ABSTRACT. Maesil (Prunus mume) has long been used as a traditional drug and healthy food in East Asian countries. It possesses a number of beneficial biological activities including potential antimicrobial effects against pathogens. Probiotics also have antibacterial effects. Moreover, some probiotics have an important role in regulating the immune system. The present study evaluated the immune enhancing effects of fermented Msesil with probiotics (Saccharomyces cerevisiae, Bacillus subtilis and Lactobacillus acidophilus) in mice, especially against Bordetella bronchiseptica, as an initial step towards the development of feed supplements for the promotion of immune activity and prevention of disease, especially in pigs. Continuous ingestion of fermented Msesil with probiotics markedly increased the macrophage ratio in peripheral blood and the T lymphocyte ratio in the spleen. In addition, antibody production against formalin-killed B. bronchiseptica significantly increased in the mice fed fermented Maesil compared with the control group. The number of leukocytes was significantly higher in the bronchio-alveolar lavage obtained from the fermented Msesil-fed animals compared to it in the control group at day 3 (maximal peak time) after experimental B. bronchiseptica infection. Moreover, at 7 day post-infection, relative messenger RNA expression levels of tumor necrosis factor-α and interferon-γ were significantly increased in splenocytes of mice fed fermented Msesil compared with those in the control group. Taken together, these findings suggest that feed containing fermented Msesil with probiotics enhances immune activity in mice, especially against B. bronchiseptica, via the potent stimulation of non-specific immune responses.

KEY WORDS: Bordetella bronchiseptica, immune enhancement, Msesil, probiotics.

Veterinary drugs and, especially, antimicrobials have been used as feed supplements in industrial animals to improve the health and physical performance by suppression of subclinical disease challenge [7, 45]. However, there is worldwide concern about overuse of antibiotics including the development and spread of antimicrobial resistant strains of bacteria and resistance genes from animals to humans through consumption of food products of animal origin that contain antimicrobial residues [24]. Multi-resistance against other antibiotics may be due to the promotion of resistance genes located on the same plasmid DNA [15, 17]. For these reasons, antibiotics as animal feed supplements have been banned from European Union beginning January 1, 2006, by Regulation 1831/2003/EEC [11]. This ban of antibiotic feed supplements has focused increasing attention on the development of alternative feed supplements.

Maesil (Latin name: Prunus mume Siebold & Zucc; Japanese name: Ume) is a deciduous tree of the genus Rosaceae that is native to China. The flower, leaf, branch, seed, and root of the plant have long been popular as a traditional drug and healthy food in East Asian countries. In particular, the unripe fruit is used in traditional Korean medical preparations as a remedy for coughs and dyspepsia [19]. It has recently been reported that Maesil possesses a number of beneficial biological activities that include suppression of cancer cell proliferation [21, 37, 38], inhibition of Helicobacter pylori [36], and improvement of blood fluidity [6]. In addition, Kim et al. [25] screened for antioxidant and free radical scavenging activities in methanol aqueous extracts of 100 plants. Maesil may be a potential natural source of free radical scavenger. Of particular relevance to the present study, it has potential antimicrobial effects against pathogens such as H. pylori [39] and human influenza A virus [52].

Since the early 1900s, foods containing certain live bacteria have been believed to be good for health [32]. The term “probiotics” was first used in 1965 to describe substances secreted by one organism that stimulate the growth of another [27]. According to the currently adopted definition by Food and Agriculture Organization of the United Nations/World Health Organization, probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [12]. Various bacterial genera most commonly used in probiotic preparations are Lactobacillus, Bifidobacterium, Escherichia, Enterococcus, Bacillus and Streptococcus. Some fungal strains belonging to Saccharomyces have also been used [14]. Reported health benefits upon consumption of probiotics include alleviation of diarrhea [8, 10, 31, 49], reduc-
tion of *H. pylori* colonization [1, 48], and improvement of inflammatory bowel disease [30, 35]. In addition, probiotics prevent or delay development of tumor in the intestine and urinary bladder [2, 41]. Of particular relevance to the present study, probiotics have antibacterial effects against several pathogens including *Listeria monocytogenes* [40], *Salmonella* typhimurium [9] and *E. coli* [29]. Moreover, some probiotics have an important role in regulating the immune system [50]. Previous studies have reported that *Lactobacillus* strains induce maturation of dendritic cells [5], and *Lactobacillus* and *Streptococcus* stimulate mononuclear cells to promote the production of interferon (IFN)-γ, interleukin (IL)-12, and IL-18 [34]. Miettinen *et al.* [33] also reported that probiotics such as *Lactobacillus* and *Streptococcus* activate NF-kappa B and STAT signaling pathways in human macrophages.

