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Effects of Allicin on the Immune Performance and Expression of Immune-related Genes in Muchuan Black-bone Chickens

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ABSTRACT

Background: In response to China's ban on medicinal feed additives with growth-promoting functions in livestock and poultry feed, there is an urgent need in modern animal husbandry for alternatives to antibiotics to sustain livestock health and improve feed conversion efficiency. Allicin, known for its safety, efficiency, lack of drug resistance and absence of residue, regulates the intestinal flora structure; inhibits the growth of harmful bacteria; improves intestinal health, immunity and stress resistance; and improves animal production.

Methods: To investigate the impact of allicin on immune performance and immune-related gene expression in Muchuan black-bone chickens, diets were supplemented with varying allicin levels (50, 100, 200 mg/kg), designated as test groups A, B and C, respectively. Initially, growth performance was assessed. Subsequently, immunoglobulins were measured *via* enzyme-linked immunosorbent assay (ELISA) and colorimetry; immune titers for avian influenza and Newcastle disease were evaluated using hemagglutination and hemagglutination inhibition tests and the expression of immune genes was analyzed *via* real-time quantitative PCR. Finally, serum biochemical indices in each group were determined.

Result: Compared to the control group, allicin supplementation resulted in increased average fasting body weight in the experimental group and significantly ($P<0.05$) higher thymus and musogastric indices in groups A and C, respectively. Serum levels of immunoglobulin M in groups A and B and immunoglobulin G levels were significantly higher in all experimental groups than in controls. Antibody titers against avian influenza H5 and H7 strains were higher in all test groups, with Newcastle disease antibody titers in groups B and C significantly higher than those in controls. The mRNA expression levels of IFN-γ were lower across all experimental groups, whereas the expression levels of TLR3 in groups B and C and TLR15 in group B were higher. Serum levels of triglycerides in group A were significantly higher and blood urea nitrogen levels were significantly lower in groups B and C than in controls. These findings indicate that dietary allicin (50-200 mg/kg) significantly improves growth performance, immunity and protein and lipid metabolism in Muchuan black-bone chickens, offering valuable data for the development and use of allicin as an antibiotic alternative.

Key words: Allicin, Growth performance, Immune function, Muchuan black-bone chicken, Serum biochemical index.

INTRODUCTION

Notice No. 194 issued by the Ministry of Agriculture and Rural Affairs of China mandates that from July 1, 2020, the inclusion of pharmaceutical feed additives with growth-promoting functions in livestock and poultry feed is prohibited. Consequently, the advancement of modern animal husbandry urgently requires alternatives to antibiotics to preserve livestock health and improve feed conversion efficiency. Natural plant extracts emerge as a solution to the misuse of antibiotics in aquaculture, offering unique benefits (Yuan *et al.*, 2023). Allicin, a natural plant additive, is an oily substance produced by the catalytic action of allinase on allicin cells. It is an organic sulfur compound, with allicin oil being the active component (Ahn *et al.*, 2022; Borlinghaus *et al.*, 2014). Derived from nature and easy to extract, allicin has no toxic side effects or drug resistance. Due to its distinctive odor, along with antibacterial, antioxidant, immunomodulatory and anti-inflammatory properties, it can be used to regulate feed flavor, improve livestock and poultry appetite, increase feed intake and promote the synthesis and utilization of amino acids and vitamins in the intestines. Additionally, allicin stimulates the olfactory and gustatory senses of animals, enhancing gastric secretion and gastrointestinal peristalsis, thereby improving appetite, feed intake and feed conversion rates in livestock and poultry (Choo *et al.*, 2020; Zhang *et al.*, 2020).

