FULL PAPER *Immunology*

Improvement of bacterial clearance and relief of clinical signs of *Salmonella enterica* serovar Typhimurium infection in pigs through upregulation of Th 1-specific responses by administration of a combination of two silicate minerals, biotite and bentonite

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ABSTRACT. Biotite and bentonite are phyllosilicate minerals that were originally used in industrial applications. Several beneficial activities of them have recently been reported, especially regulation of the immune system and antimicrobial effects. Therefore, we investigated the immune-enhancing and bacterial clearance effects of a biotite and bentonite mixture (BBM) on experimental infection of *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) to determine whether the BBM could be used as an alternative antibiotic. We administered 1% or 2% BBM as a feed supplement. We then evaluated the bacterial clearance effects of the BBM against *S*. Typhimurium. We also evaluated the immune-enhancing effect of the BBM through several immunological experiments that included examination of the lysozyme activity, $CD4^+/CD8^+$ T lymphocyte ratio and the T-helper type 1 (Th 1) cytokine profile. The clinical signs of *S*. Typhimurium and the number of viable bacteria in feces and tissues were significantly decreased in both BBM groups, especially in the 2% BBM group. The BBM also markedly enhanced the lysozyme activity, $CD4^+/CD8^+$ T lymphocyte ratio and expression levels of IFN-γ and IL-12 in *S*. Typhimurium-challenged pigs. Therefore, the BBM could be a good candidate as an alternative antibiotic that improves Th 1-specific immune responses and the bacterial clearance effect.

KEY WORDS: bacterial clearance, bentonite, biotite, immune enhancing, *Salmonella enterica* serovar Typhimurium

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In the swine industry, antibiotics or chemical therapeutics have been traditionally used as growth promoters to improve growth performance and prevent subclinical disease infection [15]. However, indiscriminate use of antibiotics has increased, and the spread of antibiotic-resistant pathogens as well as public concern with regard to their cross-transfer to humans through the consumption of pork that contains antibiotic resistance [4]. As a consequence, there is considerable attention on the development of alternative antibiotics that can be used for preventive feeding in pig nutrition and do not endanger the environment with their residues [19]. Naturally acquired compounds have been recently highlighted as promising alternative antimicrobial agents that could strengthen defense mechanisms and promote resistance to microbial infection by enhancing nonspecific systemic immunity [4, 10].

Salmonella enterica serovar Typhimurium (*S*. Typhimurium) is a frequently isolated *Salmonella* serotype in pigs and a well-known public health risk in commercial *Salmonella*-infected pork products [5]. The clinical signs of *S*. Typhimurium infection in pigs are increased body temperature accompanied by diarrhea and enterocolitis [26]. After recovering from the illness, the pigs are often stunted and grow slowly or remain as asymptomatic carriers in which the bacteria can persist without triggering any clinical signs [1, 3]. Therefore, *Salmonella* infection has economic concerns in swine production systems and negative implications in human public health [7]. Hence, limiting *Salmonella* infection in pigs could improve livestock production and human food safety [12].

Biotite and bentonite, which are silicate minerals, were originally used in various industrial applications based on their properties as catalysts, ion exchangers and absorbents [6, 18, 23]. Our previous studies have shown that dietary supplementation with biotite promotes the expression of several cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and promotes concanavalin A (T-cell mitogen)-induced lymphocyte proliferation in pigs [11, 13]. Biotite also shows antiviral activity against several viral agents, such as porcine circovirus, porcine reproduc-

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tive and respiratory syndrome virus, and bovine herpesvirus type 1 [8, 11]. Furthermore, bentonite has several beneficial biological effects that are associated with its adjuvant-like action with several antibacterial agents. Bugla-Płoskońska *et al*. [2] reported that a bentonite and lysozyme therapy efficiently killed *Salmonella enterica* spp. and *Salmonella bongori spp*. Bentonite also shows an enhanced bactericidal effect against *Escherichia coli* when combined with metal or metal ion nanoparticles, such as silver (Ag), zinc oxide (ZnO), titanium oxide (TiO_2), tungsten trioxide (WO_3), tin dioxide (SnO_2), cupric oxide (CuO) and magnesium oxide (MgO) [14, 17, 22].

These collective observations suggest that biotite combined with bentonite could be an alternative to antibiotics as a feed additive. Therefore, we investigated the bacterial clearance effect of biotite and bentonite mixture (BBM) on experimental infection with *S*. Typhimurium from the perspective of fecal shedding and the patterns of *Salmonella* distribution in disease-relevant tissues (cecum, colon and mesenteric lymph nodes (MLN)). We also examined several immune responses under normal and challenged conditions in the present study.

MATERIALS AND METHODS

Preparation of an experimental diet containing a biotite and bentonite mixture (BBM): A BBM supplement was provided by Hongik Bio (Damyang, Republic of Korea) that combined biotite and bentonite at a ratio of 1:1. Experimental diets containing 1% and 2% BBM were prepared by mixing BBM with a commercial pig diet for at least 4 hr in a rotating pan so that the BBM and commercial diet mixed uniformly.

