aims of the present study were to evaluate the immune enhancing effects of a probiotic fermented four-herb combination (PFH) in broiler chicks and to demonstrate the prophylactic effect of PFH against *Salmonella Gallinarum* in experimentally infected broiler chicks as an initial step towards the development of feed supplements for promotion of immune activity and disease prevention. Continuous ingestion of PFH markedly increased lysozyme activity in serum and the spleen, peripheral blood mononuclear cell (PBMC) proliferation, the CD4⁺:CD8⁺ T lymphocyte ratio in the spleen and antibody production level in broiler chicks. Conversely, prostaglandin E₂ synthesis in serum and PBMC culture medium was significantly decreased in the PFH-fed chicks compared with the control group in a dose-dependent manner. In the chicks experimentally infected with *S. Gallinarum*, mortality was delayed in the 2% PFH-fed chicks. Moreover, the survival rates in the 2% PFH-fed group remained the highest among all the trial groups throughout the experimental period. Taken together, these findings suggest that PFH enhances immune activity in broiler chicks and increases survivability against *Salmonella Gallinarum* in experimentally infected broiler chicks, likely because of potent stimulation of nonspecific immune responses.

KEY WORDS: herb, immune enhancement, probiotics, *Salmonella Gallinarum*.

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Veterinary drugs, especially antibiotics, are used as feed supplements to improve physical performance by suppression of subclinical disease challenge in industrial animals [7]. However, there is worldwide concern about overuse of antibiotics in regard to such things as the development and spread of antibiotic resistant strains of bacteria and resistance genes from animals to humans through consumption of food products of animal origin that contain antibiotic residues [18]. Hence, there is an urgent need to identify eco-friendly feed supplements to reduce antibiotic use. One of the most promising methods of reducing antibiotics in industrial animals is strengthening the defense mechanisms of animals through prophylactic administration of natural immunostimulants including probiotics, clay minerals and herbs [15, 17, 24]. Immunostimulants increase resistance to infectious diseases by enhancing nonspecific immunity [12].

Herbs including Ulgum (*Curcuma longa*), Yagmomil (*Houttuynia cordata*), Maesil (*Prunus mume*) and Bokbunja (*Rubus coreanus*) have long been popular as a traditional drug and health food, especially in Korea. It has recently been reported that these herbs have potential immune enhancing and antimicrobial effects. Dietary administration of Ulgum increases the total white blood cell count, circulating antibody titer as well as phagocytic activity of macrophages in mice [2]. Water extract of Yagmomil induces nitric

oxide and cytokine production by lipopolysaccharide (LPS)-activated macrophages [19]. Moreover, Maesil has potential antimicrobial effects against a number of pathogens such as *Helicobacter pylori* [29] and human influenza A virus [34]. Bokbunja is active against hepatitis B virus [20].

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host [4]. The bacterial genera most commonly used in probiotic preparations are *Lactobacillus*, *Bifidobacterium*, *Escherichia*, *Enterococcus*, *Bacillus* and *Streptococcus*. Some fungal strains belonging to the genus *Saccharomyces* have also been used [11]. Probiotics have an important role in regulating the immune system. *Lactobacillus* strains induce maturation of dendritic cells [6], and *Lactobacillus* and *Streptococci* stimulate mononuclear cells to promote the production of interferon (IFN)- γ , interleukin (IL)-12 and IL-18 [28]. Probiotics such as *Lactobacillus* and *Streptococci* activate the NF-kappa B and STAT signaling pathways in human macrophages [27]. Moreover, they have potential antimicrobial effects against several pathogens including *Listeria monocytogenes* [30], *Salmonella* Typhimurium [8] and *Escherichia coli* [25].

These collective observations suggest that probiotics and herbs including Ulgum, Yagmomil, Maesil and Bokbunja may provide an alternative way to reduce use of antibiotics and simultaneously may avoid the development of antibiotic resistant bacteria through the promotion of immune activity and prevention of diseases.

Fowl typhoid caused by *Salmonella enterica* serovar

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Gallinarum (*S. Gallinarum*) is a septicemic bacterial disease affecting galliform birds of all ages. The disease is characterized by anemia, leukocytosis and hemorrhage [31]. Although this disease has been eradicated from Australia, North America and most European countries, it is still a significant problem in Asia, Africa, Central America and South America [3, 31]. In Korea, outbreaks of fowl typhoid have been reported since 1992, and this disease has become the most serious problem in the poultry industry [23].

In the present study, *Saccharomyces cerevisiae*, *Bacillus subtilis* and *Lactobacillus acidophilus* were used as probiotics in a probiotic fermented four-herb combination (PFH) that included Ulgum, Yagmomil, Maesil and Bokbunja. The aims of the present study were to evaluate the immune enhancing effects of PFH in broiler chicks and to demonstrate the prophylactic effect of this dietary supplement against *S. Gallinarum* in experimentally infected broiler chicks as an initial step towards development of feed supplements for promotion of immune activity and disease prevention, especially in chicks. The effects of dietary PFH on lysozyme activity in serum and the spleen, peripheral blood mononuclear cell (PBMC) proliferation, prostaglandin E₂ (PGE₂) in circulation and in PBMCs, spleen T-cell subpopulations and antibody production in serum of broiler chicks were examined. In addition, survivability against *Salmonella* Gallinarum was monitored in experimentally infected broiler chicks.