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Black-bone chicken is increasingly recognized by consumers for its high nutritional and medicinal values (Sehrawat *et al.*, 2021; Li *et al.*, 2020). Melanin in this type of chicken has antioxidant, anti-aging and free radical scavenging functions (Khumpeerawat *et al.*, 2021; Li *et al.*, 2019). Muchuan black-bone chicken, a local specialty from Sichuan Province, is an important strain from the mountainous region of southern Sichuan with a historical origin in Muchuan County. It is noted for its distinctive black skin, beak, crown, mane, oral cavity, meat, bones and internal organs (Yu *et al.*, 2018; Wang *et al.*, 2018). However, the effect of allicin on immune function and serum biochemical indexes of Muchuan black-bone chicken is still lacking. This study examines the impact of varying allicin concentrations added to the feed of day-old Muchuan black-bone chickens. After a specific feeding period, we assessed the effects of different allicin levels on the growth performance, immune function indices and serum biochemical indices of the chickens, providing a scientific reference for the development and application of allicin as an antibiotic alternative in their production. The goal is to promote the development and utilization of excellent local breeds and enhance economic benefits. Our technical approach is depicted in Fig 1.

MATERIALS AND METHODS

Materials

One-day-old Muchuan black-bone chickens (n = 800; 400 males and 400 females) were housed at the Experimental Chicken Farm of Leshan Normal University, Leshan, China. Allicin, sourced from Henan Laifute Feed Technology Co., LTD., came in 1000 g bags with a concentration of 25%.

Experimental design

The chickens were housed at the farm mentioned above and randomly assigned to four groups. Each group contained five replicates, with 20 chickens per replicate. The chickens were fed from 1 to 21 days of age and from 22 to 42 days of age. The control group received a basic diet (Table 1) whereas test groups A, B and C were fed the basic diet supplemented with 50, 100 and 200 mg/kg of allicin, respectively. All groups were raised and managed under identical conditions. The duration of the experiment was 42 days.

Determination of indicators

Growth performance

Feed and water were withheld starting at 18:00 on the day before both the beginning and end of the feeding experiment. The next day at 8:00, the feed intake for each group was recorded, along with the initial and final weights and survival rates. The average daily feed intake, average daily weight gain and feed-to-gain ratio were calculated as follow:

$$\text{The average daily feed intake} = \frac{\text{Total feed intake}}{\text{Number of days in the trial}}$$

$$\text{Average daily weight gain} = \frac{\text{Final weight - initial weight}}{\text{Number of days in the trial}}$$

$$\text{Feed-to-gain ratio} = \frac{\text{Average daily feed intake}}{\text{Average daily weight gain}}$$

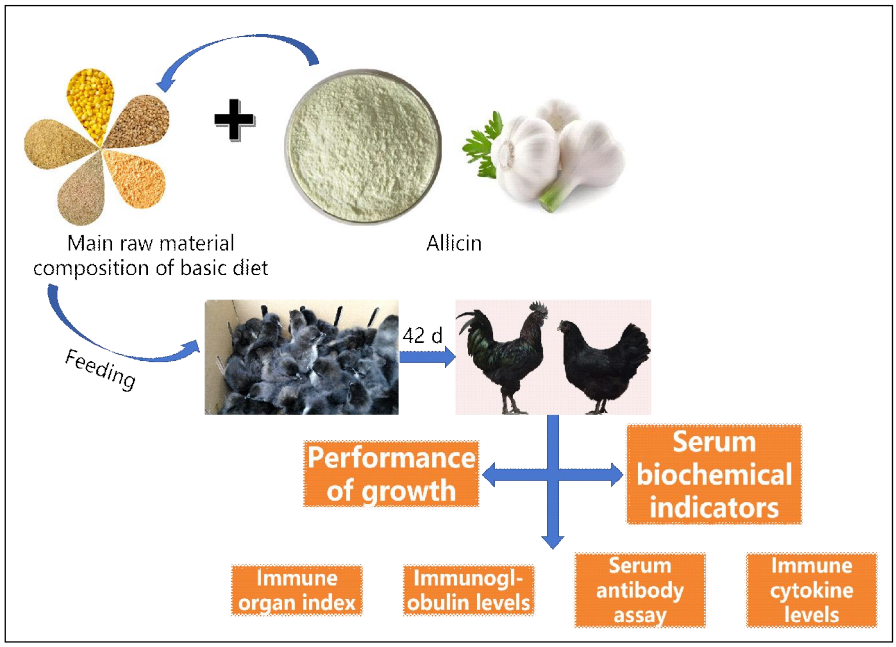


Fig 1: Technical roadmap of this study.

