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Immune-enhancing Effects of Chitosan-fermented Feed Additive on Broiler Chickens and Subsequent Protection Conferred against Experimental Infection with *Salmonella* Gallinarum

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Benefits chitosan-fermented feed additives (CFFAs) particularly in the regulation of the immune system and antimicrobial activity. Therefore, we investigated the immune-enhancing and bacterial clearance effects of CFFA (fermented by *Bacillus licheniformis*) on broiler chickens *Salmonella* Gallinarum challenge. We administered 2% or 4% CFFA evaluated its immune-enhancing effects using several immunological experiments, including examination of lysozyme activity, lymphocyte proliferation, and expression of cytokines. We also evaluated the bacterial clearance effects of CFFA against *S.* Gallinarum. CFFA administration markedly enhanced lysozyme activity, lymphocyte proliferation, and the expression of interleukin (IL)-2, IL-12, tumor necrosis factor alpha, and interferon gamma in the spleen. In broilers challenged with *S.* Gallinarum, the clinical signs of *S.* Gallinarum infection and the number of viable bacterial colonies in the feces and tissues decreased in both CFFA groups. Therefore, CFFAs could be good candidates for feed additive to improve nonspecific immune responses and bacterial clearance.

Key words: broilers, fermentation, immune response, insect, *Salmonella* Gallinarum

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Introduction

Maintenance of appropriate breeding environment and improvement of the immune systems of broiler chickens are essential for reducing the risk of infection and ensuring efficient production in the broiler industry. In addition, the broiler chicken industry seeks to improve meat quality and accelerate growth (Shomali and Mosleh, 2019). However, stress factors experienced by broiler chickens due to overcrowding increase the production of free radicals in the body, resulting in lowering of immunity and increase in the risk of infection by pathogenic microorganisms, which reduces productivity (Lara and Rostagno, 2013). Antibiotics are frequently added to farm feed to prevent production losses due to infectious diseases that increase livestock mortality. However, antibiotic resistance may develop, and antibiotics may be retained in broilers after slaughter, which may subsequently affect human health (Diarra and Malouin, 2014; Gao *et al*., 2017; Roth *et al*., 2019). Flavonoids and fatty acids are used to circumvent these problems and improve broiler productivity; insects with high protein content are added, and various substances, such as chitosan extracted from crustaceans, are

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used as additives. Studies on the substitution of antibiotics by combinations of specific compounds during feeding are currently underway to improve disease resistance and livestock productivity (Solis *et al*., 2009; Zhou *et al*., 2019; Benzertiha *et al*., 2020; Chang *et al.*, 2020).

The amino acid composition of insects is balanced and they contain micronutrients and various types of fatty acids, which render them potentially good sources of proteins and other nutrients, sustainable nutritional sources for livestock production, and good feed additives. In particular, they have attracted attention for the production of fish, poultry, and pig feeds (Sogari *et al*., 2019; Chia *et al*., 2020). Researchers have attempted to manufacture and utilize chitosan from the chitin components present in large amounts in insect shells. Chitosan can be separated from chitin via desalination and deacetylation using hydrochloric acid and sodium hydroxide, respectively (Said *et al*., 2020). However, the production of chitosan using these chemicals is associated with certain disadvantages, such as changes in molecular weight, low degree of deacetylation of the product, and loss of the nutritional value of proteins (Synowiecki and Al-Khateeb, 2003). To compensate for these shortcomings, chitosan production using enzymes have been extensively researched, and methods using enzymes in combination with microorganisms have emerged (Synowiecki and Al-Khateeb, 2003; Kumar *et al*., 2017; Affes *et al*., 2020).

Bacillus sp. is used as a probiotic. *Bacillus licheniformis* has been reported to secrete metabolites such as bacitracin, which show intestinal antibacterial activity (Ducluzeau *et al.*, 1976). Indeed, application of *B*. *licheniformis* to broiler feed helps increase body weight and eliminates pathogenic microorganisms such as *Clostridium perfringens* and *Salmonella* spp. Furthermore, the population of pathogenic *Escherichia coli* was reduced, improving the growth performance and intestinal morphology of the broilers. Thus, *B*. *licheniformis*-fermented products may be used as alternative antibiotic growth promoters for the preventive treatment of *C. perfringens* infection in broilers (Peng *et al*., 2019; Arif *et al*., 2021; Cheng *et al*., 2021).

Chitosan is a polysaccharide composed of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) and is the most abundant aminopolysaccharide found in nature. It has unique functional properties such as biocompatibility, biodegradability, and non-toxicity. Owing to these properties, chitosan is widely used in agriculture, biotechnology, and medicine, particularly in the production of antimicrobial compounds and bioenergy (Fenice and Gorrasi, 2021). Chitosan and its derivatives can also benefit the livestock industry. In a study by Osho and Adeola (2020), broiler chicks were administered dexamethasone to induce stress and then fed a standard diet supplemented with chitosan extracted from shrimp shells. In the chitosan group, improvements were observed in the shape of villi, crypt height, growth, and immune function of broiler chicks. Thus, the inclusion of chitosan additives in diet can alleviate oxidative stress in broiler chicks. Fermented chitosan produced using *B. licheniformis* has excellent physicochemical and structural properties and high levels of essential amino acids, and its use as a nutrient in animal feed and food has been suggested (Liu *et al*., 2020).