These collective observations suggest that Maesil and probiotics may possess a number of beneficial biological activities. Consistent with this, we previously demonstrated that fermented Maesil with probiotics (*S. cerevisiae*, *B. subtilis* and *L. acidophilus*) (hence, this supplement was designated the FM) beneficially affects atopic dermatitis-like skin lesions in mice [22]. Of particular relevance to the present study, FM 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.

*Bordetella bronchiseptica* is a small (0.2–0.5 μm × 1.5 μm), gram-negative, coccobacillus that is important as a cause of disease in the respiratory tract of dogs, pigs, laboratory rodents, and a variety of wild mammalian species [16]. Especially, it is a primary etiological agent or a predisposing factor that results in atrophic rhinitis [18], pneumonia [43], and porcine reproductive and respiratory disease complex [4] in swine. *B. bronchiseptica*-related infections have exacted millions of dollars from the swine industries of many countries due to infection-related mortality, sacrificed pigs, use of medicated feed, veterinary fees, sales losses from deformed pigs, daily weight gain loss, and poor feed conversion [13].

The present study evaluated the immune enhancing effects of FM in mice, especially against *B. bronchiseptica*, as an initial step towards the development of feed supplements for the promotion of immune activity and prevention of disease, especially in pigs. The evaluations encompassed several immunological criteria including differential ratio of peripheral leukocytes, subpopulation of splenocytes, and serum antibody production level in mice fed with or without FM. After experimental *B. bronchiseptica* infection, the number of leukocytes in broncho-alveolar lavage (BAL) was counted and relative mRNA expression levels of tumor necrosis factor (TNF)-α and IFN-γ in splenocytes were determined.

**MATERIALS AND METHODS**

**Maesil fermentation:** Maesil flesh and juice were obtained as a by-product during commercial beverage preparation. The probiotics were obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Fermentation was conducted as previously described [22]. Briefly, fruits were crushed, mixed thoroughly with non-fat rice bran, and the mixture was fermented with 1 × 10⁷ colony forming units (cfu)/ml *Saccharomyces cerevisiae* (Korea Collection for Type Cultures (KCTC) 7928) for 7 days at 25°C–30°C. The fermented products were further fermented with 1 × 10⁷ cfu/ml each of *Bacillus subtilis* (KCTC 1666) and *Lactobacillus acidophilus* (KCTC 3155) for 5 days at 45°C–50°C. After fermentation, the products were air-dried for 20 hr at 20°C. The final product containing the soluble Maesil ingredients was dark brown in colour and possessed a sweet flavour.

**Animals and diets:** Specific pathogen-free female 6-week-old ICR mice were purchased from Daehan Biolink (Daejeon, Korea). For each study, the mice were randomized into 2 groups. The control group received a commercial, nutritionally complete, extruded dry rodent feed (Superfeed, Gangwon, Korea). The experiment group received the same extruded dry rodent feed supplemented with 2% (w/w) FM (hence, the group was designated the FM-fed group). All mice were housed in an air-conditioned room, kept in polypropylene cages and allowed free access to the particular diet and tap water from drinking bottles with stainless steel sipper tubes. All animal procedures were conducted in accordance with the guidelines of the local ethical committee (Chonnam National University).

**Differential ratios of leukocytes in peripheral blood:** All mice (n=5 in each group) were fed with each particular diet for 4 weeks and then were sacrificed for collection of blood and spleen. Blood was collected in tubes coated with ethylene diaminetetraacetic acid from the retro-orbital plexus of each mouse. Blood leukocytes and differential counts were determined using an automated veterinary haematology analyzer (Oxford Science, Oxford, CT, U.S.A.) configured for mouse blood. Each sample was independently counted three times.