Immune function

Organ index determination

Upon completion of the experiment, six chickens (three males and three females) were randomly selected from each replicate. These chickens were weighed, bled and then euthanized. Their heart, liver, spleen, lung, kidney, muscular stomach, glandular stomach and thymus were harvested. The surface moisture of the immune organs was removed using clean filter paper and then the organs were weighed to calculate the immune organ index (weight of immune organ / live weight of the test chicken).

Immunoglobulin level

To assess immunoglobulin levels, 10 chickens (5 males and 5 females) from each replicate were chosen randomly for subwing vein sampling prior to slaughter. The collected non-anticoagulated blood was centrifuged at 3500 rpm for 10 min. The serum obtained was used to determine levels of immunoglobulin M (IgM) and immunoglobulin G (IgG) using enzyme-linked immunosorbent assays (ELISAs).

Serum antibody assay

Serum was extracted from the jugular vein of each chicken to analyze antibody titers against H5, H7 and Newcastle disease. These titers were identified using hemagglutination and hemagglutination inhibition tests.

Immune gene levels

This part of the study focused on the detection of specific immune-related genes, including interferon-γ (IFN-γ), toll-like receptor 3 (TLR3), toll-like receptor 15 (TLR15), interleukin-2 (IL-2) and interleukin-16 (IL-16), using RT-qPCR. Specific primers were designed using Primer Premier 5.0 software and synthesized by Chengdu Qingke Biotechnology Co., LTD. The sequences are listed in Table 2.

Serum biochemical indexes

Following the experiment, 10 chickens (5 males and 5 females) were randomly selected from each replicate to collect 5 mL blood from the jugular vein. The blood samples were left to stand for 20 min, then centrifuged at 3000 rpm for 10 min, to obtain the supernatant. The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), glucose (GLU), triglycerides (TGs), total protein (TP), albumin (ALB), globulin (GLB) and blood urea nitrogen (BUN) were determined using commercial kits.

Data analysis

Data from this experiment were collated and organized using Microsoft Excel. Intergroup variance analysis was performed with SPSS statistical software and the results are expressed as mean±standard deviation. Statistical graphs were created using GraphPad Prism version 8.0. $P<0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Effects of different levels of allicin powder on growth performance

At the beginning of the experiment, the average initial body weights of the 1-day-old chickens in groups A, B and C were not statistically different from those of controls (where $P<0.05$ unless specified otherwise) (Fig 2), ensuring comparability among the groups. After 42 days of feeding with different allicin supplementation levels, the average fasting body weights were higher in the experimental groups. Group B experienced the largest (and significant) increase. In terms of average daily feed intake and average daily weight gain, the experimental groups showed a slight increase compared to controls, while the feed conversion ratio slightly decreased; however, these differences were not statistically significant.

Effects of different levels of allicin powder on immune-related indicators

Effects of different levels of allicin powder on organ indices

Relative to controls, the gizzard index in group C was significantly higher as was the thymus index in group A. For other immune-related organs, including the heart,

Table 1: Composition indicators of the basic diet.

	Item	Content	
		1-21 days old	22-42 days old
Diet composition	corn/%	62.70	61.50
	soybean meal/%	25.00	17.00
	wheat bran/%	4.00	13.00
	fish meal/%	1.50	1.00
	rapeseed meal/%	3.00	4.00
	stone powder/%	1.20	1.20
	calcium hydrogen phosphate/%	1.30	1.00
	salt/%	0.30	0.30
	additive/%	1.00	1.00
	Total/%	100.00	100.00

liver, spleen, lungs, kidneys and bursa of Fabricius indices, no significant differences were observed between the groups (Fig 3).

Effects of different levels of allicin powder on immunoglobulins (Fig 4)

The serum levels of IgM were significantly higher in groups A and B than in controls. Similarly, IgG levels were significantly higher in all experimental groups than in controls.

Effects of different levels of allicin powder on immune performance (Fig 5)

The antibody titers against avian influenza H5 and H7 were higher in groups A, B and C than in controls, significantly so in groups B and C. Moreover, Newcastle disease antibody titers were significantly higher in groups B and C than in controls.