In this study, a chitosan-fermented feed additive (CFFA) was developed by inoculating *B. licheniformis* into the shells of black soldier flies (BSF), followed by their fermentation and drying of the decomposed chitosan. After administration of the developed CFFA to broiler chickens, changes in weight gain, serum lysozyme activity, splenic lymphocyte proliferation, and expression of inflammatory cytokines were determined. In addition, we investigated the bacterial clearance effect of CFFA in broiler chickens experimentally infected with *S.* Gallinarum by determining the patterns of *Salmonella* distribution in disease-relevant tissues (the liver, cecum, and feces). We also examined the changes in the proportions of immune system cells, such as CD4+ and CD8+ T cells.

Materials and Methods

Experiment 1. Evaluation of nonspecific immune responses

Preparation of experimental animals and feed

The experimental diet was prepared as follows: the shells of the BSF larvae were crushed and degreased. Molasses and water were added to the defatted BSF larvae, followed by inoculation with *B. licheniformis* and subsequent fermentation. The CFFA was prepared by drying the products from the shells using *B. licheniformis*. This study was conducted at the Korea University Insect Research Institute (Gokseong, Jeollanam-do, Korea). After complete fumigation of the breeding room with formalin, 30 one-day-old broiler chickens (Ross broilers) were stocked in a laboratory animal breeding room. The temperature and humidity in the breeding room were maintained at 28–30°C and 45–55%, respectively, and ventilation was allowed only through a hood to block the inflow of outside air. The broiler chickens used in the experiment were divided into three groups of 10 chickens each. The chickens in the control group received commercial, nutritionally complete, extruded, and dry chicken feed. The chickens in the two experimental groups received the same extruded dry chicken feed supplemented with 2% (w/w) CFFA (2% fed group) or 4% (w/w) CFFA (4% fed group). Chickens in each group were weighed weekly and fed an experimental diet for 21 days. After completion of the experiment, the broiler chickens were anesthetized using xylene, and blood was collected from the heart. The broilers were euthanized via exsanguination during blood sampling. Autopsies were performed to remove the liver, cecum, and spleen tissues from the chickens for analysis. Animal experiments were approved by the Animal Experiment Ethics Committee of Chonnam National University (CNU IACUC-YB-2020-62).

Lysozyme activity

The degree of macrophage activation upon CFFA administration was assessed using a lysozyme activity assay based on the method described by Lee *et al*. (2018). The separated serum was incubated with *Micrococcus lysodeikticus* (Thermo Fisher Scientific, Waltham, MA, USA), and the absorbance was measured at 540 nm using Multiskan EX (Thermo Fisher Scientific). Lysozyme activity was determined based on the final absorbance value.

Table 1. **Sequences of primers used in real-time PCR**

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	CCTAGGATACACAGAGGACCAGGTT	GGTGGAGGAATGGCTGTCA
IL1B	CACAGAGATGGCGTTCGTTC	GCAGATTGTGAGCATTGGGC
IL2	GAACCTCAAGAGTCTTACGGGTCTA	ACAAAGTTGGTCAGTTCATGGAGA
IL12	ACCAGCCGACTGAGATGTTC	GTGCTCCAGGTCTTGGGATA
TNFA	CGCTCAGAACGACGTCAA	GTCGTCCACACCAACGAG
IFNG	GAACTGGACAGGGAGAAATGAGA	ACGCCATCAGGAAGGTTGTT

Measurement of splenic lymphocyte proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed based on a previous study by Lee *et al.* (2018). After seeding the broiler spleen lymphocytes in a 96-well plate at a density of 1×10^6 cells per well, the T cell mitogen, concanavalin A (Con A) (Thermo Fisher Scientific), was added, and the cells were cultured for 36 h at 37°C in the presence of 5% CO_2. After incubation, MTT (Thermo Fisher Scientific) was added and the cells were incubated for another 4 h. Finally, the absorbance was measured at 540 nm.

Evaluation of changes in cytokine expression

To determine the mRNA expression of cytokines in splenic lymphocytes, the isolated cells were seeded in a 12-well plate at a density of 1×10^6 cells per well and incubated with Con A for 36 h at 37°C in the presence of 5% CO_2. After incubation, the cells were collected and RNA was isolated using the NucleoSpin RNA Plus kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). cDNA was synthesized from the isolated RNA using a RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific) and used for real-time PCR. Levels of interleukin (IL)-1β, IL-2, IL-12, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ were measured using cDNA as the template. The MyiQTM real-time PCR detection system (Bio Rad, Hercules, CA, USA) was used. Table 1 lists the sequences of the real-time PCR primers used in this study.