Experiment 1: Immune-enhancing effect of the BBM in pigs

Experimental protocol: Conventional 4-week-old pigs were purchased from a single healthy herd without any history of *S*. Typhimurium (Daehan Livestock and Feed, Chonnam, Republic of Korea). All pigs were housed in air-conditioned rooms and randomly divided into 3 groups (containing 5 pigs each). For 2 weeks, the control group received a nutritionally complete commercial diet, and the experimental groups received the same feed, but it was supplemented with either 1% or 2% (w/w) BBM (i.e., the 1% and 2% BBM groups). Body weight was monitored once a week throughout the entire experimental period. Before and at the end of the experiment, blood samples from the jugular vein of each pig were collected into tubes coated with ethylenediaminetetraacetic acid (EDTA). All animal procedures were conducted in accordance with the guidelines of the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences (CIOMS, c/o World Health Organization, Geneva, Switzerland) and were approved by the Institutional Animal Care and Use Committee of Chonnam National University (approval number: CNU IACUC-YB-2013-29).

Table 1. Real-time PCR primer sequences

		Sequence (5`- 3`)	Accession number
TNF-α	FW	CCCCCAGAAGGAAGAGTTTC	JF831365
	RV	CGGGCTTATCTGAGGTTTGA	
IFN-γ	FW	CAAAGCCATCAGTGAACTCATCA	X53085
	RV	TCTCTGGCCTTGGAACATAGTCT	
IL-4	FW	TTGCTGCCCCAGAGAAC	AY294020
	RV	TGTCAAGTCCGCTCAGG	
IL-12	FW	GGAGTATAAGAAGTACAGAGTGG	U08317
	RV	GATGTCCCTGATGAAGAAGC	
β-actin	FW	CAGGTCATCACCATCGGCAACG	U07786
	RV	GACAGCACCGTGTTGGCGTAGAGGT	

FW: forward primer; RV: reverse primer; IFN-γ: interferon gamma; IL-4: interleukin 4; IL-12: interleukin-12; TNF-α: tumor necrosis factor alpha. The GenBank accession numbers of cDNA and corresponding genes are available online at http://www.ncbi.nlm.nih.gov/.

Differential leukocytes counts in the peripheral blood: Differential blood leukocyte counts were determined using an automated veterinary hematology analyzer (Oxford Science, Oxford, CT, U.S.A.) configured for pig blood. Each sample was independently counted 3 times.

Determination of T lymphocyte subpopulations in peripheral blood: Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. The T lymphocyte subpopulations were analyzed to determine the ratios of $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells as previously described [10]. Lymphocytes were stained with both fluorescein isothiocyanate-conjugated anti-pig CD4 (BD Biosciences, Franklin Lakes, NJ, U.S.A.) and phycoerythrin-conjugated anti-pig CD8 (BD Biosciences). After incubation at room temperature for 30 min in the dark, the cells were washed twice with phosphate buffered saline (PBS). The lymphocyte subpopulations were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Evaluation of the relative mRNA expression levels of TNF-α, IFN-γ and IL-4 in PBMCs: Total RNA was extracted from PBMCs using a PureLink RNA Mini Kit (Invitrogen, Valencia, CA, U.S.A.), and target RNA was reverse transcribed by using LeGene cDNA Synthesis Master Mix (LeGene, San Diego, CA, U.S.A.) in accordance with the manufacturer's instructions. All samples were simultaneously transcribed to minimize variations in reverse transcriptase efficiency. The relative mRNA expression levels were determined by a real-time polymerase chain reaction (PCR) assay using a MyiQ^{TM}2 system (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with iQ^{TM} SYBR Green Supermix (Bio-Rad Laboratories). The threshold cycle (Ct; the cycle number at which the amount of amplified gene of interest reaches a fixed threshold) was subsequently determined. The quantitative PCR conditions were as follows: 5 min at 94°C, followed by 45 cycles of 30 sec at 94°C, 30 sec at 56°C and 45 sec at 72°C. The primer sequences for porcine TNF-α, IFN-γ and IL-4 are shown in

Table 1. After amplification, a melting program was run to prove the presence of only one PCR product. The relative quantitation value of the targets (TNF-α, IFN-γ and IL-4) was normalized to the endogenous control β-actin gene and relative to a calibrator. It was expressed as $2^{-\Delta\Delta Ct}$(by fold), in which ΔCt=Ct of the target gene − Ct of the endogenous control gene and ΔΔCt=ΔCt of the samples for the target gene − ΔCt of the calibrator for the target gene. All experiments were performed in a triplicate manner.