Effects of different levels of allicin powder on the expression of immune genes (Fig 6)

Relative to controls, the relative expression levels of IFN-γ mRNA were significantly lower in all experimental groups. The levels of TLR3 mRNA were significantly higher in groups B and C and those of TLR15 were significantly higher in group B. However, those of IL-2 and IL-16 were only slightly lower in all experimental groups.

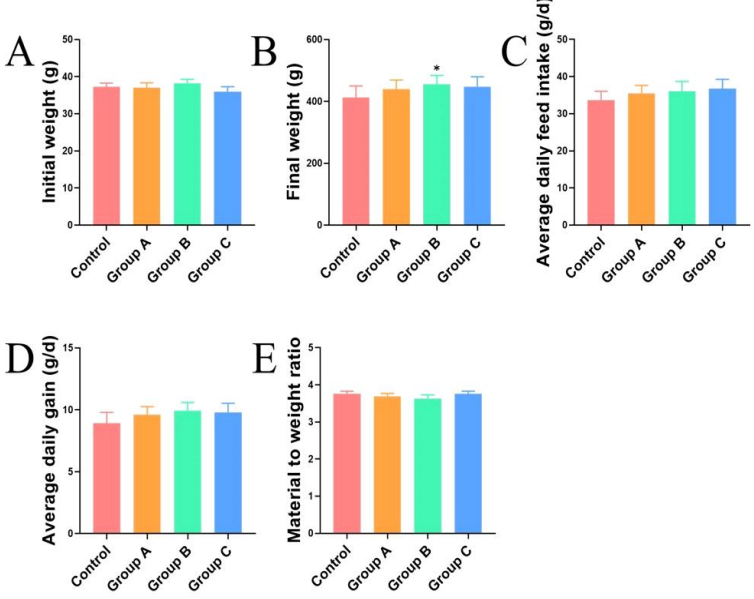
Effects of different levels of allicin powder on serum biochemical indicators (Fig 7)

Relative to controls, the serum levels of TGs were significantly higher in group A and BUN levels were significantly lower in groups B and C (being significantly lower in group B than in group C). No other indicators significantly differed among the groups.

Several studies have indicated that incorporating allicin into poultry diets can increase average daily feed intake and average daily gain, while simultaneously reducing the

Table 2: Primer parameters.

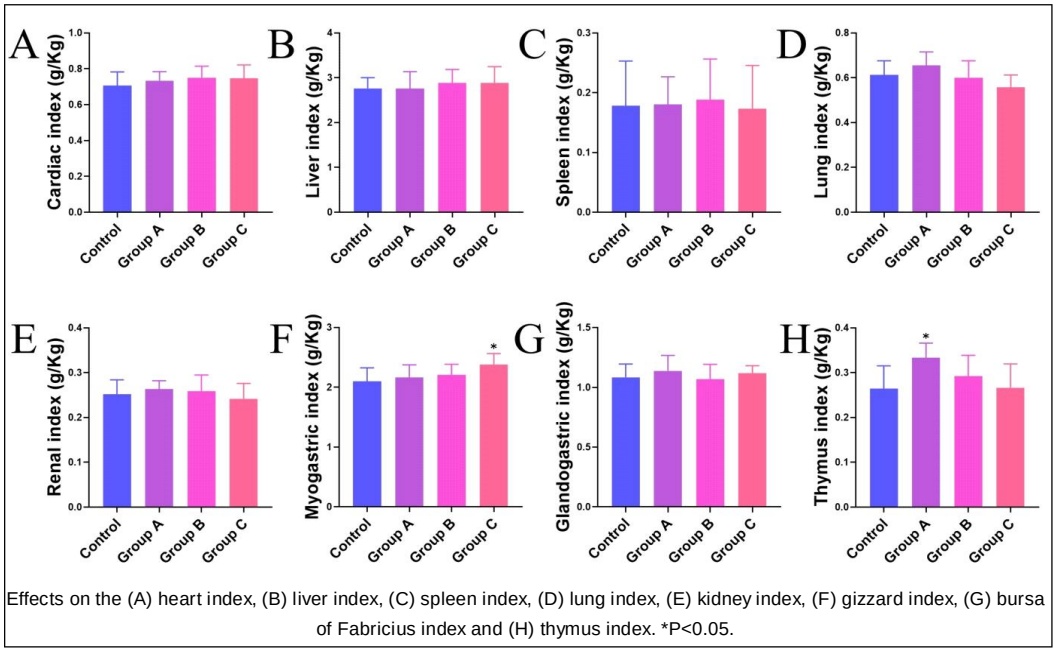
Gene	Primer (5'-3')
IFN-γ	F: AGCTGACGGTGGACCTATTATT
	R: GGCTTTGCGCTGGATTC
TLR3	F: TGGGATGCTCTATTCCTTGC
	R: CTTTAGGTGACTACAATCTGCCATT
TLR15	F: TGGGCTGTGGTATGTGAGAA
	R: AGATGCTCCTTCGTCCAGTC
IL-2	F: TCTGGGACCACTGTATGCTCT
	R: ACACCAGTGGGAAACAGTATCA
IL-16	F: TCCCTCTGCAAAATGGTCA
	R: TCGCGATCTCAGGTTGTGT
GAPDH	F: GGTGGTGCTAAGCGTGTTAT
	R: ACCTCTGTCATCTCTCCACA