**Determination of lymphocyte subpopulation in spleen:** Spleen was obtained from each aforementioned mouse. Splenocytes were isolated under sterile conditions. Isolated cells were analyzed to determine of *T* (CD3⁺/CD19⁻), *B* (CD3⁻/CD19⁺), T-helper (CD4⁺/CD8⁻), T-cytotoxic cell (CD4⁺/CD8⁺) component ratio as previously described [26]. To determine the ratio of *T* and *B* cell component, the cells were stained with both phycoerythrin (PE)-conjugated anti-CD3 (BD Biosciences, Franklin Lakes, NJ, U.S.A.) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD19 (BD Biosciences). To determine the ratios of T-helper and T-cytotoxic cell component, the cells were stained with both FITC-conjugated anti-mouse CD4 (BD Biosciences) and PE-conjugated anti-mouse CD8 (BD Biosciences). After incubation at 4°C for 15 min in the dark, the cells were washed twice with phosphate-buffered saline (PBS) and the lymphocyte subpopulation was 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 FITC or PE. Results for each lymphocyte subpopulation were expressed as percentages of events in the FSC/SSC lymphocyte gate.

Measurement of antibody production level against formalin-killed B. bronchiseptica: B. bronchiseptica was obtained from the National Veterinary Research & Quarantine Service (Gyeonggi, Korea). Immunization process was conducted as previously described [23]. All mice (n = 5 in each group) were acclimatized to the particular diet for a week before the 1st inoculation with 1 × 10⁹ cfu/ml formalin-killed B. bronchiseptica. Each mouse received 2 intraperitoneal injections (0.3 ml each time) with a 2 week interval between injections. The 1 inoculation was given with Freund’s complete adjuvant (Sigma-Aldrich, St. Louis, MO, U.S.A.) on day 7 and the second with Freund’s incomplete adjuvant (Sigma-Aldrich) on day 21. Blood samples were individually collected in microcentrifuge tubes from the retro-orbital plexus on day 28. Serum was obtained by centrifugation and separated serum was inactivated at 56°C for 30 min. Antibody production level was measured by enzyme-linked immunosorbent assay (ELISA) as previously described [23]. Briefly, 96-well plates (Iwaki, Tokyo, Japan) were coated with 100 µl of a solution containing 20 µg whole formalin-killed B. bronchiseptica (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, wells were washed three times with PBS-T. Inactivated serum samples were diluted 1:160 with PBS-T (this ratio was optimized previously) [23]. The diluted serum samples were added to the wells and incubated at room temperature for 2 hr. After three washes with PBS-T, 100 µl of a solution of a 1:5,000 dilution of horseradish peroxidase-conjugated goat antimouse IgG (Sigma-Aldrich) 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-ethyl benzthiazoline-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 absorbance of 405 nm using an ELISA plate reader (Thermo Labsystems, Helsinki, Finland). Non-immunized mice sera were used as negative control (n = 4), and each sample was tested in duplicate.

Enumeration of the number of leukocytes in BAL derived from experimentally B. bronchiseptica infected mice: All mice (n = 12 in each group) were acclimatized to the particular diet for 4 weeks before experimental B. bronchiseptica infection. Ten microliters of the B. bronchiseptica culture (1 × 10⁹ cfu/ml, dose optimized previously) was inoculated intranasally in each nostril of each mouse. At day of 1, 3, 5 and 7 post-infection, 3 mice from each group were sacrificed for collection of BAL as previously described [26]. Briefly, the BAL samples were collected by inserting a suitable cannula towards the lungs into the exposed trachea. A 2 ml syringe was attached to the cannula and the lungs were washed with 1 ml of PBS by flushing the solution in and out of the lungs several times. The wash solutions were collected into a suitable tube. The BAL samples were then washed twice with PBS. The number of leukocytes from each mouse was counted using a hemocytometer.

