Original Article

Immunological Response and Nutritional Effects of *Lactobacillus* spp.-fermented Garlic on Turkey Broilers

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ABSTRACT

In the era of free antibiotics used in animal production, the application of feed additives should be prioritized to improve poultry health and production. The present study was conducted to evaluate the influences of garlic fermented by *Lactobacillus* spp. on the growth rate, intestinal microorganisms, and immune response of turkey broilers. A completely randomized design was used, involving 90 turkey broilers aged 1-56 days, with five treatments and three replicates per treatment. The birds were given feed and water *ad libitum* for the entire experiment period. The treatments included the supplementation of aqueous extract from fermented garlic (FG) to drinking water. The results showed that broilers supplemented with 0.8% FG exhibited the largest final body weight (1,158 g/bird), body weight gain (19.64 g/bird/day), and significantly improved feed conversion ratio (1.962) while decreasing the feed intake of turkey broilers from to 1-56 days. The immune organ indices, including the spleen, thymus, and bursa of Fabricius indices, were increased in the 0.6% FG treatment group ($P<0.05$), while antibody titers (at 28 and 42 days of age) were improved in the 0.6% and 0.8% FG treatments ($P<0.05$). *Clostridium perfringens* and *Salmonella* spp. were not detected in the intestines of these birds, while the amount of *Escherichia coli* was reduced ($P<0.05$) and *Lactobacillus* spp. increased ($P>0.05$) without a significant effect. It can be concluded that supplementation with 0.8% FG improved growth performance, and 0.6% FG may enhance the immunity of turkeys. Moreover, 0.6% and 0.8% FG could be widely used for poultry production.

Keywords: Fermented garlic (FG), Growth effects, Immune response, Microorganisms, Turkey broilers

1. Introduction

Along with its growth, the poultry industry is facing significant challenges, particularly concerning disease outbreaks, including Newcastle disease, one of the most common diseases in poultry. This disease has several adverse effects on productivity and economic efficiency, and it can result in high bird mortality (1). Up to the present time, turkey flocks have been gradually expanded, contributing to overall poultry development to meet the demand for poultry meat in Vietnam. The procedure of limiting antibiotics use in livestock in Vietnam partially affects the efficiency of livestock production, including poultry. In addition, the misuse of vaccinations, antibiotics, and intensive breeding have compromised the immune system of poultry. Therefore, it is important to identify dietary supplements in poultry farming to improve immune functions during the first stage of chick growth (2). Oral, intranasal, and intraocular methods are the most frequently used vaccine administration methods. Nevertheless, because of inappropriate handling and storage, immunization has been demonstrated to be ineffective in preventing Newcastle disease in chickens, especially in the tropics. Therefore, the use of therapeutic additives, such as garlic, may be a viable option. Garlic is a rich source of important nutrients, exhibits potent antimicrobial capabilities, and can be added to animal diets to boost digestive activity and stimulate the growth of chickens and other livestock species. Garlic exerts beneficial effects on inflammation, oxidative stress, hyperlipidemia, and hypertension (3). Further, organosulfur acts as an antibacterial agent that inhibits gram-positive bacteria, such as lactic acid bacteria (4). The beneficial effects of aqueous garlic extract on the growth performance of poultry were observed previously. These advantages include disease prevention, appetite stimulation, and increasing beneficial microflora in the intestine to improve digestive functions, thereby accelerating the growth of poultry. Some studies on the effects of garlic supplementation on the growth performance of poultry demonstrated that adding fermented garlic (FG) improved the performance and blood and immune parameters. According to a previous investigation, supplementation with garlic increases growth and immunity in chickens (5). The study conducted by Hossain et al. (6) found that feed fermented by *lactobacilli* can support poultry growth, development, and immunity. *Lactobacilli* can enhance the host's immune response against pathogens and attach to intestinal epithelial cells, where they survive, reproduce, and prevent or reduce the adhesion of pathogens (7). The stimulating abilities and increase in chemical compounds of garlic are fermented by *Lactobacillus* (3). Lee et al. reported that FG increased the growth performance of grower chickens, particularly during the early phase, and enhanced intestinal morphology (8). However, the effects of dietary FG addition on turkeys are unclear yet. Considering the limited research on garlic fermented by *Lactobacillus* spp., the present study aimed to determine the suitable levels of FG on the growth rate, intestinal microorganisms, and immune response of turkeys.