(A) Comparison of the average initial body weight of 1-day-old chickens. (B) Comparison of the average fasting body weight of 42-day-old chickens. (C) Effects of different levels of allicin powder on the average daily feed intake. (D) Effects of different levels of allicin powder on average daily weight gain. (E) Effects of different levels of allicin powder on feed conversion ratio. *P<0.05.

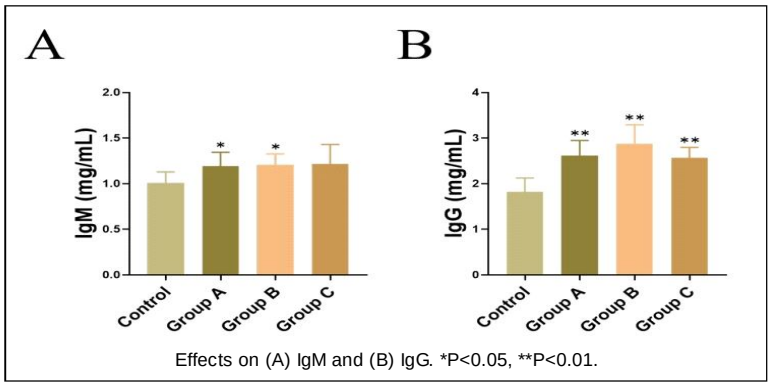
Fig 2: The effects of different levels of allicin powder on the growth performance of Muchuan black-bone chickens.

feed-to-gain ratio (Wang *et al.*, 2017; Chen *et al.*, 2021). This effect is potentially due to the ability of allicin to stimulate the oral and gastrointestinal chemoreceptors in birds, thereby activating the hypothalamic feeding center and improving feeding behaviors (Gong *et al.*, 2020). The immune organ index serves as an important metric for



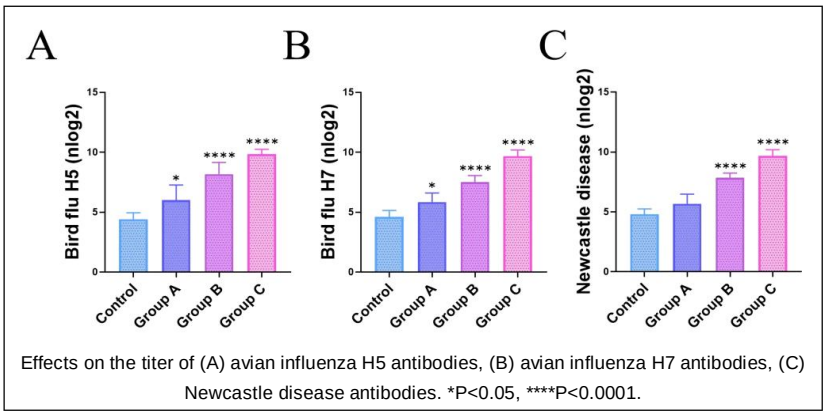
Effects on the (A) heart index, (B) liver index, (C) spleen index, (D) lung index, (E) kidney index, (F) gizzard index, (G) bursa of Fabricius index and (H) thymus index. *P<0.05.