Experiment 2: The bacterial clearance effect of BBM in pigs with S. Typhimurium infection

S. Typhimuirium *challenge experiment*: Pigs were randomly divided into 3 groups and fed the control diet, 1% BBM-supplemented diet or 2% BBM-supplemented diet. The BBM-supplemented diets were fed from 2 weeks before experimental challenge to the end of the experiment. The pigs were inoculated orally with 10 m*l* of *S*. Typhimurium diluted at 1×10^9 colony-forming units (cfu)/m*l* as previously described [9]; the strain of *S*. Typhimurium was originally isolated from a pig with naturally occurring salmonellosis (Animal and Plant Quarantine Agency, Gyeonggi, Republic of Korea). This strain is naturally resistant to kanamycin. All pigs were sacrificed at 7 days post infection (DPI), and their cecum, colon and MLN tissues were collected.

Clinical observations of the infected pigs: The typical clinical signs of *S*. Typhimurium infection, such as inappetence, depression, coughing, vomiting and diarrhea, were monitored daily after infection for each pig. Rectal body temperature and body weight were measured every 2 days. Fecal samples were collected at 1 DPI, 3 DPI, 5 DPI and 7 DPI, and the fecal conditions were recorded based on the method of Tanaka *et al*. [24] with some modifications. In brief, the fecal condition score of each pig was visually measured every day by 3 independent evaluators; the score ranged from 0 to 3 points (0 =normal feces, 1 =moist feces, 2 =mild diarrhea and 3 =severe diarrhea).

Viable S. Typhimurium counts in the fecal and tissue samples: Viable bacterial cell counts were determined in the feces at 1 DPI, 3 DPI, 5 DPI and 7 DPI. The cecum, colon and MLN tissues were also collected from each sacrificed pig at 7 DPI. The samples were homogenized (10%, w/v) in sterile PBS after serially 10-fold diluted in PBS. Each dilution was spread onto xylose lysine deoxycholate (XLD) agar (BD Biosciences) supplemented with 100 μg/m*l* kanamycin and incubated at 37°C for 48 hr. Characteristic red colonies with black centers were counted and expressed as colony-forming units per gram of feces or colony-forming units per gram of tissue, but only when 20–200 colonies per plate were observed.

Histological analysis of tissues of experimentally infected pigs: After the postmortem examination, the cecum and colon tissue samples from all pigs were fixed in 10% neutral-buffered formalin, embedded in paraffin and then cut into sections. After dewaxing and dehydration, the sections were stained with hematoxylin and eosin (H&E) for histological examination.

Serum lysozyme activity assay: Serum was obtained from blood samples that had undergone centrifugation at 2,000 × *g* for 10 min at 4°C. Lysozyme activity assays were conducted as previously described [10], with some modifications. A lysozyme standard diluent was prepared by dissolving crystalline lysozyme (Sigma-Aldrich, St. Louis, MO, U.S.A.) in 0.1 M phosphate buffer (pH 6.2) to concentrations of 1–10 μg/m*l*. Each standard solution (20 μ*l*) or prepared serum sample was added to a well of a 96-well microtiter plate in a triplicate manner. A *Micrococcus lysodeikticus* working solution (0.075% w/v in 0.1 M phosphate buffer; Sigma-Aldrich) was added to each well. After incubation at 37°C for 15 min, 30 min, 45 min and 60 min, the absorbance of each diluent at 540 nm was analyzed, and the regression coefficient between the absorbance and time was calculated from each standard diluent. The lysozyme activity of the serum samples was determined based on the regression coefficient of the standard lysozyme concentration.

Evaluation of T lymphocyte subpopulations and relative mRNA expression levels of IFN-γ and IL-12 in experimentally infected pigs: After *S*. Typhimurium infection, blood samples were individually collected from the jugular vein of the pigs at the end of the experiment. Isolation of lymphocytes and analysis of T lymphocyte subpopulations were performed by flow cytometer, as described in Experiment 1. The mRNA expression levels of T helper type 1 (Th 1) cytokines (IFN-γ and IL-12) were also evaluated, as described in Experiment 1.

Statistical analysis: The data are expressed as the mean ± standard deviation (SD). The means of the different parameters were compared between groups by one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison tests. All statistical analyses were performed using GraphPad InStat version 3.0 software (GraphPad Software, La Jolla, CA, U.S.A.). A value of $P<0.05$ indicated statistical significance.

RESULTS

The effects of the BBM on the T lymphocyte subpopulations in normal pigs: Flow cytometric analysis of peripheral blood was performed 14 days after administration of 1% BBM or 2% BBM. The percentage of $CD4^+CD8^-$ T lymphocytes in the BBM groups significantly increased compared with those of the control group (data not shown). In particular, the $CD4^+/CD8^+$ T lymphocyte ratios in the BBM groups were significantly increased ($P<0.01$) compared with those observed in the control group (Fig. 1).

The Effects of the BBM on cytokine mRNA expression levels in normal pigs: We analyzed the mRNA expression levels of TNF-α, IFN-γ and IL-4 after administration of 1% BBM or 2% BBM. There was no significant difference in the levels of TNF-α and IL-4 between the BBM groups and the control group (Fig. 2A and 2C). The relative mRNA expression level of IFN-γ was significantly increased in the 1% BBM and 2% BBM groups compared with the control group ($P<0.05$) (Fig. 2B).