2. Materials and Methods

2.1. Location

The present research was conducted at the experimental farm of the Department of Animal Science and Veterinary Medicine, Tra Vinh University, Vietnam, from December 2022 to February 2023. The study was conducted following the decision of the Committee of Education and Research at Tra Vinh University.

2.2. Trial Design

A total of 90 turkey chicks were used in this study. The experiment was a completely randomized design with five treatments (triplicates). Six turkey chicks in a 1:1 gender ratio were used per experimental unit. Freely accessible feeders and drinkers were provided to all experimental units. The birds were given water and feed *ad libitum* throughout the experiment. Five treatments were formulated using five levels of aqueous extract of FG in drinking water: 0%, 0.2%, 0.4%, 0.6%, and 0.8% FG. Aqueous extract from garlic fermented by *Lactobacillus* spp. and produced each morning could be used for only 2-3 h. Birds were kept in iron cages (surface area approximately 1.5 m^2) covered with nets to prevent the birds from leaving the cage. The cage floor was covered with husks and Balasa bio-yeast. Each cage was cleaned weekly to prevent harmful effects and to maintain hygiene.

2.3. Experimental Feed

All ingredients were purchased from a local feed store in Tra Vinh province, Vietnam. The chemical composition of the diet was checked for dry matter, crude protein, organic matter, total minerals, Ca, and P before mixing. The experimental feed was formulated according to the growth phase, that is 1-28 days and 29-56 days of age (Table 1). All birds received the first and second Newcastle vaccines at 3 and 14 days of age. The vaccines contained strain F virus, one of the most common strains infecting chicks in Vietnam. In addition, the Gumboro and highly pathogenic avian influenza vaccines were administered at 7 and 16 days of age, respectively.

2.4. Fermented Garlic Preparation

A total of 1 kg of fresh garlic was peeled and sliced. Moreover, alcohol was poured on the chopped garlic. Then, the above mixture was added to molasses and thoroughly mixed; molasses is a component of the fermentation process as a culture medium for lactic acid

Table 1. The ingredients used in the experimental diets

Ingredients	Growing phases	
	1-28 days old	28-56 days old
Broken rice	13.0	12.7
Corn	24.2	30.2
Soybean meal	22.8	18.0
Rice bran	29.0	28.3
Fish meal	8.1	7.7
Limestone	2.00	2.20
Methionine	0.10	0.10
Salt	0.3	0.3
Lysine	0.20	0.20
Vitamin-Mineral Premix*	0.30	0.30
Total	100.0	100.0
ME (Kcal/kg)	2,959	3,010
CP (%)	21.0	19.0
Lysine	1.28	1.18
Methionine	0.55	0.52
Calcium	1.28	1.34
Phosphate	0.60	0.65

*: Vitamin – mineral premix was mixed according to growing phases of chickens.

bacteria. After 10 minutes of room temperature incubation, the mixture was mixed again. Vinegar (1 L) was added at this stage to accelerate the fermentation process and create an acidic environment for the mixture (i.e., through acetic acid). *Lactobacillus* spp. was added at this stage at a concentration of 5×10^5 CFU. Fresh water was added to the final volume of 20 L; following that, it was incubated for 7-14 days at room temperature without direct sunlight. The ratio of garlic to water was 1:8 (8). The quantity of *Lactobacillus* spp. was assessed, and the pH of the mixture was recorded weekly. The mixture was not used when the quantity of *Lactobacillus* spp. decreased or the pH exceeded 4.0.

2.5. Growth Performance

Each bird's body weight (BW) was recorded on the first day. Body weight gain (BWG) was measured weekly and before feeding. Feed intake (FI) was recorded daily in the morning before feeding, based on the amount of consumed and leftover feed. Moreover, mortality was recorded daily. After accounting for mortality, the feed conversion ratio (FCR) was calculated by dividing the FI (g) by BWG (g). At 56 days of age, the birds were slaughtered, and immune organs were assessed.

2.6. Immune Organ Indices

On the final day of the experiment, the birds were chosen randomly and slaughtered (each replicate included one female and one male bird). To measure internal organ sizes, the carcasses were physically plucked and eviscerated. The total weight and that of immunological organs (the spleen, bursa of Fabricius, and thymus) of the carcasses were recorded using a digital scale. The following formula was used to obtain the immune organ index values: 1,000×(immune organ weight [g]/BW [g]).