Fig 3: Effects of different levels of allicin powder on immune organ indices in Muchuan black-bone chickens.



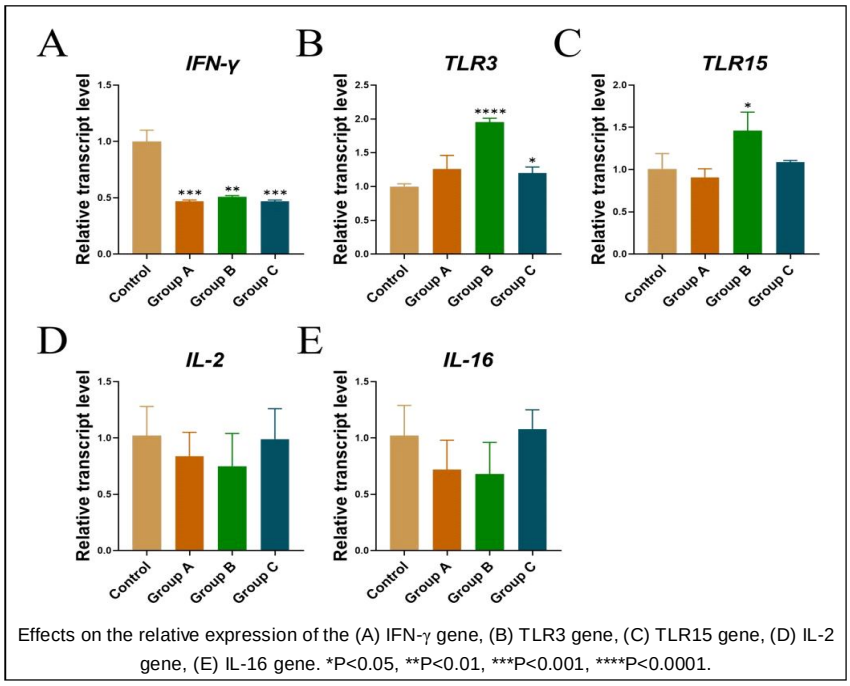
Effects on (A) IgM and (B) IgG. *P<0.05, **P<0.01.

Fig 4: Effects of different levels of allicin powder on immunoglobulins in Muchuan black-bone chickens.



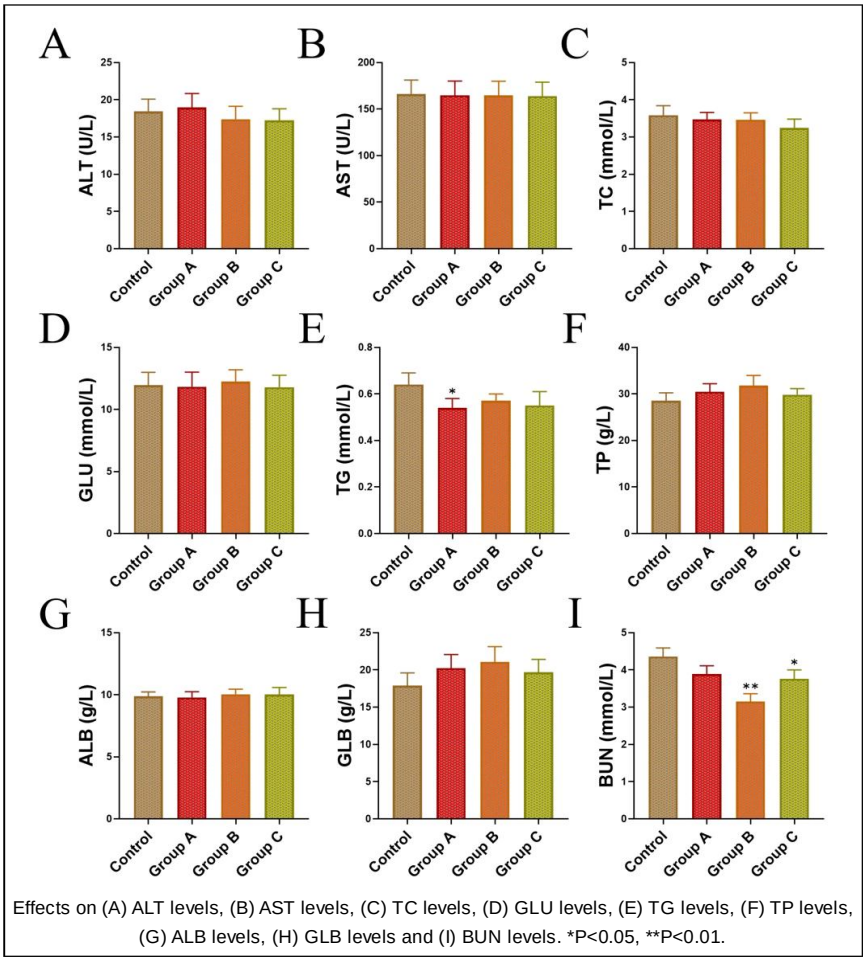
Effects on the titer of (A) avian influenza H5 antibodies, (B) avian influenza H7 antibodies, (C) Newcastle disease antibodies. *P<0.05, ****P<0.0001.