MATERIALS AND METHODS

Herbal fermentation: Flesh and juice of Ulgum, Yagmomil, Maesil and Bokbunja were obtained as a by-product during commercial product preparation. The probiotics (*Saccharomyces cerevisiae*, *Bacillus subtilis* and *Lactobacillus acidophilus*) were obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Fermentation was conducted as previously described [16]. Briefly, 15% (w/w) each of the four herbs was crushed and mixed thoroughly with 40% (w/w) non-fat rice bran, and the mixture was fermented with 1×10^7 colony forming units (cfu)/ml of *S. cerevisiae* [Korea Collection for Type Cultures (KCTC) 7928] for 7 days at 25–30°C. The fermented products were further fermented with 1×10^7 cfu/ml each of *B. subtilis* (KCTC 1666) and *L. acidophilus* (KCTC 3155) for 5 days at 45–50°C. After fermentation, the products were air-dried for 20 hr at 20°C. The final product (PFH) containing the soluble herbal ingredients was dark brown in color and possessed a sweet flavor.

Chicks and diets: Three independent studies including the immunological assays, antibody production assay and monitoring of survivability against experimental infection described below were conducted with 20-day-old Ross broiler chicks from a single healthy *Salmonella*-free parent stock (Hanvit Bio, Chonnam, Republic of Korea). Prior to the experiment, the birds destined for use in the survivability experiments were confirmed to be *Salmonella*-free by bacteriological culture of fecal samples obtained by cloacal

swabs [14] and a serum plate agglutination test using *S. Gallinarum* antigen [22]. In each independent study, chicks were randomized into three feeding groups. The control group received a commercial, nutritionally complete antibiotic-free chicken feed (Hanvit Bio). The experiment groups received the same chicken feed supplemented with either 1% (w/w) PFH (1% PFH-fed group) or 2% (w/w) PFH (2% PFH-fed group).

All birds were housed in separate air-controlled rooms and allowed free access to tap water and their particular diet. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (Approval number: CNU IACUC-YB-2010-1).

Immunological assays: All chicks (n=6 in each group) were fed each particular diet for 2 weeks. The birds were subjected to four immunological assays including the assay for determination of lysozyme activity, PBMC proliferation assay, PGE₂ assay and assay for determination of the spleen T lymphocyte subpopulations described below. Body weight was monitored every 2 days.

Determination of lysozyme activity in serum and the spleen: Blood was collected from the wing vein of each chick on day 15. Serum was obtained by centrifugation at $2,000 \times g$ for 10 min at 4°C and used for determination of lysozyme activity [21]. Briefly, a standard dilution series was produced by dissolving crystalline lysozyme (Sigma-Aldrich, St. Louis, MO, U.S.A.) in a phosphate buffer (pH 6.2) to a concentration of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 or 6.0 µg/ml. Twenty microliters of each solution was added to two wells of a 96-well microtiter plate. Two hundred microliters of phosphate buffer was added to one well, and 200 µl of a *Micrococcus lysodeikticus* solution (600 mg/l phosphate buffer; Sigma-Aldrich) was added to the other well. The absorbance was determined at 540 nm after 15, 30, 45 and 60 min of incubation at 41°C. For each concentration, the regression coefficient (*b*) between absorbance and time was calculated. Serum samples were treated in a manner similar to the standard solutions. The activity of lysozyme (lysis of *M. lysodeikticus*) was determined based on the correlation of coefficient *b* and the lysozyme concentration. For determination of lysozyme activity in the spleen, all chicks were sacrificed by cervical dislocation, and the spleen was removed. After the fat and membrane were trimmed, spleen tissue (0.5 g) was homogenized in 5 ml ice-cold phosphate buffered saline (PBS). The homogenate was diluted to make a concentration of 1 mg/ml. The lysozyme activity of the diluted homogenate was determined as described for serum. Each sample was tested in duplicate.

Evaluation of PBMC proliferation: *In vitro* PBMC proliferation response was determined using a previously described method [35] with some modifications. Blood collected into a tube containing ethylenediaminetetraacetic acid was diluted with an equal volume of PBS. The diluted blood mixture was layered over half its volume of Lymphoprep™ (Axis-Shield, Oslo, Norway) and separated by density-gradient centrifugation at $800 \times g$ for 20 min at room temperature to recover PBMCs at the interface. The

PBMCs were washed with PBS three times prior to resuspension in 2 ml RPMI-1640 medium (Lonza, Basel, Switzerland) with 2% (v/v) antibiotic-antimycotics (Invitrogen, Carlsbad, CA, U.S.A.). Live cells were detected by microscopic observation of trypan blue dye exclusion. Cell suspensions were diluted to a final concentration of 1×10^7 cells/ml in RPMI-1640 medium with 2% (v/v) antibiotic-antimycotics. One milliliter of cell suspension and 1 ml RPMI-1640 medium with 2% (v/v) antibiotic-antimycotics and 100 $\mu\text{g}/\text{ml}$ lipopolysaccharide (LPS; Sigma-Aldrich) were added to wells of a 24-well culture plate (Iwaki, Tokyo, Japan). The cultures were set up in triplicate. After 24 hr of incubation in a 5% CO_2 incubator at 41°C , 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium salt (MTT; Sigma-Aldrich) was added to the cell culture to a final concentration of 500 $\mu\text{g}/\text{ml}$. The cells were incubated for a further 4 hr and then 300 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to the culture, which was subsequently vibrated for at least 10 min to fully dissolve the colored material. Each sample was diluted 10-fold with PBS, and 100 μl of each diluted sample was transferred to wells of a 96-well plate (Iwaki). The absorbance of each sample was read using an enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Labsystems, Helsinki, Finland) at an optical density of 540 nm ($\text{OD}_{540\text{nm}}$).