2.7. Antibody Titer

At 28, 42, and 56 days of age, 2-5 mL blood was collected from the wing veins (according to regulation QCVN 01-83:2011/BNNPTNT of Vietnam). The blood was placed in a syringe, the plunger was pulled to aspirate air (or the blood was injected into a sterile test tube), the sample was identified on the syringe and was placed on its side in the sample container to clot for 1-2 h at room temperature, avoiding direct sunlight. The samples were placed in a storage box at 2-8°C for transportation to the sample analysis center. Moreover, they were packaged and transferred to a sample analysis center to evaluate antibody titers in response to the Newcastle vaccine at 28, 42, and 56 days of age.

The serum was stored in a refrigerated container until analysis. Individual serum samples were tested for Newcastle disease virus-specific antibody titers using an ELISA kit according to the manufacturer's instructions (IDvet, Innovative Diagnostics, France). Approximately 100 µL of serum was added to the wells. Antibody titers of 993 or higher after vaccination indicated active protection (i.e., positive).

2.8. Intestinal Bacteria

After slaughter, the intestine was removed and collected. The intestine was weighed and placed in cooling bags for transport to the laboratory, where bacterial counts were assessed. To culture *Clostridium perfringens*, 100 µL of each diluted solution was distributed into sterilized media using TSC-Perfringens Agar Base (Oxoid Ltd., UK), and the plates were incubated at 37°C for 24 h to determine bacterial counts. In addition, to quantify *Escherichia coli*, TBX-Tryptone Bile Glucuronic agar was used and incubated at 44°C for 24 h. To quantify *Salmonella* spp., BPW-Buffered Peptone Water was employed and incubated at 38°C for 48 h. For *Lactobacillus* spp. bacterial counts, MRS Agar (Himedia, USA) was used and incubated at 37°C for 72 h. The bacterial colony count was determined as the common logarithm of colony-forming units/g of wet intestine material.

2.9. Data Analyses

The data were preliminarily processed using Microsoft Excel 365, and an analysis of variance (ANOVA) was performed using a GLM with Minitab software (version 17.0). The graphs were written using GraphPad Prism (version 9.0). Tukey’s test was employed to compare means with 95% confidence. In addition, statistical significance was reported at $P < 0.05$.

3. Results

3.1. Growth Performance of Turkeys

All groups were affected by the supplementation with FG (Table 2). The initial weights did not differ significantly, indicating that the results were not affected by the initial weights. The addition of FG to the diets increased final weight and BWG and improved the FCR, while it decreased FI ($P<0.05$). The highest BW (1,158 g/bird) and BWG (19.64 g/bird/day) occurred in the 0.8% FG diet. The highest amount of FI was recorded in 0%, 0.2%, and 0.4% FG diets ($P<0.05$). Dietary addition with 0.8% FG improved the FCR of birds from 1-28 days old (1.962) and for the entire trial time, compared to that in other treatments ($P<0.05$).

3.2. Immune Organ Indices

Aqueous extract of FG affected the immune organ indices positively (Figure 1). The highest index was observed in the 0.6% FG treatment with respect to all immune organs ($P<0.05$). The spleen index was 1.185 in the 0.6% FG treatment, compared to 0.846 in the control. The thymus and bursa of Fabricius indices were 2.311 and 2.788 in the 0.6% FG treatment, respectively, compared to those of 1.665 and 1.996 in the control.

3.3. Antibody Titers

Antibody titers gradually decreased over time (Figure 2). The FG supplementation increased antibody titers (4,019) in the 0.8% FG treatment, while the lowest titer was 1,808 in the control at 28 days of age ($P<0.05$). In the 0.6% FG treatment at 42 days of age, the antibody titer was the highest (2,650), in comparison to that in the other FG supplement treatments ($P<0.05$). No significant effects on antibody titers occurred at 56 days of age ($P>0.05$); however, the 0.6% FG treatment produced the highest antibody titer.

3.4. Intestinal Microorganism Abundances at 56 Days of Age

No *C. perfringens* and *Salmonella* spp. were detected in the turkeys' intestines. However, *Escherichia coli* and *Lactobacillus* spp. were detected in all treatments. The FG supplementation reduced the amount of *E. coli* (Figure 3), especially in the 0.8% FG treatment ($P<0.05$). The amount of *E. coli* was 3.333×10^4 CFU/g in the treatment of 0.8% FG, compared to the control with 6.300×10^4 CFU/g. The highest abundance of *Lactobacillus* spp. in the intestine was observed in the treatment with 0.8% FG; however, no statistically significant effect was observed ($P>0.05$).