Experiment 2. S. Gallinarum challenge inoculation into broiler chickens

After complete disinfection of the room with formaldehyde, 30 one-day-old broiler chickens were stocked in a laboratory animal breeding room. The temperature and humidity in the room were maintained at 28–30°C and 45–55%, respectively, and ventilation was allowed only through the hood to block the inflow of outside air. Broiler chickens were divided into three groups in the same manner as in Experiment 1 and provided free access to feed and water. Experimental infection with *S.* Gallinarum was performed 21 days after feeding. *S.* Gallinarum was cultured overnight at 37°C in tryptic soy broth (Thermo Fisher Scientific), and 5 mL of this culture was inoculated orally at a concentration of 1×10^9 colony-forming units (CFUs)/mL per animal. The broiler chickens were observed for 14 days after bacterial challenge and survivability was recorded. An autopsy was performed 14 days after the challenge inoculation to confirm the presence of pathological lesions. Pathological lesions were evaluated by counting the number of white spots visible in the livers of broilers during autopsy and recording the score. The number of anterior white spots in both the left and right lobes of the liver was counted. The evaluation criteria were as follows:

No white spots: 0
Less than 50 white spots: 1
More than 50 white spots and less than 100 white spots: 2
More than 100 white spots: 3

Number of colonies of S. Gallinarum bacteria isolated from broiler chickens after challenge inoculation

On day 14 after the *S.* Gallinarum challenge inoculation, all broiler chickens were euthanized, and an autopsy was performed to measure the number of *S.* Gallinarum in the liver, cecum, and feces, which are the representative target organs of *Salmonella* infection. The liver was collected from the lower end of the right lobe, and the cecum was cut approximately 5 cm upward based on the bursa. Each tissue sample was homogenized using a Precellys®24 (Bertin Technologies, Montigny-le-Bretonneux, France). The homogenate was then serially diluted 10-fold in phosphate-buffered saline (PBS), and 100 μL of each dilution was spread onto xylose lysine deoxycholate agar plates and incubated at 37°C for 24 h. Characteristic black-colored colonies were counted and expressed as log CFU/g tissue as described previously (Lee *et al*., 2018).

Identification of lymphocyte subpopulations in broiler chickens after S. Gallinarum challenge inoculation

On day 14 of the *S.* Gallinarum challenge inoculation, the splenic lymphocytes were isolated, and the T lymphocyte subpopulations were analyzed to determine the ratios of CD4+ CD8− and CD4− CD8+ T cells, as previously described (Jung *et al*., 2013). Briefly, fresh spleen tissue isolated from broilers was disassembled using a strainer and separated into individual cells. The cells were washed with ice-cold PBS and stained with PE/Cy7-anti-chicken CD3 (T cell marker; Thermo Fisher Scientific; 1:200), FITC-anti-chicken CD4 (helper T cell marker; Thermo Fisher Scientific; 1:200), and PE-anti-chicken CD8 (cytotoxic T cell marker; Thermo Fisher Scientific; 1:200). After incubation at 25°C for 30 min in the dark, the cells were washed twice with PBS and the lymphocyte subpopulations were analyzed using a flow cytometer (BD Accuri Flow Cytometry, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Statistical analysis

The experimental results obtained from each sample were

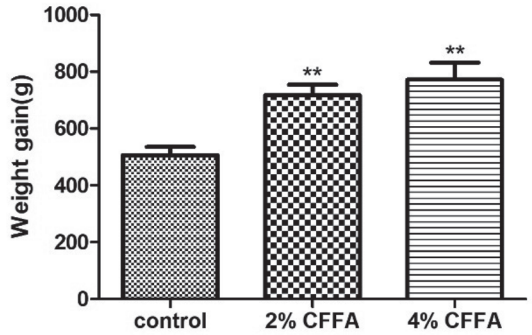

Fig. 1. **Comparison of weight gain in chickens fed chitosan-fermented feed additives (CFFA).** Values are presented as mean ± SD and were compared with those of the control group using Dunnett's multiple comparison test (**$p < 0.01$).

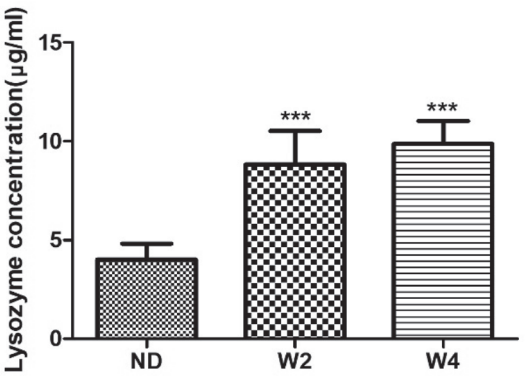

Fig. 2. **Comparison of lysozyme activity.** The serum collected from each group was incubated with *Micrococcus lysodeikticus*, and the absorbance was measured at 540 nm. Values are presented as mean ± SD and were compared with those of the control group using Dunnett's multiple comparison test. (***$p < 0.001$).

tested for significant differences using analysis of variance (ANOVA) and Dunnett's multiple tests at p value < 0.05 using GraphPad Prism software, version 5.0. The test results were displayed graphically as means and standard deviations.