Measurement of PGE_2 in serum and PBMC culture medium: Serum and PBMC culture medium were assayed for PGE_2 synthesis. PBMCs were prepared as described above. Lymphocytes (1×10^7 per well) were cultured in wells of 24-well culture plates (Iwaki). Cells were coincubated with 50 $\mu\text{g}/\text{ml}$ LPS (Sigma-Aldrich) for 24 hr at 41°C in a 5% CO_2 atmosphere. The culture medium was collected, and the supernatant was obtained by centrifugation at $2,000 \times g$ for 10 min at 4°C . The concentrations of PGE_2 in the PBMC culture medium and serum were measured using a ParameterTM Prostaglandin E_2 Immunoassay Kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions.

Determination of T lymphocyte subpopulations in the spleen: The spleen was obtained from each chick, and single-cell suspensions were prepared by pushing the tissue through a 40- μm nylon mesh (BD Biosciences, Franklin Lakes, NJ, U.S.A.). Isolated cells were analyzed to determine the component ratio of T helper cells ($\text{CD3}^+\text{CD4}^+$) and T cytotoxic cells ($\text{CD3}^+\text{CD8}^+$) as previously described [9] with some modifications. To determine T helper cells, the cells were stained with both fluorescein isothiocyanate (FITC)-conjugated mouse anti-chicken CD3 (Southern Biotech, Birmingham, AL, U.S.A.) and phycoerythrin (PE)-conjugated mouse anti-chicken CD4 (Southern Biotech). To determine T cytotoxic cells, the cells were stained with both FITC-conjugated mouse anti-chicken CD3 (Southern Biotech) and PE-conjugated mouse anti-chicken CD8 (Southern Biotech). After incubation at room temperature for 30 min in the dark, the cells were washed twice with PBS, and the lymphocyte subpopulations were analyzed using a FACSort flow cytometer (BD Biosciences). Viable

lymphocytes were gated by forward and side-scatter characteristics (FSC/SSC), and 10,000 events were analyzed for positive staining with both FITC and PE. Results for each lymphocyte subpopulation were expressed as percentages of events in the FSC/SSC lymphocyte gate.

Measurement of antibody production level in serum after antigen inoculation regimen: All chicks ($n=6$ in each group) were acclimatized to their particular diet for a week before the first inoculation with formalin-killed *Pasteurella multocida* type A (National Veterinary Research & Quarantine Service, Gyeonggi, Republic of Korea). Each chick received two subcutaneous injections (1 ml each time) with a 2-week interval between injections. The first inoculation was given with Freund's complete adjuvant (Sigma-Aldrich) on day 7, and the second was given with Freund's incomplete adjuvant (Sigma-Aldrich) on day 21. Two blood samples were collected individually into microcentrifuge tubes from the wing vein, one before immunization and one on day 28. Serum was obtained by centrifugation at $2,000 \times g$ for 10 min at 4°C , and the separated serum was inactivated at 56°C for 30 min. Antibody production level was measured by ELISA as previously described [17] with some modifications. Briefly, 96-well plates (Iwaki) were coated with 100 μl of a solution containing 20 μg whole formalin-killed *P. multocida* type A (used as antigen) in 1 ml of 0.1 M carbonate-bicarbonate buffer (pH 9.6) and left overnight at 4°C . After three washes with PBS containing 0.05% Tween 20 (PBS-T), the wells were saturated with 200 μl of 5% skim milk (BD Biosciences). After incubation for 2 hr at room temperature, the wells were washed three times with PBS-T. Inactivated serum samples were diluted 1:160 with PBS-T. The diluted serum samples were added to wells and incubated at room temperature for 1 hr. After three washes with PBS-T, 100 μl of a 1:5,000 dilution of horseradish peroxidase-conjugated rabbit anti-chicken IgY (Jackson ImmunoResearch, West Grove, PA, U.S.A.) was added to each well. After incubation at room temperature for 1 hr, the plates were washed, and 100 μl of substrate consisting of 0.05 M citrate buffer (pH 4.0), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Bio Basic, Markham, ON, Canada) and 30% hydrogen peroxide was added to each well. After incubation for 10 min at room temperature in the dark, the reaction was stopped with 5% sodium dodecyl sulfate (AppliChem, Darmstadt, Germany). Reactions were read at an $\text{OD}_{405\text{nm}}$ using an ELISA plate reader (Thermo Labsystems). Pre-immunized chicks sera were used as negative control, and each sample was tested in duplicate.