4. Discussion

The increase in growth performance was in line with the findings reported by Fadlalla et al. (5) and Lee et al. (8). A previous study indicated that > 4% garlic in broiler diets adversely affected growth (9). The results of the current study were within the normal range (<4%) of supplementation with garlic for poultry. The main reason that FG can boost growth performance is the increase in nutrient absorption in the intestine. The alcohol in the FG mixture (10) combined with allicin affected the gut function and its microbiota. *Lactobacillus* can break down sugars and proteins in organic matter, converting them into lactic acid after a period of fermentation. *Lactobacillus* creates an acidic environment that inhibits the growth of harmful microorganisms and bacteria. Garlic extracts stimulate the growth of *Lactobacillus* spp. in addition to exhibiting antimicrobial effects, and certain *Lactobacillus* strains can inhibit pathogenic bacteria (5).

Table 2. The effects of fermented garlic on growth performance of turkeys from 1-56 days old

Criteria	Treatments					SEM	p-value
	FG0	FG1	FG2	FG3	FG4		
BW, g/bird							
Initial weight	55.97	56.33	56.93	60.30	58.63	1.491	0.282
At 28 days old	358.3^{b}	363.6^{b}	373.2^{b}	392.3^{ab}	425.3^{a}	7.350	0.001
At 56 days old	1068^{b}	1089^{b}	1128^{ab}	1192^{ab}	1268^{a}	32.74	0.009
BWG, g/bird/day							
At 1-28 days old	10.80^{b}	10.98^{b}	11.30^{b}	11.86^{b}	13.10^{a}	0.262	0.001
At 28-56 days old	25.34^{b}	25.92^{ab}	26.96^{ab}	28.55^{ab}	30.11^{a}	0.947	0.029
At 1-56 days old	18.07^{b}	18.45^{b}	19.13^{ab}	20.21^{ab}	21.60^{a}	0.589	0.010
FI, g/bird							
At 1-28 days old	25.88^{a}	23.86^{b}	24.62^{ab}	23.20^{b}	23.08^{b}	0.353	0.001
At 28-56 days old	69.98^{a}	67.51^{a}	66.44^{ab}	65.44^{ab}	61.26^{b}	1.267	0.008
At 1-56 days old	47.98^{a}	45.68^{ab}	45.53^{abc}	44.32^{bc}	42.17^{c}	0.742	0.004
FCR							
At 1-28 days old	2.64^{a}	2.33^{abc}	2.36^{ab}	2.13^{bc}	1.91^{c}	0.094	0.003
At 28-56 days old	3.08	3.28	3.05	2.92	2.60	0.174	0.156
At 1-56 days old	2.86^{a}	2.81^{a}	2.70^{ab}	2.52^{ab}	2.25^{b}	0.098	0.009

Means in a row without a common superscript letter differ ($P<0.05$); SEM Standard error of the mean. BW Body weight; BWG Body weight gain; FI Feed intake; FCR Feed conversion ratio