Results

Experiment 1. Evaluation of nonspecific immune responses Comparison of weight gain

The weight gain of the broilers was calculated as the difference between the weight at the end of the experiment and that at the beginning. A significant increase in weight gain was observed in chickens of the CFFA-fed groups compared to those in the control ($p < 0.01$). The weight gain did not differ significantly between the 2% and 4% CFFA groups. However, the higher the CFFA concentration, the greater was the weight gain (Fig. 1).

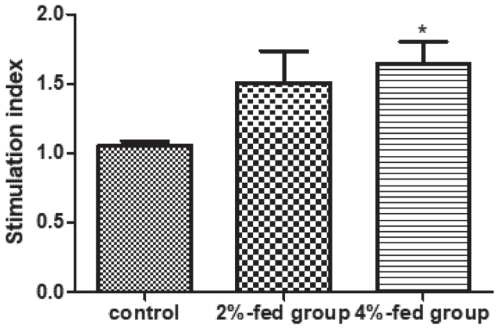

Fig. 3. **Comparison of the results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).** Values are presented as mean ± SD and were compared with those of the control group using Dunnett's multiple comparison test (*$p < 0.05$).

Lysozyme activity

Compared with the control, a highly significant increase in lysozyme activity was observed in the groups fed 2% and 4% CFFA ($p < 0.001$). Additionally, CFFA administration activated macrophages and increased innate immune responses in broiler chickens (Keshav *et al.*, 1991; Gajda E and Bugla-Płoskońska, 2014) (Fig. 2).

Measurement of splenic lymphocyte proliferation

The results of the MTT assay showed that the proliferative capacity of lymphocytes tended to increase in the CFFA-fed groups. Compared with the control, the group fed with 4% CFFA showed a significant increase in lymphocyte proliferation ($p < 0.01$) (Fig. 3).

Evaluation of the changes in cytokine expression

The expression levels of IL-1β, IL-2, IL-12, TNF-α, and IFN-γ in broiler spleen tissues were measured. No significant change was observed in the expression of IL-1β in the CFFA-fed groups compared with that in the control group (Fig. 4a), although the expression level of IL-2 increased significantly in the 2% and 4% CFFA-fed groups ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 4b). The expression level of IL-12 increased significantly ($p < 0.01$) in the 2% and 4% CFFA-fed groups compared with that in the control (Fig. 4c). The expression level of TNF-α in the 2% and 4% CFFA-fed groups was significantly higher than that in the control group ($p < 0.001$) (Fig. 4d). The expression level of IFN-γ in the 2% and 4% CFFA-fed groups was significantly higher than that in the control group ($p < 0.05$, and $p < 0.01$, respectively) (Fig. 4e).

Experiment 2. S. Gallinarum challenge inoculation into experimental animals Evaluation of clinical symptoms in broiler chickens after S. Gallinarum challenge inoculation

Mortality was observed in the control and 2% CFFA-fed groups during the 14 day period after *S.* Gallinarum challenge. Four broiler chickens died in the control group, one died in the 2% CFFA-fed group, and none died in the 4% CFFA-fed group.