Experimental *S. Gallinarum* infection and survivability in broiler chicks: All chicks ($n=20$ in each group) were acclimatized to their particular diet for 2 weeks before experimental bacterial infection. The *Salmonella Gallinarum* (SG3001) used in the present study was originally isolated from a chick with naturally occurring fowl typhoid (National Veterinary Research & Quarantine Service). *S. Gallinarum* was prepared using a previously described method [32] with some modifications. Briefly, the original

stock of *S. Gallinarum* was cultured overnight on MacConkey agar (BD Biosciences). A single colony was selected and inoculated on Luria-Bertani broth (BD Biosciences) in a universal bottle and incubated at 250 rpm for 16 hr at 37°C. Viable counts of the broth culture were made using the pour plate method. Inoculated broth was diluted with PBS to obtain an infective dose of 1×10^{10} cfu/ml (the optimal dose as determined in preliminary experiments). Three milliliters of the diluted bacteria was used to orally inoculate each chick. The chicks were observed for 17 days after the bacterial challenge, and their survivability was recorded. Fecal samples from cloacal swabs were collected at 1, 5, 10 and 15 days post-infection (DPI). The liver, spleen and cecum were also collected from all remaining chicks at the end of the experiment. The viable bacteria count was determined in fecal samples and each tissue as previously described [32].

Statistical analysis: The data are expressed as means \pm standard deviation (SD). A paired Student's *t*-test was performed for statistical analysis of the data. All statistical analyses of data were performed using the SPSS version 17.0 software (SPSS, Chicago, IL, U.S.A.). $P < 0.05$ was considered to be the level of significance.

RESULTS

Body weight changes: No significant differences in body weight change were found among the groups for 2 weeks (data not shown).

Lysozyme activity in serum and the spleen: The serum lysozyme concentration in the 2% PFH-fed group ($3.04 \pm 1.57 \mu\text{g/ml}$) was significantly higher than in the control group ($0.79 \pm 0.60 \mu\text{g/ml}$) ($P < 0.05$). Moreover, the serum lysozyme concentration in the 1% PFH-fed group ($2.43 \pm 1.86 \mu\text{g/ml}$) also showed a tendency to increase compared with the control group, although the difference was not significant ($P = 0.07$; Fig. 1a). The spleen lysozyme concentration in the 2% PFH-fed group ($3.95 \pm 0.90 \mu\text{g/mg tissue}$) was significantly higher than in the control group ($2.24 \pm 0.31 \mu\text{g/mg tissue}$; $P < 0.01$) and the 1% PFH-fed group ($2.40 \pm 0.42 \mu\text{g/mg tissue}$; $P < 0.05$). However, no significant difference in the spleen lysozyme concentration was evident between the control group and the 1% PFH-fed group (Fig. 1b).

PBMC proliferation: PBMCs were coincubated with mitogen (LPS), and PBMC proliferation was determined ($\text{OD}_{540\text{nm}}$) after incubation with MTT. The PBMC proliferation of the 2% PFH-fed group (0.526 ± 0.08) was significantly enhanced compared with the control group (0.379 ± 0.05 ; $P < 0.05$). Moreover, the PBMC proliferation of the

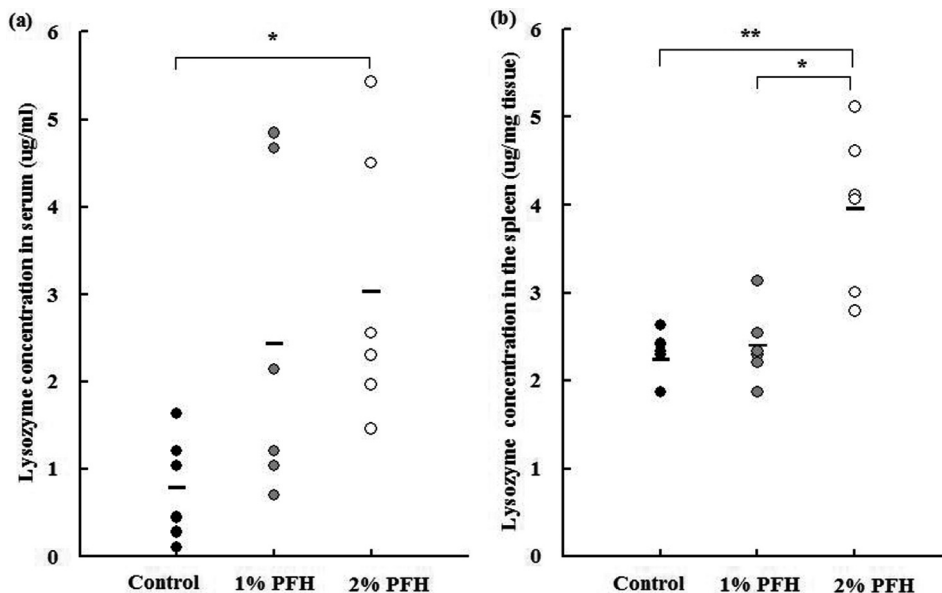


Fig. 1. Effect of PFH on lysozyme activity in serum and the spleen in broiler chicks. Chicks were fed with each particular diet for 2 weeks and then sacrificed for collection of blood and the spleen. (a) The serum lysozyme concentration of the 2% PFH-fed group was significantly higher than that of the control group ($*P < 0.05$). Moreover, the serum lysozyme concentration of the 1% PFH-fed group also showed a tendency to increase compared with the control group, although the differences were not significant ($P = 0.07$). (b) The spleen lysozyme concentration of the 2% PFH-fed group was significantly higher than those of the control group ($**P < 0.01$) and the 1% PFH-fed group ($*P < 0.05$), although no significant difference in the spleen lysozyme concentration was observed between the control group and the 1% PFH-fed group. Each point represents and individual lysozyme concentration ($\mu\text{g/ml}$ or $\mu\text{g/mg tissue}$), and each line represents the mean value of six chicks in each group.