Evaluation of relative mRNA expression levels of TNF-α and IFN-γ in spleen of experimentally B. bronchiseptica infected mice: At day 7 after experimental infection, spleen was obtained from 3 mice of each group as described above. Splenocytes were isolated under sterile conditions. Total RNA extraction was performed using a RNeasy Mini kit (Qiagen, Valencia, CA, U.S.A.) and target RNA was reverse transcribed using Superscript II reverse transcriptase enzyme (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. To minimize variations in reverse transcriptase efficiency, all samples were transcribed simultaneously. Primers and probes for murine TNF-α, IFN-γ, and β-actin were designed as previously described [42]. Their sequences are shown in Table 1. The probes were dual-labelled with the reporter dye 6-carboxyfluorescein (FAM) at the 5’ end and the quencher dye 6-carboxytetr methylrhodamine (TAMRA) at the 3’ end. TNF-α and IFN-γ mRNA levels were determined by a real-time PCR assay using a Rotor-gene 6000 (Corbett Research, Sydney, Australia) with 0.5 µg of cDNA. The threshold cycle (Ct; the cycle number at which the amount of amplified gene of interest reaches a fixed threshold) was determined subsequently. Relative quantitation of TNF-α and IFN-γ mRNA expression was calculated by a comparative Ct method as previously described [28]. The relative quantitation value of target (TNF-α or IFN-γ) was normalized to an endogenous control β-actin gene and relative to a calibrator. It was expressed as 2 ΔΔCt (fold), where ΔCt = Ct of target gene – Ct of endogenous control gene and ΔΔCt = ΔCt of samples for target gene – ΔCt of the calibrator for the target gene.

Statistical analysis: The data are expressed as mean ± standard deviation (SD). A paired Student’s t-test was performed for statistical analysis of the data. All statistical analysis of data performed using SPSS version 17.0 software (SPSS, Chicago, IL, U.S.A.). P < 0.05 was considered as the level of significance.

RESULTS

Differential ratio of leukocytes in peripheral blood: The ratio of monocytes in peripheral leukocytes of the FM-fed group was significantly increased as compared to the control group (P < 0.05), although the ratio of other cells (neutrophils, lymphocytes, eosinophils and basophils) in peripheral leukocytes of the FM-fed group showed a slightly decreased but insignificant tendency compared with the control group.
Subpopulation of lymphocytes in spleen: The T lymphocyte (CD3+/CD19–) ratio in spleen of the FM-fed group (37.76 ± 2.01) was significantly increased compared to the control group (33.19 ± 2.06) (P<0.05). Moreover, the ratio of T-helper (CD4+/CD8–) and T-cytotoxic (CD4–/CD8+) cells in spleen of the FM-fed group showed a slightly increased tendency compared with the control group, although the difference was not significant (P=0.12 and P=0.56, respectively). Conversely, the ratio of B lymphocyte (CD3–/CD19+) in spleen of the FM-fed group (51.17 ± 3.13) showed a slightly decreasing tendency compared with the control group (53.92 ± 3.15), although in this instance the difference was not significant (P=0.06). Control: control group; FM: FM-fed group. Data are presented as mean ± SD (n=5) and each value expressed in percent (%) leukocytes. *P<0.05 versus control.

Antibody production level against formalin-killed B. bronchiseptica: Antibody production level (determined as optical density value) against formalin-killed B. bronchiseptica of the FM-fed group (0.570 ± 0.046) was significantly increased compared to the control group (0.477 ± 0.058) (P<0.05) (Fig. 2). Non-immunized mouse sera displayed a clearly negative reaction (0.048 ± 0.003) against the B. bronchiseptica antigen. Control: control group; FM: FM-fed group. Data are presented as mean ± SD (n=5).

