

## RESEARCH ARTICLE

# Effect of Antimicrobial Peptides from Fly Maggots on Immunity of Yellow-feathered Broilers

Zhengli WANG<sup>1,a</sup> Jiayu YAN<sup>1,b,†</sup> Shengjie GAO<sup>1,c,†</sup> Xiancun ZENG<sup>1,d</sup>  
Liyang DAI<sup>1,e</sup> Jungang WANG<sup>1,f(\*)</sup> Hong SHEN<sup>1,g(\*)</sup>

† These authors contributed equally to this study

<sup>1</sup> Shihezi University, Collage of Animal Science & Technology, Xinjiang 832003, P. R. CHINA

ORCID: <sup>a</sup> 0000-0002-6541-4111; <sup>b</sup> 0000-0001-5291-9818; <sup>c</sup> 0000-0002-0598-0840; <sup>d</sup> 0000-0002-4250-7817; <sup>e</sup> 0000-0002-6142-0952

<sup>f</sup> 0000-0002-5007-3123; <sup>g</sup> 0000-0002-6672-5480

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**Abstract:** The aim of this experiment was to investigate the effect of dietary supplementation of fly maggot antimicrobial peptides on immune biochemical indicators, the effect of immune organ index, small intestinal bacteria and small intestinal mucosal cell count in yellow-feathered broilers. Three hundred clinically healthy 1-day-old yellow-feathered broilers were randomly divided into 3 treatment groups, 10 replicates in each group and 10 broilers in each replicate. The groups were called hereafter as basal diet group (control group), basal diet + 100 mg/kg fly maggot antimicrobial peptide (AMPs) group, basal diet + 15% bacitracin zinc group. The test period was 42 d. The results showed that the contents of albumin, IgG and IgM in the antimicrobial peptide group were significantly higher than those in the control group ( $P<0.05$ ). The spleen index and thymus index in the AMPs group were higher than those in the control group and the bacitracin zinc group ( $P<0.05$ ). The number of *E. coli* in each segment of the small intestine of broilers in the control group was significantly higher than that in the AMPs group and the bacitracin zinc group ( $P<0.05$ ). The number of goblet cells in each segment of the small intestine of broilers in the bacitracin zinc group was higher than that in the AMPs group, but there was no significant difference ( $P>0.05$ ). In conclusion, these findings that revealed maggot antimicrobial peptides as dietary supplementation can improve the immunity of the yellow-feathered broilers.

**Keywords:** Broilers, Immune system organ index, Immunobiochemical parameters, Intestinal bacteria, Maggot antimicrobial peptide

## Sinek Larvalarından Elde Edilen Antimikrobiyal Peptidlerin Sarı Tüylü Piliçlerin Bağışıklığı Üzerine Etkisi

**Öz:** Bu çalışmanın amacı, sinek larvalarından elde edilen antimikrobiyal peptitlerinin sarı tüylü piliçlerin diyetine takviyesinin bağışıklık biyokimyasal göstergeleri, bağışıklık organ indeksi, ince bağırsak bakterileri ve ince bağırsak mukozal hücre sayısı üzerine etkisini araştırmaktır. Klinik olarak sağlıklı 300 adet 1 günlük sarı tüylü civciv, her grupta 10 hayvan ve her grubun 10 tekrarı olmak üzere rastgele 3 gruba ayrıldı. Gruplar böylelikle, bazal diyet grubu (kontrol grubu), bazal diyet + 100 mg/kg sinek larvası antimikrobiyal peptid (AMP) grubu ve bazal diyet + %15 basitrasin çinko grubu olarak adlandırıldı. Deney süresi 42 gün olarak gerçekleştirildi. Sonuçlar, AMP grubunun albümin, IgG ve IgM seviyesinin kontrol grubuna oranla önemli ölçüde yüksek olduğunu gösterdi ( $P<0.05$ ). AMP grubunun dalak ve timus indeksi, kontrol ve basitrasin çinko grubundan daha yüksekti ( $P<0.05$ ). Kontrol grubuna ait piliçlerin ince bağırsak kısımlarındaki *E. coli* sayısı, AMP ve basitrasin çinko grubundan önemli ölçüde daha yüksekti ( $P<0.05$ ). Basitrasin çinko grubuna ait piliçlerin ince bağırsağının kısımlarındaki goblet hücrelerinin sayısı AMP grubundan daha fazlaydı, ancak anlamlı bir fark yoktu ( $P>0.05$ ). Sonuç olarak, diyet takviyesi olarak kullanılan larval antimikrobiyal peptitlerin ortaya çıkaran bu etkinlikleri, sarı tüylü piliçlerde bağışıklığı güçlendirebilir.