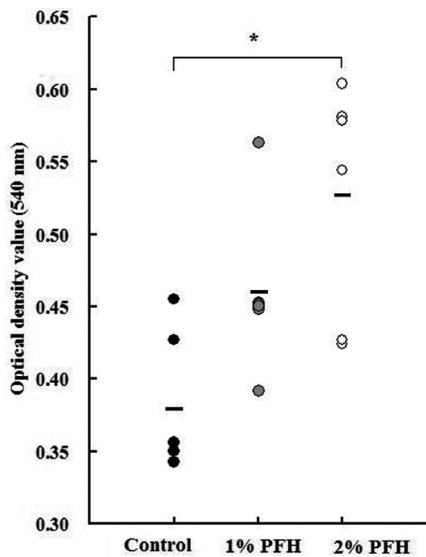


Fig. 2. Effect of PFH on PBMC proliferation in broiler chicks. PBMCs were coincubated with mitogen (LPS), and PBMC proliferation was determined as an OD_{540nm} value of colored material after incubation with MTT. PBMC proliferation of the 2% PFH-fed group was significantly enhanced compared with that of the control group (* $P < 0.05$). Moreover, PBMC proliferation of the 1% PFH-fed group also showed a slightly enhanced tendency compared with the control group, although the difference was not significant ($P = 0.09$). Each point represents an individual OD_{540nm}, and each line represents the mean value of six chicks in each group.

1% PFH-fed group (0.460 ± 0.06) also showed a slightly enhanced tendency compared with the control group, although the difference was not significant ($P = 0.09$; Fig. 2).

PGE₂ synthesis in serum and PBMC culture medium: Systemic PGE₂ synthesis in the PFH-fed groups (1% and 2% PFH: 231.98 ± 7.22 pg/ml and 209 ± 7.33 pg/ml, respectively) was significantly decreased compared with the control group (247.37 ± 13.47 pg/ml) in a dose-dependent manner ($P < 0.05$, control vs. 1% PFH-fed group; $P < 0.01$, 1% PFH-fed group vs. 2% PFH-fed group; $P < 0.001$, control vs. 2% PFH-fed group; Fig. 3a). Moreover, peripheral blood lymphocytic PGE₂ synthesis in the PFH-fed groups (1% and 2% PFH: 145.80 ± 11.89 pg/ml and 110.90 ± 8.36 pg/ml, respectively) was also significantly decreased compared with the control group (166.19 ± 16.16 pg/ml) in a dose-dependent manner ($P < 0.01$, control vs. 1% PFH-fed group; $P < 0.001$, 1% PFH-fed group vs. 2% PFH-fed group; $P < 0.001$, control vs. 2% PFH-fed group; Fig. 3b).

Subpopulations of T lymphocytes in the spleen: The percentages of CD3⁺CD4⁺ T lymphocytes in the spleens of the PFH-fed groups (1% and 2% PFH: 16.10 ± 1.00 and $17.80 \pm$

1.57% , respectively) were significantly increased compared to those of the control group ($14.51 \pm 1.16\%$) in a dose-dependent manner ($P < 0.01$, control vs. 1% PFH-fed group; $P < 0.01$, 1% PFH-fed group vs. 2% PFH-fed group; $P < 0.001$, control vs. 2% PFH-fed group). Conversely, the percentages of CD3⁺CD8⁺ T lymphocytes in the spleens of the PFH-fed groups (1% and 2% PFH: 22.79 ± 4.99 and $22.15 \pm 2.66\%$, respectively) were significantly decreased compared with those of the control group ($26.13 \pm 3.66\%$; $P < 0.01$, control vs. 1% PFH-fed group; $P < 0.01$, control vs. 2% PFH-fed group). However, no significant difference in spleen CD3⁺CD8⁺ T lymphocytes was observed between the 1% and 2% PFH-fed groups (Fig. 4a). The ratios of CD4⁺:CD8⁺ cells in the spleens of the PFH-fed groups (1% PFH: 0.73 ± 0.14 ; 2% PFH: 0.81 ± 0.05) were significantly increased compared with that of the control group (0.56 ± 0.06 ; $P < 0.01$, control vs. 1% PFH-fed group; $P < 0.001$, control vs. 2% PFH-fed group). However, no significant difference in the ratio of CD4⁺:CD8⁺ splenocytes was observed between the 1% and 2% PFH-fed groups (Fig. 4b).