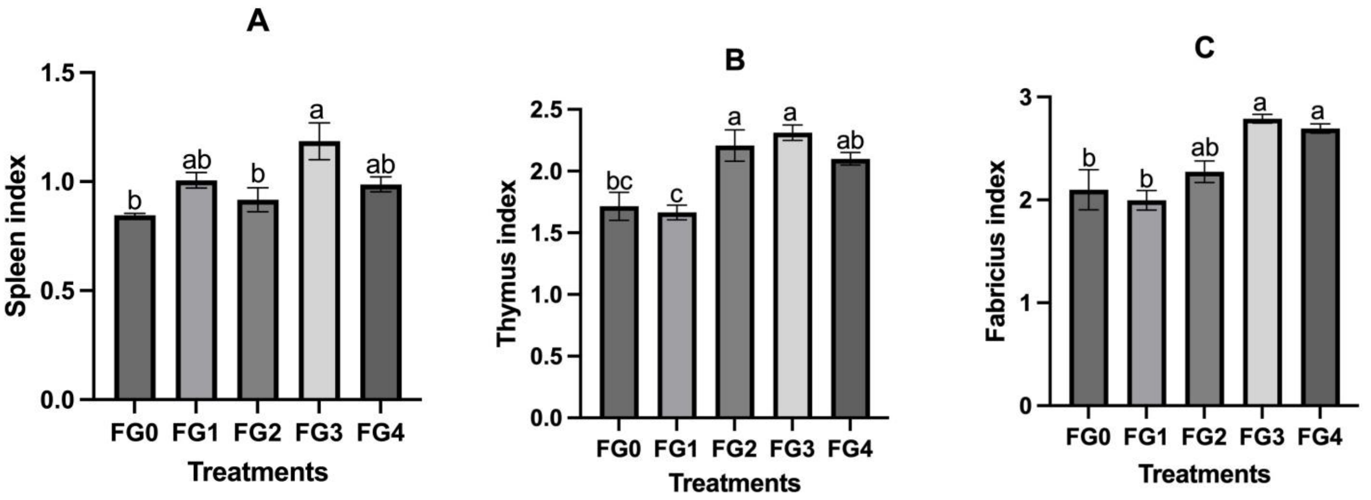

Figure 1: The impact of FG on the immune organs index of 56-day-old turkeys. A: a significant FG influence on the spleen organs index (P < 0.05); B: a significant FG effect on the thymus organs index (P < 0.05); and C: a significant FG effect on the Fabricius organs index (P < 0.05). Means in a row without a common superscript letter differ (P<0.05)

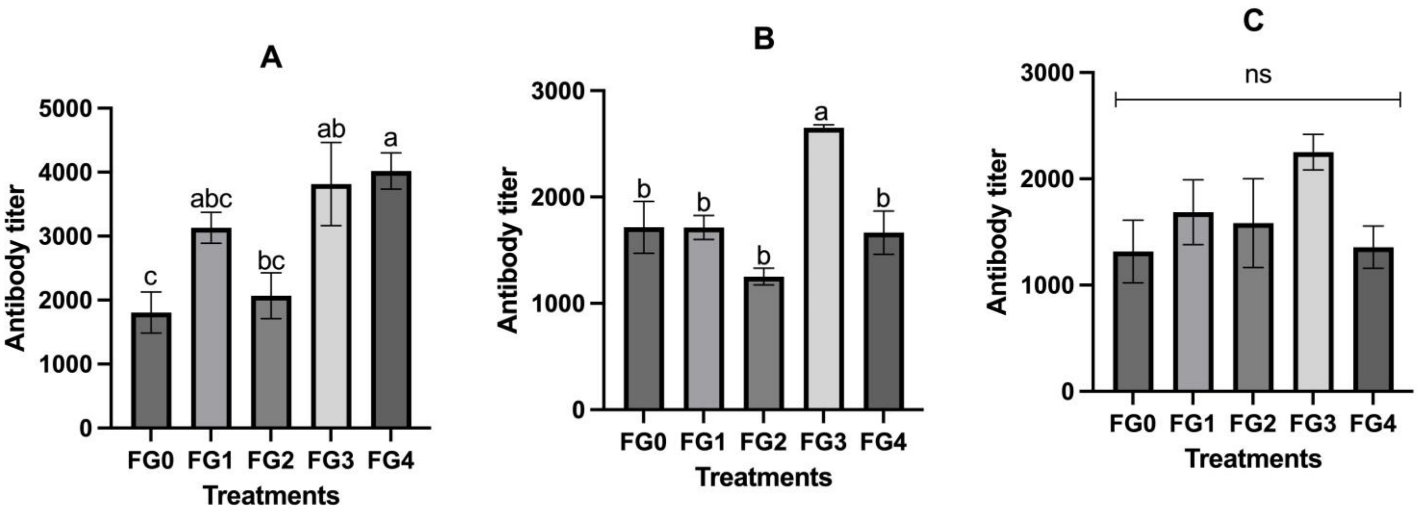

Figure 2: The effects of FG on antibody titre of turkeys from 1-56 days old; A: Significant effects of FG on antibody titre at 28 days old (P < 0.05); B Significant effects of FG on antibody titre at 42 days old (P <0.05); C: Non-significant of FG on antibody titre at 56 days old (P > 0.05). Means in a row without a common superscript letter differ (P<0.05).