Changes of clinical signs in pigs infected with S. Typhimurium: After the experimental challenge with *S.* Ty-

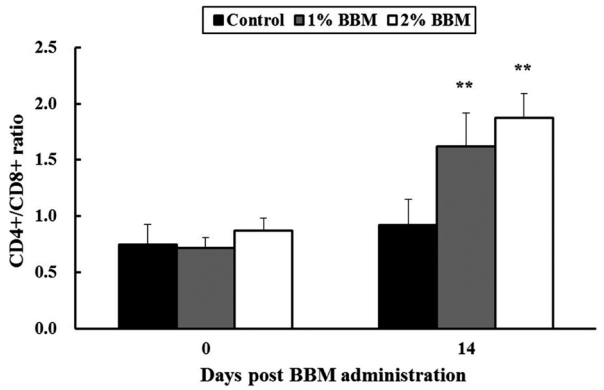

Fig. 1. Effects of the biotite and bentonite mixture (BBM) on T lymphocyte subpopulations in normal pigs. After BBM administration to pigs for 2 weeks, peripheral blood mononuclear cells (PBMCs) were isolated, and the $CD4^+/CD8^+$ T lymphocyte ratio was evaluated using flow cytometry. The BBM group has a significant increase in the $CD4^+/CD8^+$ T lymphocyte ratio compared with the control group. The data are presented as the mean ± SD of 5 pigs per group. $^{**}P< 0.01$ *vs.* control group.

phimurium, the rectal body temperature and fecal condition scores were measured to evaluate the severity of infection. The rectal body temperature of all groups rapidly increased to more than 39.5°C at 3 DPI. There was no significant difference between the pigs fed the BBM and the control pigs during the experimental period (data not shown). All infected animals showed mild diarrhea (scores 1–2) at 1 DPI, and the diarrhea symptom became severe at 3–5 DPI (scores 1–3). The fecal consistency was back to normal at 7 DPI (score of approximately 1) (Fig. 3A). The diarrhea score tended to decrease during the experimental period in both BBM groups compared with the control group; however, there were no significant differences between the control group and BBM groups.

Bacterial clearance of feces and tissues in pigs infected with S. Typhimurium: Viable bacterial cells in feces were counted at 1 DPI, 3 DPI, 5 DPI and 7 DPI. In all *Salmonella*-affected tissues (MLN, colon and cecum), viable bacterial cells were counted at the end of the experiment. The number of viable bacteria in both the feces and tissues tended to decrease in both BBM groups during the experimental infection period compared with the control group. The differences from the control group were significant at 3 DPI ($P<0.05$ in the 1% BBM group; $P<0.01$ in the 2% BBM group), 5 DPI ($P<0.01$ in the 2% BBM group) and 7 DPI ($P<0.05$ in the 2% BBM group) (Fig. 3B). The number of viable bacteria in the MLN tissue was also significantly lower in the 2% BBM group than in the control group ($P<0.05$). The number of viable bacteria in the colon tissue was notably lower in both BBM groups than in the control group ($P<0.01$). Similarly, viable bacterial counts in the cecum tissue were markedly decreased in both BBM groups compared with the control group ($P<0.05$) (Fig. 3C).

Histopathological observations in Salmonella-infected pigs: All infected pigs showed typical histopathological