Antibody production level in serum following antigen administration: The antibody production level (determined as OD_{405nm}) of the 2% PFH-fed group (2.40 ± 0.14) was significantly increased compared with the control group (1.71 ± 0.21 ; $P < 0.01$). Moreover, the antibody production level of the 1% PFH-fed group (1.97 ± 0.16) also showed a tendency to increase compared with the control group, although the difference was not significant ($P = 0.06$; Fig. 5). Sera of pre-immunized chicks displayed a clear negative reaction (Control, 1% PFH-fed and 2% PFH-fed groups: 0.36 ± 0.03 , 0.33 ± 0.13 , 0.34 ± 0.08 , respectively; data not shown in the figure) against the *Pasteurella multocida* type A antigen.

Survival rates in chicks experimentally infected with *Salmonella Gallinarum*: Survival rate trends for all the trial groups are shown in Fig. 6. Mortality was first observed at 5-days post-infection (DPI) in the control group and 1% PFH-fed group, whereas mortality in the 2% PFH-fed group was delayed for 2 days compared with the other groups. The survival rates in the 2% PFH-fed group remained the highest among all the trial groups throughout the experimental period. By 17 DPI, the survival rates were 15% (3 of 20 chicks) in the control group, 35% (7/20) in the 1% PFH-fed group and 45% (9/20) in the 2% PFH-fed group. The differences in the final survival rates were significant between the control group and the PFH-fed groups ($P < 0.05$). However, no statistical difference in the final survival rates was observed between the 1 and 2% PFH-fed groups. The number of the viable bacteria in the fecal samples was not significantly different among the groups during the overall period, and the level was also not significantly different in tissues among the groups at the end of experiment (data not shown).

DISCUSSION

Lysozyme, which is secreted by some phagocytes such as macrophages and polymorphonuclear leukocytes, is highly

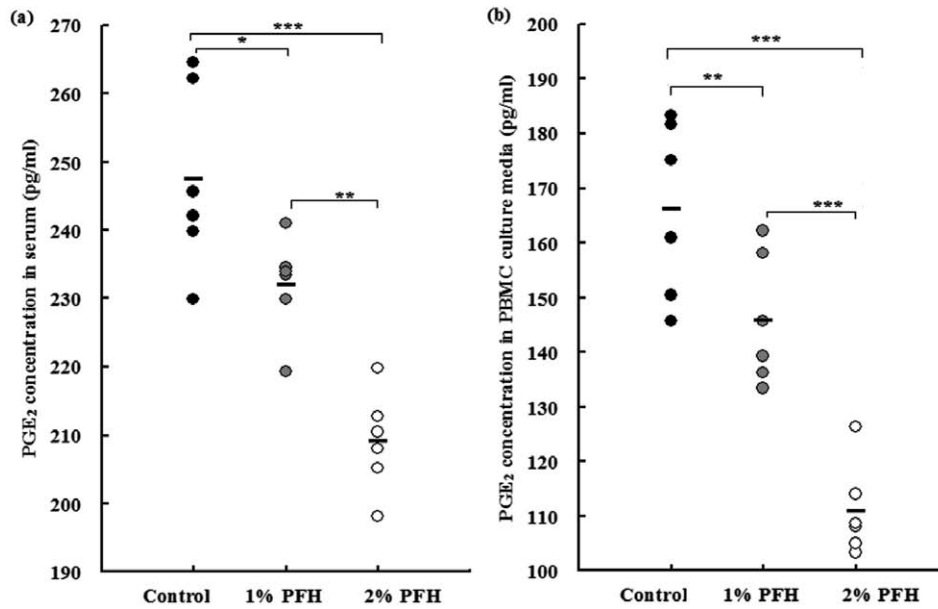


Fig. 3. Effect of PFH on PGE₂ synthesis in serum and PBMCs culture medium in broiler chicks. (a) The concentrations of PGE₂ in serum of the PFH-fed groups were significantly decreased in a dose-dependent manner compared with the control group (* $P < 0.05$, control vs. 1% PFH-fed group; ** $P < 0.01$, 1% PFH-fed group vs. 2% PFH-fed group; *** $P < 0.001$, control vs. 2% PFH-fed group). (b) The concentrations of PGE₂ in PBMC culture medium of the PFH-fed groups were also significantly decreased in a dose-dependent manner compared with the control group (** $P < 0.01$, control vs. 1% PFH-fed group; *** $P < 0.001$, 1% PFH-fed group vs. 2% PFH-fed group; *** $P < 0.001$, control vs. 2% PFH-fed group). Each point represents an individual PGE₂ concentration (pg/ml), and each line represents the mean value of six chicks in each group.