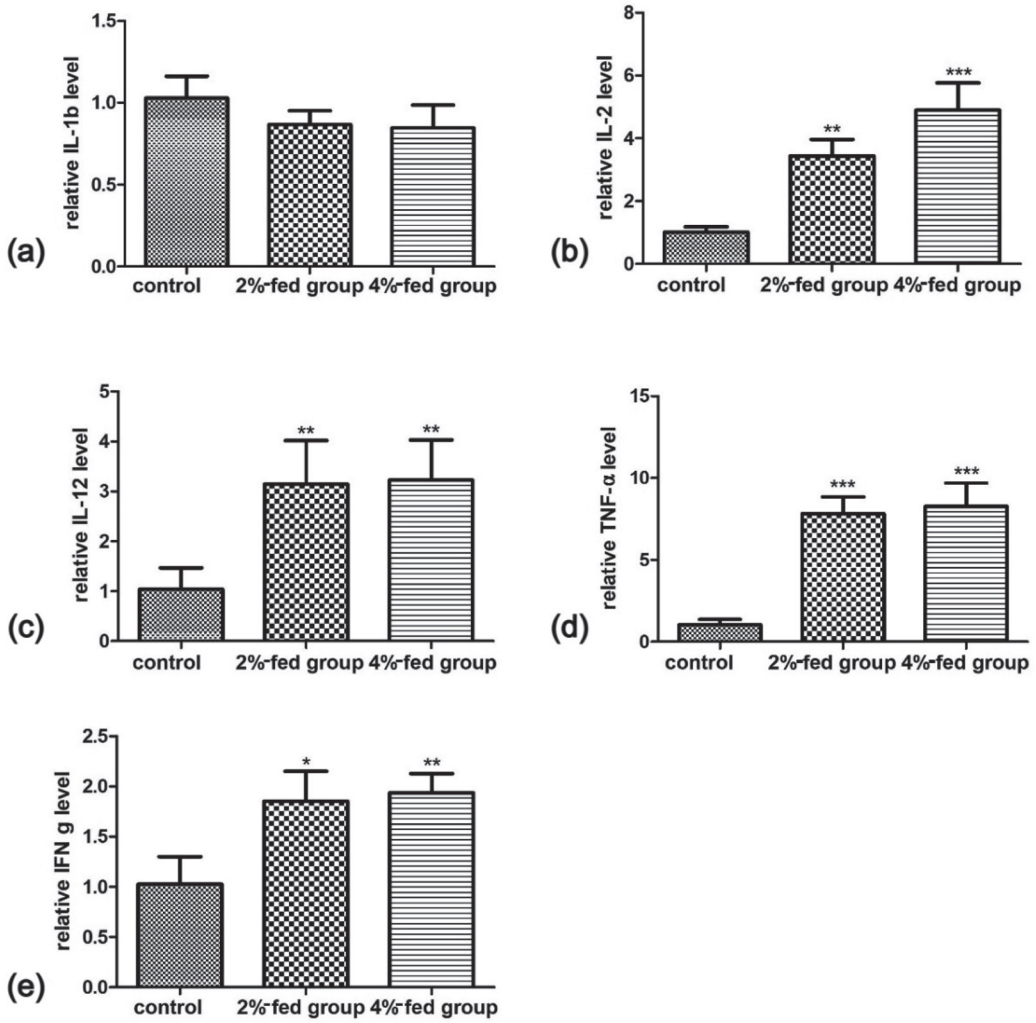

Fig. 4. **Production of inflammatory cytokines.** IL-1β (a), IL-2 (b), IL-12 (c), TNF-α (d) and IFN-γ (e). Values are presented as mean ± SD and were compared with those of the control group using Dunnett's multiple comparison test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

All surviving broilers were euthanized and autopsied. In the livers of infected broiler chickens, clear "white spots," which are typical symptoms of *S.* Gallinarum infection, were observed (Fig. 5). The number of white spots visible in the liver was counted and evaluated by conversion into scores. The number of spots in the 2% and 4% CFFA-fed groups was lower than that in the control group (Fig. 5d). The degree of lesions caused by *S.* Gallinarum infection tended to decrease significantly in the CFFA-fed groups.

Number of S. Gallinarum bacteria isolated from broiler chickens after challenge inoculation

The viable bacterial cell counts in the liver, cecum, and fecal samples collected from each sacrificed broiler 14 days post-infection were determined. The number of viable *S.* Gallinarum cells in the tissues of the CFFA-fed groups tended to be lower than that in the control group. Significant differences were observed between viable *S.* Gallinarum counts in the liver tissues of broiler chickens in the 2% and 4% CFFA-fed groups, and those in the control group ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 6a). Moreover, *S.* Gallinarum was detected to a significantly lesser extent in the ceca of broilers in the 2% CFFA-fed group and 4% CFFA-fed group than in the control group ($p < 0.001$) (Fig. 6b). The bacterial count of *S.* Gallinarum in the feces of broilers in the 2% and 4% CFFA-fed groups was significantly lower than that in the control group ($p < 0.05$) (Fig. 6c).

Identification of lymphocyte subpopulations in broiler chickens after S. Gallinarum challenge inoculation

Upon confirming the lymphocyte subpopulations in the spleen tissues of broiler chickens after challenge inoculation, we observed that the proportion of CD4+ T cells was significantly