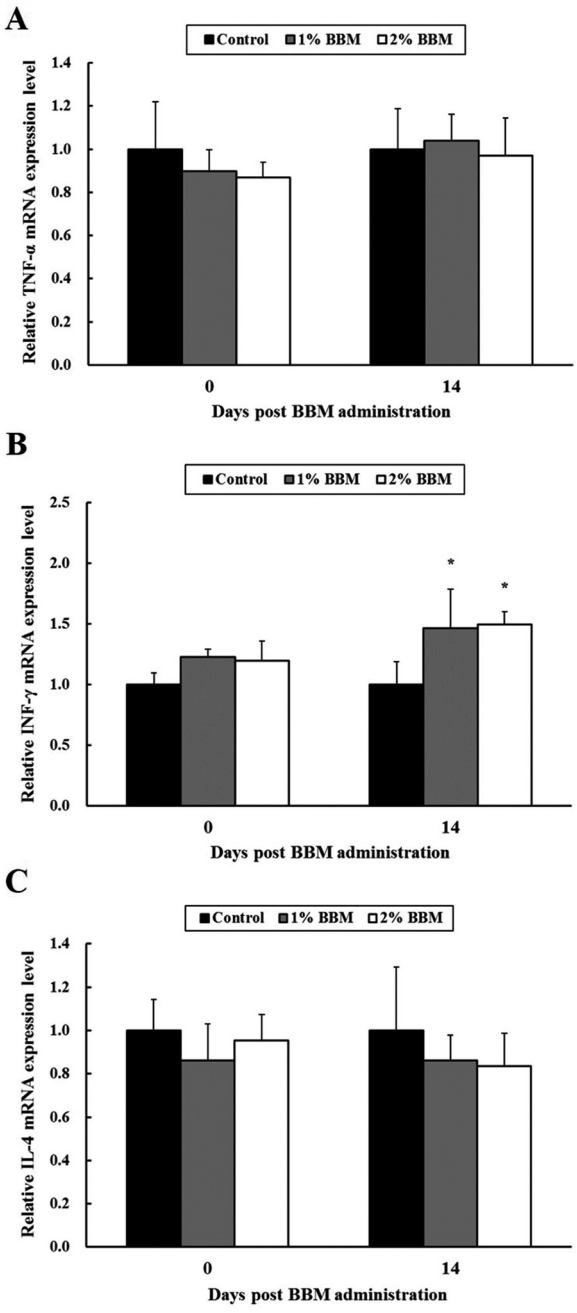

Fig. 2. The effects of the BBM on the relative mRNA expression levels of TNF-α, IFN-γ and IL-4 in normal pigs. The mRNA expression levels of (A) TNF-α, (B) IFN-γ and (C) IL-4 from the peripheral blood were measured through quantitative real-time PCR and normalized to that of β-actin. The BBM group shows a significant increase in the IFN-γ level. The data are presented as the mean ± SD of 5 pigs per group. $^{*}P<0.05$ *vs.* control group.

lesions of salmonellosis, such as severe desquamation of epithelial cells and infiltration of inflammatory cells into the mucosal layer. However, the 1% BBM group and 2% BBM group showed mild histopathological lesions in a dose-

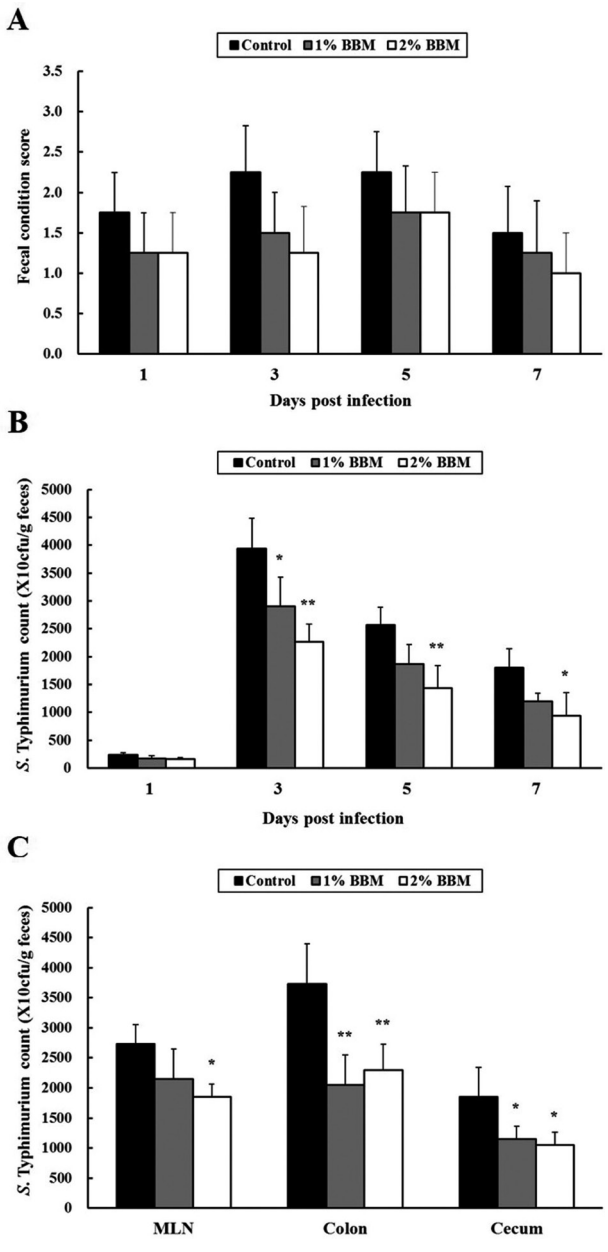

Fig. 3. The effects of the BBM on the fecal condition score and bacterial clearance in pigs challenged with *S.* Typhimurium infection. The pigs were fed a supplemented diet and then orally inoculated with *S.* Typhimurium. The fecal condition score and viable bacterial cell counts were measured after *S.* Typhimurium infection at 1, 3, 5 and 7 days. All pigs were sacrificed at the end of the experiment, and the cecum, colon and mesenteric lymph node (MLN) tissues were collected and used for viable bacterial cell counting. (A) The fecal condition scores are lower in the BBM groups than in the control group. The numbers of viable bacteria in the (B) feces and (C) tissues are notably decreased in the BBM groups, especially in the 2% BBM group. The data are presented as the mean ± SD of 5 pigs per group. **P*<0.05 *vs.* control group. ***P*<0.01 *vs.* control group.

dependent manner in the cecum and colon tissue compared with the control group (Fig. 4).