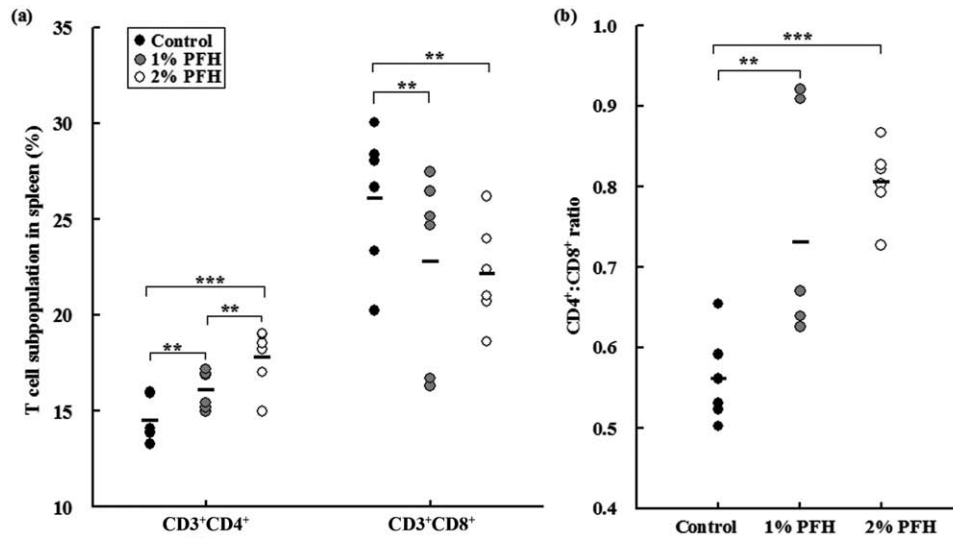


Fig. 4. Effect of PFH on the spleen T lymphocyte subpopulations in broiler chicks. (a) The percentages of spleen CD3⁺CD4⁺ T lymphocytes in the PFH-fed groups were significantly increased in a dose-dependent manner compared with that those of the control group (** $P < 0.01$, control vs. 1% PFH-fed group; ** $P < 0.01$, 1% PFH-fed group vs. 2% PFH-fed group; *** $P < 0.001$, control vs. 2% PFH-fed group). Conversely, the percentages of CD3⁺CD8⁺ T lymphocyte in the spleens of the PFH-fed groups were significantly decreased compared with that of the control group (** $P < 0.01$, control vs. 1% PFH-fed group; ** $P < 0.01$, control vs. 2% PFH-fed group). However, no significant difference in spleen CD3⁺CD8⁺ T lymphocytes was observed between the 1% and 2% PFH-fed groups. (b) The ratios of spleen CD4⁺:CD8⁺ cells in the PFH-fed groups was significantly increased compared with that of the control group (** $P < 0.01$, control vs. 1% PFH-fed group; *** $P < 0.001$, control vs. 2% PFH-fed group). However, no significant difference in the ratio of CD4⁺:CD8⁺ splenocytes was observed between the 1% and 2% PFH-fed groups. Each point represents individual data, and each line represents the mean value of six chicks in each group.

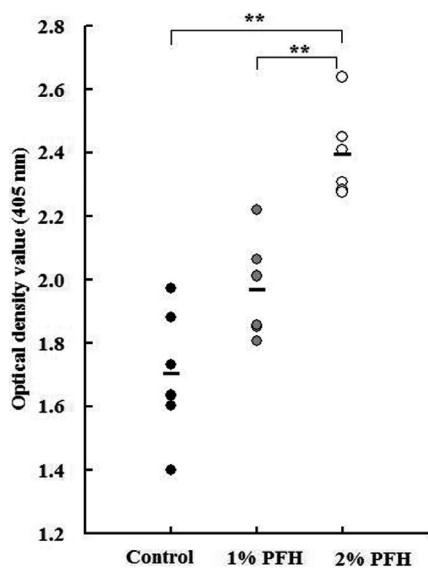


Fig. 5. Effect of PFH on antibody production level in the serum of broiler chicks. Chicks received two subcutaneous injections of formalin-killed *P. multocida* type A. The serum antibody production level was determined (OD_{405nm}) using an ELISA plate reader. The serum antibody production level of the 2% PFH-fed group was significantly increased compared with that of the control group (** $P < 0.01$). Moreover, the antibody production level of the 1% PFH-fed group also showed a tendency to increase compared with the control group, although the difference was not significant ($P = 0.06$). Each point represents an individual OD value, and each line represents the mean value of six chicks in each group.

active against Gram-positive bacteria. After the internalization of antigens, destruction is likely accomplished by the lysosomal reservoir of hydrolytic enzymes and by a respiratory burst (i.e., O_2^- and H_2O_2) [26]. Hydrolytic enzymes such as lysozyme and acid phosphatase can destroy glucosidic bonds in the cell walls of *Escherichia coli* and *Staphylococcus* as a result of their phagocytic activity [10]. The phagocyte is an important kind of accessory cell in the immune response and antigen presentation. In the present study, the lysozyme activity of the 2% PFH-fed group was significantly higher than those of the control group in serum and the spleen. Moreover, the serum lysozyme activity of the 1% PFH-fed group also showed a tendency to increase compared with the control group. These results suggest that continuous ingestion of PFH can stimulate activation or antigen presentation in a phagocyte, thus enhancing the immune function of the body. This is consistent with a previous report demonstrating that dietary administration of Ulgum can increase the phagocytic activity of macrophages in mice [2] and supports previous reports that Maesil [29, 34], Bokbunja [20] and some probiotics [8, 25, 30] have potential antimicrobial effects against a number of pathogens.