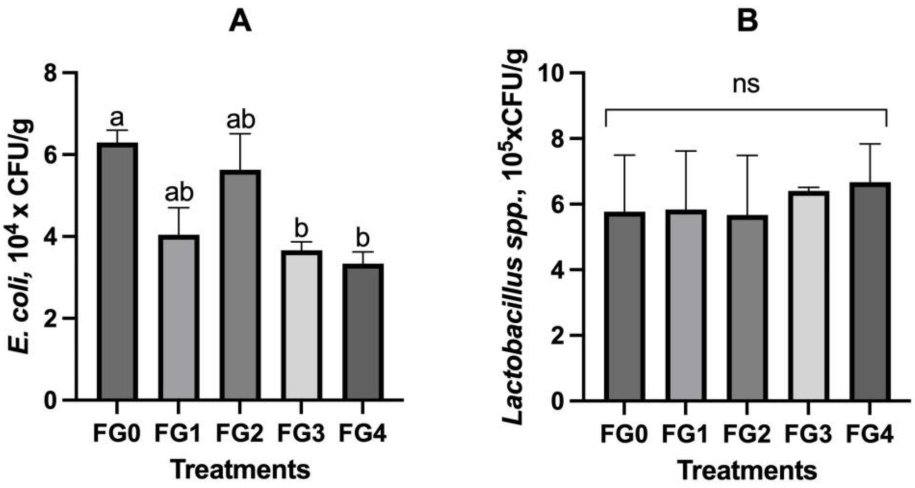

Figure 3: The effects of FG on quantity of *Escherichia coli* and *Lactobacillus spp.* in the intestine; A: Significant effects of FG on reduction of *Escherichia coli* (P < 0.05); B: Non-significant effects of FG on *Lactobacillus spp.* (P > 0.05). Means in a row without a common superscript letter differ (P<0.05).

Using garlic and *Lactobacillus* for the fermentation process may improve poultry performance and health. Moreover, lactic acid produced from fermentation by *Lactobacillus* may decrease the pH of water and the diet, and subsequently decrease the pH of the digestive tract to create an acidic environment conducive to the formation of beneficial bacteria communities. This result was also reported by Ahmed et al. (4); they found that beneficial microorganisms in fermented aqueous extract could produce various metabolites, such as antibacterial substances, lactic acid, bacteriocin, and higher alcohols, which could lower the pH of the digestive tract, kill or inhibit pathogenic bacteria, and improve the digestion and absorption capacity of the intestines. The FI decreased due to the increased level of FG in the drinking water. Garlic, whether fermented or unfermented, reduces the FI of broiler chicks. Consequently, when nutrient digestibility is at its highest, the starter phase is likely to be the underlying mechanism of the fermented-induced increase in DWG (6). In the present study, the effects of FG on birds' digestive systems were prominent. In addition to increasing the activity of enzymes, including proteases, amylases, lipases, and phytases, fermentation by lactic acid bacteria, such as *Lactobacillus*, reduce the levels of anti-nutrients, including tannins and phytic acid in food, thereby increasing the bioavailability of proteins, simple sugars, and iron (11). According to a previous investigation, sulfur compounds (e.g., 1-propenyl allyl thiosulfate and allyl methyl thiosulfate) found in genus *Allium* plants can influence FI, FCR, and BW of broilers (12).