Serum lysozyme activity in serum: We analyzed the serum lysozyme activity to evaluate the effect of the BBM on phagocytic cells, which are a first-line defense mechanism for bacterial infections. The serum lysozyme concentration was significantly higher in both BBM groups compared with in the control group ($P<0.05$ in the 1% BBM group; $P<0.01$ in the 2% BBM group). However, no significant difference in serum lysozyme concentration existed between the 1% BBM and 2% BBM groups (Fig. 5A).

The effects of the BBM on the T lymphocyte subpopulations and the Th 1 cytokine profiles in Salmonella-infected pigs: We specifically analyzed the $CD4^+/CD8^+$ T lymphocyte ratio and the mRNA expression levels of Th 1 cytokines (IFN-γ and IL-12) based on the results of Experiment 1, which demonstrated the effect of the BBM on T helper cells. The $CD4^+/CD8^+$ T lymphocyte ratio in the peripheral blood was significantly higher in both BBM groups than in the control group ($P<0.05$ at 1% BBM group, $P<0.01$ at 2% BBM group) (Fig. 5B). The relative mRNA expression levels of IFN-γ and IL-12 were also significantly increased in the 1% BBM group and 2% BBM group compared with the control group (Fig. 6). The significance was much more pronounced in the 2% BBM group compared with the control group.

DISCUSSION

To evaluate the immune-enhancing effect of BBM in normal conditioned pigs, we analyzed several cellular immunity-related parameters, such as the $CD4^+/CD8^+$ T lymphocyte ratio, and the mRNA expression levels of TNF-α (pro-inflammatory cytokine), IFN-γ (Th 1 type cytokine) and IL-4 (Th 2 type cytokine) after administration of a 1% BBM-supplemented diet or 2% BBM-supplemented diet. The ratio of 2 primary T lymphocyte subsets-$CD4^+$ and $CD8^+$ T lymphocyte-are generally used as the most meaningful parameters to measure immune functions and responses [28]. Therefore, high $CD4^+/CD8^+$ T lymphocyte ratios are associated with increased immune capacity in pigs, whereas low ratios are usually associated with immune deficiency and many infectious diseases [9]. In the present study, the $CD4^+/CD8^+$ T lymphocyte ratio and level of IFN-γ (which is a representative cytokine of Th 1) were significantly increased in the 1% BBM and 2% BBM groups. These results implied that dietary supplementation of BBM mainly stimulated Th 1-specific responses, which include increased IFN-γ production. We reported similar results in our previous study, which showed an increase in the level of $CD4^+$ T lymphocytes and IFN-γ after administration of a biotite-supplemented diet [13].

Based on the effects of the BBM on normal pig immunity, we also evaluated the bacterial clearance effect of the BBM by using the *S.* Typhimurium challenge model to determine whether the BBM could be used as an alternative antibiotic. After the Salmonella challenge, all pigs showed clinical signs of Salmonellosis (fever and diarrhea). Necropsy and histopathological results also revealed that all pigs had the