LPS is a constituent of the outer membrane of Gram-negative bacteria. The molecule acts as an endotoxin and elicits

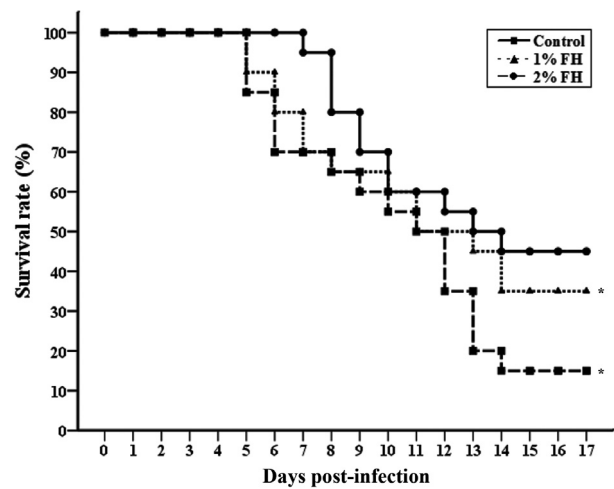


Fig. 6. Prophylactic effect of PFH against *Salmonella Gallinarum* in experimentally infected broiler chicks. Mortality was first observed at 5 DPI in the control group and 1% PFH-fed group. The first indication of mortality in the 2% PFH-fed group was delayed for 2 days compared with the other groups. The survival rates in the 2% PFH-fed group remained the highest among all the trial groups throughout the experimental period. By 17 DPI, the survival rates were 15% (3 survivors among 20 chicks) in the control group, 35% (7/20) in the 1% PFH-fed group and 45% (9/20) in the 2% PFH feeding group. The differences in the final survival rates were significant between the control group and the PFH-fed groups (* $P < 0.05$). However, no statistical difference in the final survival rates was observed between the 1% and 2% PFH-fed groups.

a potent immune response in animals. In the present study, PFH enhanced LPS-induced lymphocyte proliferation in broiler chicks. This indicates that the mitogenicity of lymphocytes is enhanced by ingestion of PFH in broiler chicks, especially against Gram-negative bacteria. This finding is consistent with a previous report demonstrating that water extracts of Yagmomil induce nitric oxide and cytokine production by LPS-activated macrophages [19].

PGE_2 , one of the most important eicosanoids, is an endogenous inhibitor of the immune response. PGE_2 suppresses T-helper 1 cell differentiation and B cell functions [13]. In the present study, the PGE_2 synthesis in serum and PBMC culture medium of the PFH-fed groups was significantly decreased in a dose-dependent manner compared with the control group. The reduction of PGE_2 was consistent with the increment of the antibody production level and enhancement of PBMC proliferation.

The $CD4^+ : CD8^+$ ratio is used as a measure of immune function and response. Low ratios are usually observed in individuals with acute viral diseases and hemophilia [5], whereas high ratios have been associated with an increase in the immunofunctional ability of chicks [1, 9]. In the present study, the percentages of spleen $CD3^+ CD4^+$ T lymphocytes in the PFH-fed groups were significantly increased in a dose-dependent manner compared with the control group. Conversely, the percentages of spleen $CD3^+ CD8^+$ T lymphocytes

phocytes in the PFH-fed groups were significantly decreased compared with that of the control group. Therefore, the ratios of spleen CD4⁺:CD8⁺ cells in the PFH-fed groups were significantly increased compared to that of the control group. These results imply that PFH confers a benefit on the immune function in broiler chicks and may enhance the resistance of broiler chicks to infectious diseases.

Antibody titer is the indicator of humoral immunity [33]. Accordingly, the effects of PFH on humoral immunity in boiler chicks were measured in the present study by the antibody production level in serum of broiler chicks after an antigen administration regimen. Antibody production significantly increased in the 2% PFH-fed group compared with the control group, although no significant difference was observed between the control group and the 1% PFH-fed group. This indicates that humoral immunity is also enhanced by ingestion of PFH in broiler chicks.

The beneficial effects of PFH on immune activity in broiler chicks led us to evaluate the prophylactic effect of PFH against *Salmonella* Gallinarum, which is the most serious problem in the Korean poultry industry [23], in experimentally infected chicks. Mortality was first observed at 5 DPI in the control group and the 1% PFH-fed group, whereas that in the 2% PFH-fed group was delayed for 2 days compared with the other groups. Moreover, the survival rates in the 2% PFH-fed group remained the highest among all the trial groups throughout the experimental period. These results indicate that ingestion of PFH prolongs survival and increases survival rates in broiler chicks experimentally infected with *Salmonella* Gallinarum, perhaps as a consequence of a general immune enhancing effect.

Taken together, these findings suggest that some component(s) of PFH enhances immune activity in broiler chicks and increases survivability against *Salmonella* Gallinarum in experimentally infected broiler chicks, probably because of a potent stimulation of nonspecific immune responses. Hence, PFH may be a good candidate as an alternative feed supplement to reduce use of antibiotics through promotion of immune activity and prevention of diseases, especially in chicks. Before this potential is realized, much work remains to be done. PFH contains a complex array of compounds; these and the probiotic microorganisms could become modified during the fermentation process. Therefore, precise knowledge of the major component(s) of PFH responsible for its immune enhancing effect is needed. Such studies are currently in progress.

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