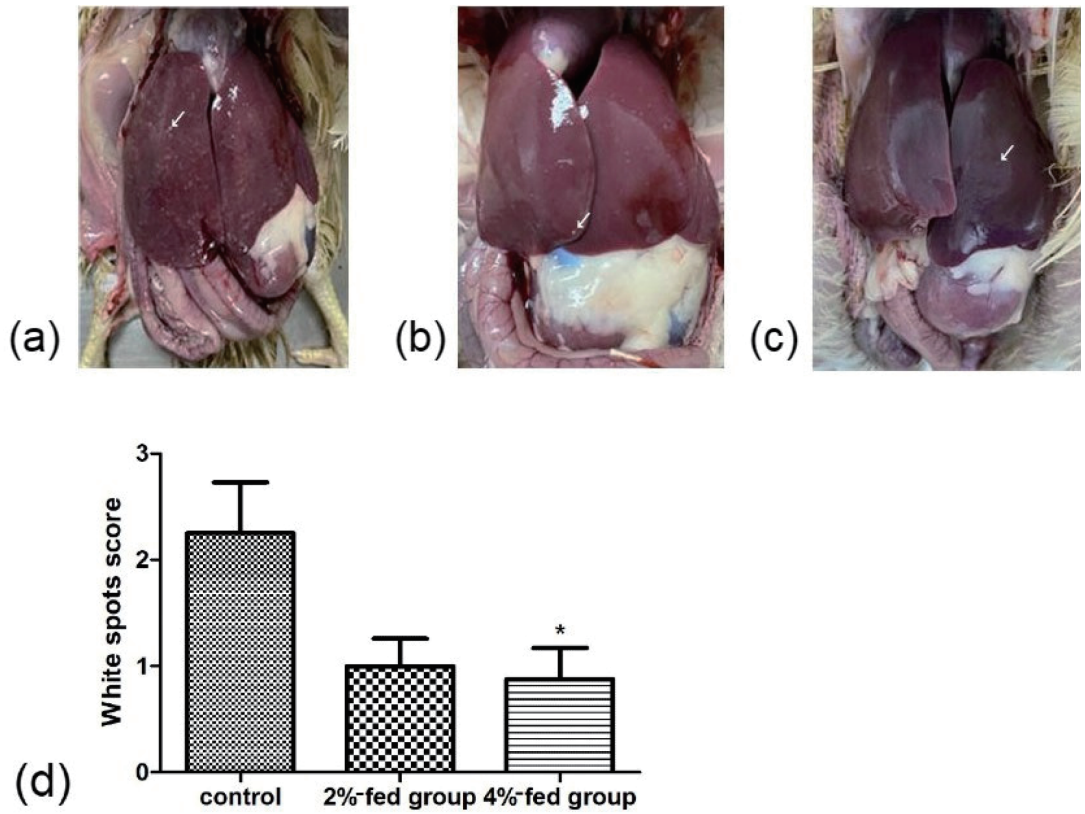

Fig. 5. **Gross appearances and white spot scores of the liver tissues after inoculation with *Salmonella* Gallinarum.** The numbers of spots in the control group (a), The numbers of spots in the 2% CFFA-fed group (b) and 4% CFFA-fed group (c). The numbers of white spots visible were counted and evaluated by converting them into scores (d). Values are presented as mean ± SD and were compared with those of the control group using Dunnett's multiple comparison test (*p < 0.05).

higher in the 2% and 4% CFFA-fed groups than that in the control group ($p < 0.001$) (Fig. 7a). Conversely, the population of CD8+ T cells in the spleens of the CFFA-fed groups was significantly lower than that in the control group ($p < 0.001$) (Fig. 7b). Therefore, the ratio of CD4/CD8+ T cells in the spleens of the CFFA-fed chickens was significantly higher than that in the control chickens ($p < 0.001$) (Fig. 7c).

Discussion

In this study, BSF was fermented with *B. licheniformis* and was used as a feed additive for broiler chickens. We hypothesized that feeding CFFA would increase the growth rate and improve immunity and defense against *S.* Gallinarum pathogens.

In the present study, CFFA, prepared by fermenting the shells of BSF larvae with *B. licheniformis*, was administered to broiler chickens to determine the effects on weight gain, resistance to infection, and immunity. A significant increase was observed in all the aforementioned parameters in the 2% and 4% CFFA-fed groups compared with those in the control group. Lysozyme, an antibacterial enzyme found in the granules of neutrophils and macrophages, hydrolyzes the peptidoglycans in the cell walls of Gram-positive bacteria, which leads to bacterial cell death. This enhances the release of bacterial products, including peptidoglycans, which activate pattern recognition receptors in the host cells. Thus, lysozyme is an important component of the innate immune system (Ragland and Criss, 2017) and an indicator of macrophage activation, and high levels of lysozymes are secreted when immunologically specific or nonspecific stimuli are administered (Keshav *et al*., 1991; Gajda and Bugla-Płoskońska, 2014). A significant increase in lysozyme activity was observed in the 2% and 4% CFFA-fed groups compared to that in the control group. This suggested that CFFA administration has the potential to activate innate immunity in broiler chickens.

The spleen, the site of T and B cell maturation and storage,acts as a secondary lymphoid organ (Chapman *et al*., 2021). The MTT assay detects only living cells. In addition, the degree of detection differs according to the degree of cell activation. Owing to these characteristics, the results of the MTT assay can be used to test whether cells are proliferating, activated, or cytotoxic (Mosmann, 1983). As assessed using the MTT assay, the proliferative