A study on broilers indicated that the anti-inflammatory and antibacterial activities of garlic might exert significantly positive effects on growth performance and enhance feed efficiency by promoting the development of intestinal villi (3). The phytochemical components of garlic mentioned above have a variety of positive impacts on broiler production and physiological biochemistry, which may account for its capacity to increase chicken productivity (9). Similarly, by increasing the size, height, and cell area of the intestinal villus and facilitating the absorption process, garlic can improve the FCR in broiler chicks (5). Moreover, adding garlic improves enzyme activity in the pancreas and creates an environment conducive to feed digestion (13). Moreover, it includes strong antioxidants and has other positive effects on gut health (14). Therefore, garlic extract fermented with *Lactobacillus* improved the growth performance of turkey chicks. In addition to the proportionate weight of lymphoid organs, various indicators of immunity must be considered when assessing an animal's immune system. The present study was performed using various data on the immune system, including immune organ indices and antibody titers. Broilers fed with fermented feed did not significantly experience changes in the relative weights of the thymus, spleen, or bursa of Fabricius (15). Significant differences in immune organ indices were found, similar to an investigation conducted by Stefaniak et al. (16), in which the addition of feed fermented by *Lactobacillus* increased the immune organ index (thymus index). As indicated above, chicken lymphoid compartments respond differentially to fermented feed because B cells differentiate in the bursa of Fabricius while T cells differentiate in the spleen (17). Changes in lymphoid organ function may be linked to variations in thymus mass. Therefore, increasing exposure to infections or a decreased ability to maintain production potential to meet hygienic requirements could lead to an increased thymus index (18). The inclusion of *Lactobacillus* in the fermentation process is a potential reason for this improvement. The spleen and thymus indices of broilers exhibit the strongest effects of *Lactobacillus* (19); it also increases the weight of these organs in broilers (16). The antibody titers against the Newcastle disease virus in turkeys at 28 and 42 days of age were increased in the present study, which was similar to the results of previous studies, reporting that garlic supplementation in broiler diets increased the production of anti-Newcastle disease virus antibody (9,13,20). Because of the elevated CD4/CD8 cell count, garlic enhanced antibody production after immunization against the Newcastle disease virus (9,13). Garlic supplementation increased the spleen and thymus' relative weights, associated with a rise in white blood cell production and lymphocyte proliferation. Additionally, it has been demonstrated that garlic extract increases the formation of reactive oxygen species in macrophages, spleen, and thymocytes, as well as interleukin-2 and interferon-gamma gene expression (21). Therefore, it could be an explanation for the increase in antibody titers. As discussed above, FG has antibacterial and anti-inflammatory properties. Enhanced immunological regulation by garlic intake has been demonstrated in broilers (22), and it is believed to be the potential mechanism underpinning garlic's ability to alleviate *Eimeria*-induced growth depression (8) and *C. perfringens*- and *Salmonella* spp.-induced growth depression observed in the present research. Since it can limit the growth of pathogenic bacteria while boosting the growth of non-pathogenic bacteria through the synthesis of various metabolites, *Lactobacillus* in the FG mixture is correlated as a probiotic for animals, enhancing the intestinal micro-ecological environment (7). In addition, normal gut microbes (such as *Lactobacillus*) ferment the nutritious components to produce hydrogen peroxide and lactic acid as end products (23). The production of lactic acid decreases the pH of the gastrointestinal tract, thus limiting the growth of pathogenic microbes (7). Garlic supplementation decreased the caecal burden of *C. perfringens* since garlic contains organosulfur compounds (9). The most prevalent pathogenic or zoonotic bacteria in chicken farming are *E. coli*, *C. perfringens, and Salmonella enterica* (24). Supplementation with garlic fermented by *Lactobacillus* spp. in water reduced *E. coli* and increased *Lactobacillus* spp. quantitites. Salem et al. (25) reported that adding *Lactobacillus acidophilus*, *Lacticaseibacillus casei*, and *Bifidobacterium* to water decreased *Salmonella* and *E. coli* and increased *Lactobacillus* quantities. Garlic may reduce multidrug-resistant variations by inhibiting *Salmonella* invasion, antimicrobial resistance, and biofilm formation. It can be concluded that supplementation with garlic fermented by *Lactobacillus* spp. at 0.8% in drinking water increased the growth performance of turkeys of 1-56 days of age. Colonization by *E. coli* was reduced in the treatment with 0.8% FG, whereas the amount of *Lactobacillus* spp. in the small intestine increased. Moreover, better immune organ indices of the spleen, thymus, and bursa of Fabricius were observed after treatment with 0.6% FG. The antibody titers indicated higher levels in the treatment with 0.6% FG at 28 and 42 days.

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Authors' Contribution

Study concept and design: N. H. Q. and N. T. L.
Acquisition of data: K. N., N. H. P., and B. N. M.
Analysis and interpretation of data: N. H. Q., N. T. L., and D. D. L.
Drafting of the manuscript: N. H. Q., N. T. A. T., and A. G.
Critical revision of the manuscript for important intellectual content: N. H. Q. and N. T. L.
Statistical analysis: N. T. L., N. T. A. T., and A. T.
Administrative, technical, and material support: N. H. Q., N. T. L., N. T. A. T, A. T., and D. D. L.
Study supervision: N. H. Q., N. T. L., N. T. A. T, A. T., and D. D. L.

Ethics

The study followed all protocols approved by the Committee of Education and Research at Tra Vinh University, Vietnam (Approval No. 401/2022/HĐ.HĐKH&ĐT-ĐHTV).

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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