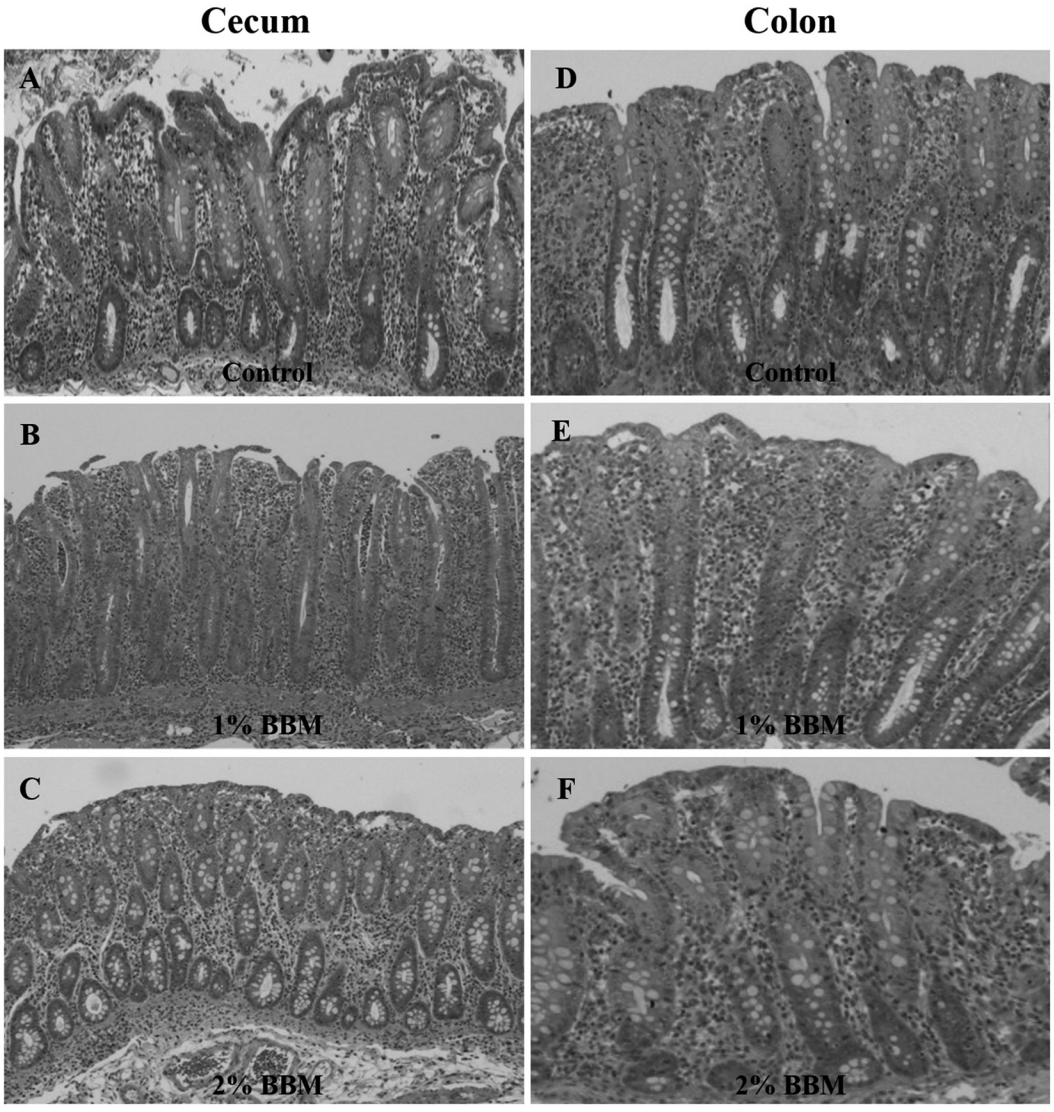

Fig. 4. Histopathological features of the cecum and colon tissues in pigs challenged with *S.* Typhimurium infection. Representative pictures of the cecum (A, B and C) and colon (D, E and F) stained with H&E in the control group, 1% BBM group and 2% BBM group. In both tissues, infiltration of lymphoid cells and desquamation of the epithelial cells are less severe in the 1% and 2% BBM groups than in the control group. The magnification in all images is 100 ×.

typical signs of enterocolitis. However, these clinical signs tended to be lower in both BBM groups than in the control group. The numbers of viable bacteria in feces and tissues also notably decreased in the BBM groups, especially in the 2% BBM group. These results are similar to the results of a previous study by Szabó *et al*. [21], who showed the *Salmonella* clearance effect of a probiotic strain of *Enterococcus faecium* in relieving clinical signs, fecal shedding and internal organ dissemination of *S*. Typhimurium.

We also demonstrated several cellular immunity-associated components, such as the lysozyme activity, $CD4^+/CD8^+$ T lymphocyte ratio and Th 1 type cytokine profiles, which are critically associated with host defense mechanisms encountered in bacterial infection. *Salmonella* has a unique mechanism for overcoming host immune systems through suppression of the intracellular generation of bactericidal enzymes and secretion of Th1-related cytokines, such as IFN-γ and IL-12 [27]. In particular, lysozyme is a key bactericidal enzyme secreted by phagocytes, such as macrophages and polymorphonuclear leukocytes. It directly degrades the peptidoglycan wall of gram-positive bacteria by hydrolyzing glucosidic bonds and causing bacterial cell wall lysis, which could also contribute to the stimulation of macrophages [20, 25]. The Th 1 cytokines are considered as key components necessary for the intracellular clearance of *Salmonella* through the priming of T lymphocytes [16]. We previously reported the *S.* Typhimurium clearance effect by using several naturally acquired agents through the upregulation