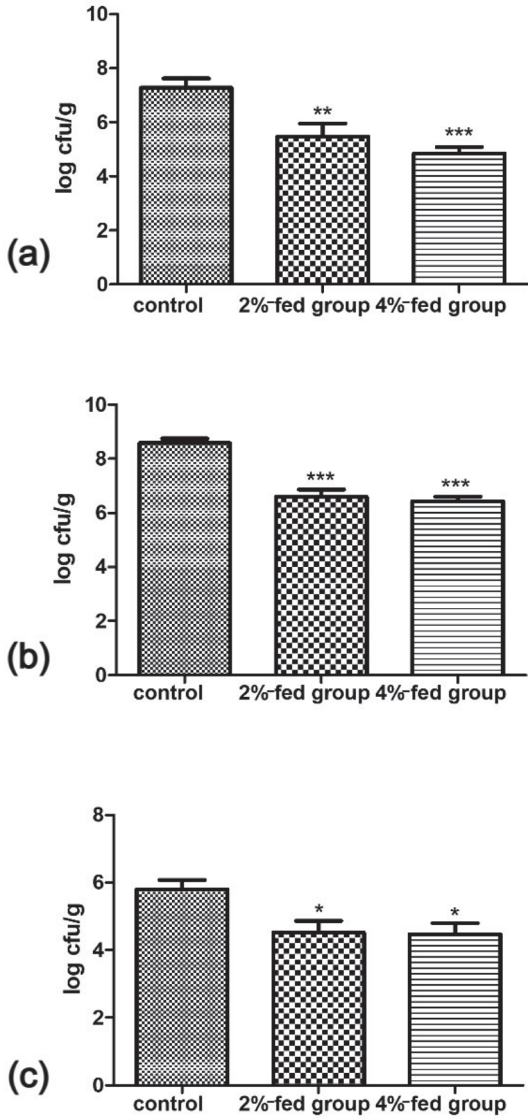

Fig. 6. **Counts of *Salmonella* Gallinarum in the liver, cecum, and feces after inoculation.** liver tissues (a), ceca (b) and feces (c). Values are presented as mean ± SD and were compared with those of the control group using Dunnett's multiple comparison test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

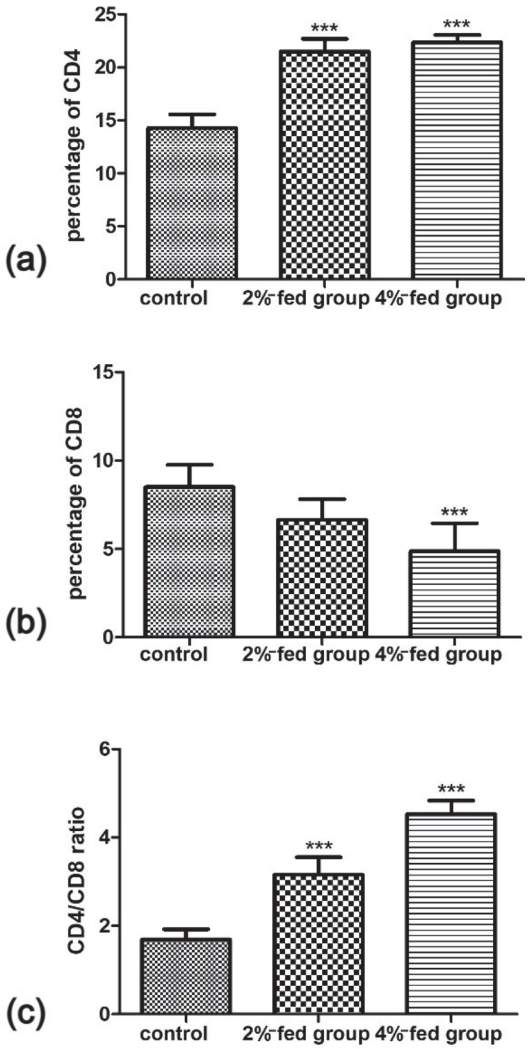

Fig. 7. **Determination of the proportions of CD4+ and CD8+ cells, and the CD4+/CD8+ ratio in splenic lymphocytes after challenge inoculation.** The proportion of helper T cells (CD4) (a), CD8 (b) and the ratio of CD4/CD8 (c). Values are presented as mean ± SD and were compared with those of the control group using Dunnett's multiple comparison test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

capacity of lymphocytes increased significantly in the 4% CFFA-fed group compared to that in the control group. This indicated that CFFA administration increased splenic activity in broiler chickens, thereby increasing the activity of immune cells.

To evaluate the immune-enhancing effect of CFFA in broiler chickens, we analyzed the mRNA levels of *TNFA* and *IFNG* (Th1-type cytokines) after administration of CFFA. *IL2, IL12, TNFA,* and *IFNA* mRNA levels were significantly higher in the 2% and 4% CFFA-supplemented groups than in the control group. These results implied that dietary supplementation with CFFA mainly stimulated Th1-specific responses, including increased IL-2, IL-12, and IFN-γ production. IL-12, secreted by phagocytes, binds to T and natural killer cells, and is involved in signaling via the signal transducer and activator of transcription 4 (STAT4) and production of IFN-γ. It induces the proinflammatory function of CD4+ T cells and links the innate and adaptive immune responses. IFN-γ is an important mediator of cellular immunity mediated by Th cell populations (Costers *et al*., 2009).