Fig 5: Effects of different levels of allicin powder on immune performance of Muchuan black-bone chickens.



Effects on the relative expression of the (A) IFN-γ gene, (B) TLR3 gene, (C) TLR15 gene, (D) IL-2 gene, (E) IL-16 gene. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Fig 6: Effects of different levels of allicin powder on the immune genes of Muchuan black-bone chickens.



Effects on (A) ALT levels, (B) AST levels, (C) TC levels, (D) GLU levels, (E) TG levels, (F) TP levels, (G) ALB levels, (H) GLB levels and (I) BUN levels. *P<0.05, **P<0.01.

Fig 7: Effects of different levels of allicin powder on serum biochemical indicators of Muchuan black-bone chickens.

assessing immune function in poultry, offering insight into the development of the immune system (Gong *et al.*, 2020). Additionally, the levels of immunoglobulins and the expression of immune genes are indicative of humoral and cellular immune functions, respectively (Silveira *et al.*, 2018). Blood biochemical indices provide a direct measurement of various physiological activities within the body (Xu *et al.*, 2015). Previous research has demonstrated the beneficial role of allicin in regulating serum characteristics and improving serum biochemical indices (Pathak *et al.*, 2020).

In the present study, compared to controls, the average fasting body weight of Muchuan black-bone chickens increased in the experimental groups. Notably, the increase was significant in the group fed a basal diet supplemented with 100 mg/kg allicin. The musogastric index in the group that received 200 mg/kg allicin and the thymus index in the group supplemented with 50 mg/kg allicin significantly increased. Serum IgM levels in the groups supplemented with 50 mg/kg and 100 mg/kg allicin were significantly higher than in controls and IgG levels were significantly higher across all experimental groups vs. controls. Moreover, the antibody titers for avian influenza H5 and H7 were higher in all test groups, with the increases in Newcastle disease antibody titers in the 100 mg/kg and 200 mg/kg allicin groups being significant. Compared to controls, the mRNA relative expression levels of IFN-γ in all experimental groups decreased, while those of TLR3 increased in the groups fed a basal diet supplemented with 100 mg/kg and 200 mg/kg allicin. Furthermore, TLR15 expression was upregulated in chickens that received 100 mg/kg allicin. In terms of serum biochemistry, TG levels in chickens on a basal diet with 50 mg/kg allicin significantly increased and BUN levels in groups supplemented with 100 mg/kg and 200 mg/kg allicin were significantly decreased. These findings suggest that allicin improves the immune function of Muchuan black-bone chickens, although further research is needed to fully understand the impact of different allicin concentrations on immune organ indices.

CONCLUSION

Incorporating 50-200 mg/kg allicin into the diet of Muchuan black-bone chickens improves blood nutrient metabolism, increases growth and improves immune performance to a degree. These results provide a scientific basis for the development and application of allicin as an alternative to antibiotics in the production of these chickens. Furthermore, the findings lay a theoretical foundation for promoting the development and utilization of local poultry varieties, thereby potentially improving economic benefit.

ACKNOWLEDGMENT

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Conflict of interest

The author declares that they have no conflicts of interest in the research presented in this manuscript.

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