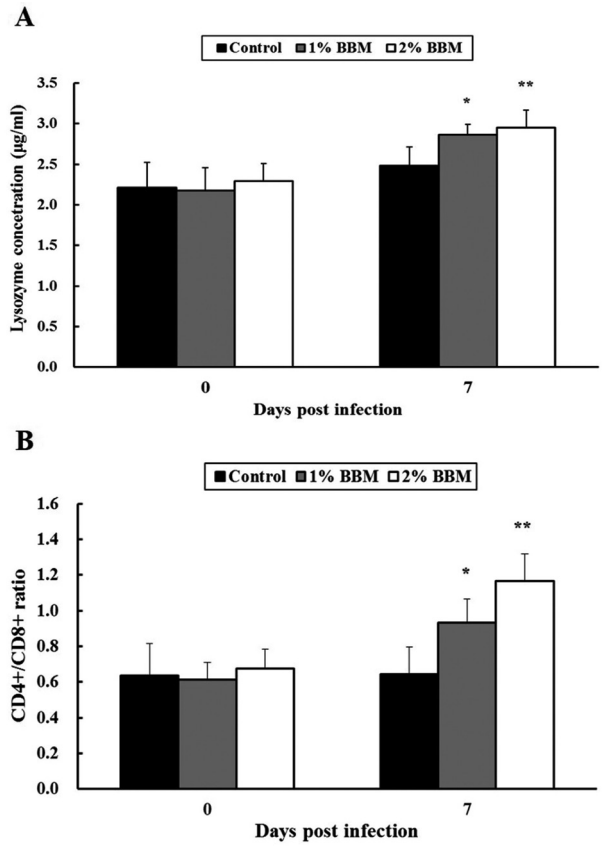

Fig. 5. The effects of the BBM on the lysozyme activity and $CD4^+$/$CD8^+$ T lymphocyte ratio in pigs challenged with *S.* Typhimurium infection. Serum samples and PBMCs were isolated from each blood sample. (A) The serum lysozyme concentration and (B) $CD4^+/CD8^+$ T lymphocyte ratio are significantly higher in the 1% and 2% BBM groups than in the control group. The data are presented as the mean ± SD of 5 pigs per group. **P*<0.05 *vs.* control group. ***P*<0.01 *vs* control group.

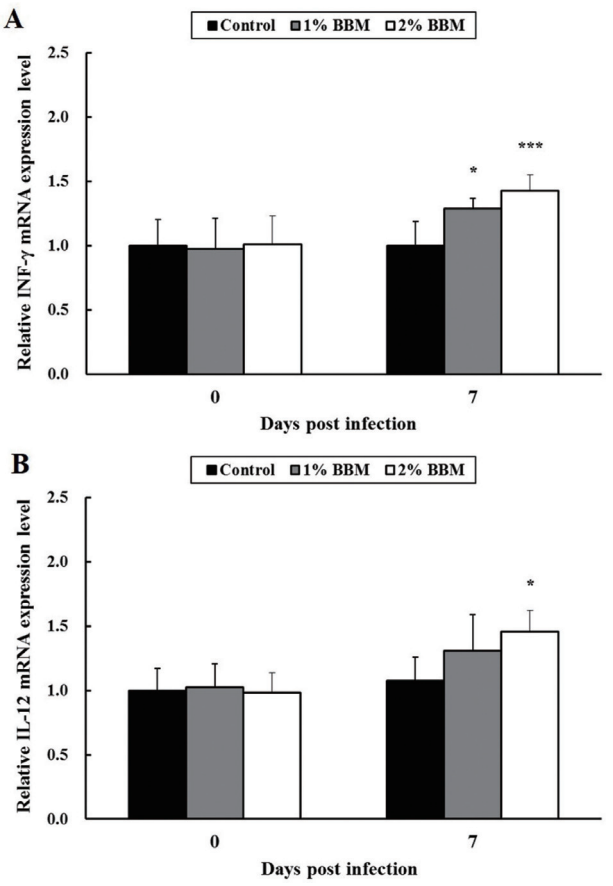

Fig. 6. The effects of the BBM on the relative mRNA expression levels of IFN-γ and IL-12 in pigs challenged with *S.* Typhimurium infection. PBMCs were isolated from the blood samples, and then, mRNA expression levels of (A) IFN-γ and (B) IL-12 were measured through quantitative real-time PCR. The BBM group shows a significant increase in the IFN-γ and IL-12 levels. The data are presented as the mean ± SD of 5 pigs per group. **P*<0.05 *vs.* control group. ****P*<0.001 *vs.* control group.

of lysozyme activity and Th 1-specific immune responses [9, 10]. Our data similarly showed that dietary supplementation with the BBM significantly enhanced the lysozyme activity, $CD4^+/CD8^+$ T lymphocyte ratio and expression levels of IFN-γ and IL-12 in *S.* Typhimurium-challenged pigs. Therefore, these results implied that the BBM can stimulate phagocytic action and Th 1-specific responses after *S.* Typhimurium infection and that these immune-boosting effects may be connected to the bacterial clearance effect of the BBM. Similar results were also noted in a study by Bugla-Płoskońska *et al.* [2], who showed that bentonite effectively killed *Salmonella enterica* spp. by stimulating lysozyme activity. Our previous studies also reported strong stimulation of secretion after administration of a biotite-supplemented diet $CD4^+$ T lymphocytes and IFN-γ [8, 13].

In conclusion, the present study demonstrated that administration of the BBM may effectively improve Th 1-specific immune responses and bacterial clearance against *S.* Typhimurium in experimentally infected pigs. This bacterial clearance activity may be especially associated with strong stimulation of Th 1-specific immune responses. Daily feeding of the BBM as a feed additive did not induce any toxic symptoms in normal pigs or *S.* Typhimurium-infected pigs. However, our study did not determine the precise mechanisms of the BBM or what component of the BBM is mainly associated with the immune-enhancing and bacterial clearance effects. Hence, the present knowledge in conjunction with further elucidation to clarify these mechanisms could lead to new candidates as alternative antibiotics in the swine industry.

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