After the *S.* Gallinarum challenge, mortality was observed in the control and 2% CFFA-fed groups. Four broiler chickens died

in the control group, one died in the 2% CFFA-fed group, and none died in the 4% CFFA-fed group. Autopsies were performed to measure the number of white spots in the liver, which is a typical symptom of *S.* Gallinarum infection. Upon visual confirmation in the autopsy, many specific white spots were observed in the livers of the control chickens. However, the number of white spots in the livers of CFFA-fed chickens decreased significantly (Fig. 5). In addition, the isolation rates of *Salmonella* from the liver, cecum, and feces of broiler chickens were significantly lower in the 2% and 4% CFFA-fed groups than in the control group. We identified several cellular immunity-associated factors such as lysozyme activity, CD4+/CD8+ T lymphocyte ratio, and Th1-type cytokine profiles, which are critically associated with activation of the host defense mechanisms during bacterial infection. *Salmonella* uses a unique mechanism for overcoming the host immune system: suppression of the intracellular generation of bactericidal enzymes and secretion of Th1-related cytokines, such as IFN-γ and IL-12 (Wick, 2004). The Th1 cytokines are considered key components necessary for the intracellular clearance of *Salmonella* via the priming of T lymphocytes (Meurens *et al.*, 2009). We have previously reported that *Salmonella typhimurium* uses several naturally acquired agents by upregulating lysozyme activity and activating Th1-specific immune responses (Jung *et al*., 2012; Jung *et al*., 2013). Our data showed that dietary supplementation with CFFA significantly increased the CD4+/CD8+ T lymphocyte ratio and the expression levels of IFN-γ and IL-12 in *S.* Gallinarum-infected broiler chickens. Therefore, these results implied that CFFA can stimulate phagocytic action and Th1-specific responses after *S.* Gallinarum infection, and that these immune-boosting effects may be attributed to the bacterial clearance property of CFFA.

In many cases, an increase or decrease in the immune function is evaluated by measuring the number, percentage, or ratio of white blood cells. The ratios of helper and cytotoxic T cells (CD4+ and CD8+, respectively) are commonly used to assess the immunoregulatory balance of T cells (Erf *et al.*, 1998; Abdukalykova *et al.*, 2008; Makau *et al*., 2020). Therefore, we believe that the administration of CFFA changed the CD4+/CD8+ ratio in the spleen tissue, resulting in an increase in immune function.

In this study, the splenic CD4+ T lymphocyte subpopulations of broiler chickens challenged with *S.* Gallinarum increased significantly in the 2% and 4% CFFA-fed groups compared with those in the control. The CD4+/CD8+ T cell ratio, a measure of immune function and response, was significantly higher in the 2% and 4% CFFA-fed groups than in the control group. Low ratios are usually observed in individuals with acute viral diseases and hemophilia (Chakravarti, 1995), whereas high ratios are associated with increase in the immunofunctional abilities of chicks (Erf *et al*., 1998; Abdukalykova *et al*., 2008). In the present study, the ratio of splenic CD4+/CD8+ cells in the CFFA-fed groups was significantly higher than that in the control group. These results implied that CFFA improved the immune function in broilers and may enhance their resistance to infectious *S.* Gallinarum.

In this study, we used chitosan as a fermentation material in addition to the immune-enhancing fermented *B. licheniformis*. We observed increase in the levels of serum hormones and growth factors, immune organ weights, and concentrations of immunoglobulins and cytokines upon administration of chitosan to the chickens. Chitosan induced the expression of IL-6 and IL-10 in the jejunal mucosa of chickens, thereby improving immune function. Miao *et al.* (2020) has shown that chitosan improves the growth performance and immune function of broiler chicks, while another study showed that feeding chitosan by adding it to the diet potentially alleviated oxidative stress in immunosuppressed broiler chicks (Osho and Adeola, 2020). Thus, the application of chitosan as a feed additive is expected to enhance immunity in livestock production. Therefore, immunological synergy of the two targets can be expected by fermenting chitosan with *B. licheniformis*.

Taken together, these findings suggested that some components of CFFA enhanced immune activity in broiler chickens and increased the bacterial clearance of *S.* Gallinarum in experimentally infected broilers, possibly because of the potent stimulation of nonspecific immune responses. Hence, CFFA may be a good alternative feed supplement that can reduce the use of antibiotics by promoting immune activity and preventing diseases. However, we did not investigate the exact mechanisms underlying CFFA-mediated protection against *S.* Gallinarum in broilers in this study. Furthermore, as CFFA contains a complex array of compounds, knowledge regarding the major components responsible for their immune-enhancing effects is required. These studies are currently underway in our laboratory.

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Author Contributions

The authors who participated in this paper made the following contributions:

Bo Mi Park conducted the experiments and wrote the paper.
Jina Lee and Young Kyu Park analyzed the data.
Young Cheol Yang designed the experiments.
Bock Gie Jung edited the paper.
Bong Joo Lee supervised and edited the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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