Number of leukocytes in BAL of experimentally B. bronchiseptica infected mice: After experimental intranasal infection of B. bronchiseptica, the number of leukocytes in BAL of the FM-fed group explosively increased to a maximal peak at 3 day post-infection, and then rapidly decreased under the level of the control group at 7 day post-infection. However, the number of leukocytes in BAL of the control group showed a weakly increasing then decreasing pattern compared with the FM-fed group. Of note, the number of leukocytes in BAL significantly increased in the FM-fed group (3.7 × 10^5 cells/ml) compared with the control group (2.3 × 10^5 cells/ml) at maximal peak day (P<0.05) (Fig. 3).
**MAESIL IMMUNE-ENHANCING EFFECT**

Relative mRNA expression levels of TNF-α and IFN-γ in spleen of experimentally B. bronchiseptica infected mice: Relative mRNA expression levels of TNF-α and IFN-γ in splenocytes obtained from three mice of each group at 7 day post-infection were measured by a real-time polymerase chain reaction assay. The relative mRNA expression levels of TNF-α and IFN-γ significantly increased in the FM-fed group compared with the control group (*P<0.05) (Fig. 4).

**DISCUSSION**

In the present study, the ratio of monocytes in peripheral leukocytes of the FM-fed group was significantly increased as compared to the control group. Moreover, the ratio of T lymphocyte in spleens of FM-fed mice was also significantly increased as compared to the control group. These results imply that continuous ingestion of FM markedly reinforces mitogenicity and immune responses of macrophages and T lymphocytes. This may relate to previous studies that reported that some probiotics have an important role in regulating the immune system [50]. Especially, Miettinen et al. [34] reported that Lactobacillus and Streptococci stimulate mononuclear cells to promote the production of IFN-γ, IL-12, and IL-18. These organisms activate NF-kappa B and STAT signaling pathways in human macrophages [33]. Besides, probiotics induce maturation of dendritic cells [34] and the production of T-helper 1-classified cytokines [44]. These reports suggest that probiotics including Lactobacillus strains may up-regulate
innate immunity [47], consistent with the results of the present study that showed enhancing effects of FM on mitogenicity and immune responses of macrophages and T lymphocytes.

Antibody titer is the indicator reflecting the state of humoral immunity [51]. Hence, to evaluate effects of FM on humoral immunity, especially against *B. bronchiseptica*, the present study measured antibody production level in serum after formalin-killed *B. bronchiseptica* (antigen) administration regimen. Antibody production level significantly increased in the FM-fed group of mice compared with the control group. This indicates that humoral immunity is also enhanced by ingestion of FM in mice.

The above observations concerning the immune enhancing effects of FM led us to evaluate the immune activity of this supplement against experimental infection of *B. bronchiseptica*, which is important as a cause of disease in the respiratory tract of a variety of mammalian species, especially swine [4, 16, 18, 43]. Mice were experimentally infected with *B. bronchiseptica* through the nasal cavity. At day 7 post-infection, the number of leukocytes in BAL of the FM-fed group explosively increased to a maximal peak at 3 day post-infection. Although leukocyte numbers in control mice also increased to a maximal peak at day 3 post-infection, the number of leukocytes was significantly higher (*P*<0.05) in BAL samples obtained from FM-fed mice compare to mice in the control group. This increase is believed to be due to the infiltration of immunocytes [26]. At day 7 post-infection, relative mRNA expression levels of TNF-α and IFN-γ were significantly increased in the splenocytes of mice in the FM-fed group compared with those of the control group. TNF-α and IFN-γ are mainly produced by activated macrophages and stimulates T cells [20]. These cytokines play an important role in the host defence against infection by viral and microbial pathogens, and induce a variety of physiologically significant responses that contribute to immunity [3, 46]. Hence, these results indicate that continuous ingestion of FM enhances immune activity against experimentally *B. bronchiseptica* infection in mice.

Taken together, these findings suggest that some component(s) of FM enhances immune activity in mice, especially against *B. bronchiseptica*, probably via a strong stimulation of non-specific immune responses. Hence, in combination with probiotics, fermented Maesil, which is readily obtained as a by-product during commercial beverage preparation, may be a good alternative feed supplement to reduce use of antibiotics through the promotion of immune activity and prevention of diseases, especially in pigs. However, FM 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 FM responsible for its immune enhancing effect is needed, and studies are currently in progress. Confirmation of the immune enhancing effect of FM as a feed supplement is now required in swine.

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