**Anahtar sözcükler:** Piliç, İmmün sistem organ indeksi, İmmünobiyokimyasal parametreler, Bağırsak bakterileri, Larval antimikrobiyal peptid

## INTRODUCTION

In the poultry breeding industry, the massive use of antibiotics will cause increased drug resistance of strains, and drug residues appear in by-products such as eggs, which

seriously threaten human health. Therefore, a green, safe and pollution-free antibiotic alternative is eagerly sought. During the last years, host antimicrobial peptides have been recognized as key mediators of the innate immune response in many vertebrate species, providing the first line

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(\*) Corresponding Author

Tel: +86 13289936976 (H. Shen) +86 13289937169 (J. Wang) Fax: +86 09932058077

E-mail: shenhong98@163.com (H. Shen) wangjung98@163.com (J. Wang)



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of defense against potential pathogens <sup>[1,2]</sup>. Antimicrobial peptides (AMPs) are important small peptides of the innate immune system of the body, which are widely present in organisms in nature and can effectively improve the performance of livestock and poultry and enhance immunity <sup>[3-5]</sup>, and they have a very broad application prospect as a new antibiotic alternative. Currently, there are 2961 antimicrobial peptides in the Antimicrobial Peptide Database (APD), of which as many as 40 fly antimicrobial peptides have been isolated. Antimicrobial peptides from fly maggots have the advantages of high antibacterial activity, wide antibacterial spectrum, and green safety without residues <sup>[6,7]</sup>. We also show that the CATH peptides 1, 2, 3 and their amide-modified structures possess potent antimicrobial activities against both Gram-positive and Gram-negative pathogens, with these bacteria being affected to different extents <sup>[8]</sup>. The presence of the antimicrobial peptides in a broad range of tissues and their largely enhanced expression during development is suggestive of their potentially important role in early host defense and disease resistance of chickens. Nowadays, fly maggot antimicrobial peptides have been widely used in poultry disease treatment and aquatic research, but they have not been found to be used as feed additives in broiler humoral immunity and intestinal health <sup>[9]</sup>.

Therefore, this experiment intends to add fly maggot antimicrobial peptide into the diet of yellow-feathered broilers, study its effects on immune biochemical parameters, immune organ index, small intestinal bacteria and small intestinal mucosal cell count in broilers, determine its appropriate amount in the diet, and finally lay a theoretical foundation for the application of fly maggot antimicrobial peptide replacement antibiotics in the poultry breeding industry.

## MATERIAL AND METHODS

### Ethical Statement

The study was approved by the Animal Experimentation Ethics Committee of the School of Animal Science and Technology, Shihezi University. All chickens were kept experimentally and euthanized in strict accordance with the guidelines of the committee. During the test, all efforts were made to minimize the suffering of the animals.

### Animals, Experimental Design and Feed

In this study, 300 yellow-feathered broilers with similar healthy body weight were randomly divided into 3 treatment groups, 10 replicates in each group, 10 broilers in each group, which were called hereafter as basal diet group (control group), basal diet + 100 mg/kg fly maggot antimicrobial peptides group (AMPs), and basal diet + 15% bacitracin zinc group. Corn-soybean meal diet was used in the experiment, and the basal diet was prepared according

to The National Research Council (NRC) (1994) broiler nutritional standard, and its composition and nutritional level were listed in *Table 1*. All tests were performed in the same chicken house, and the house temperature was monitored thermostatically throughout the study. The temperature, which was 32-35°C on the first day, was lowered and maintained gradually at 22°C for the last two weeks. The artificial light program was implemented in accordance with commercial conditions (23 h of lighting throughout the experiment per day). The chickens had free access to food and water. Other immunization and disinfection measures were performed in strict accordance with the farm procedures, and the test period was 42 days. The basal diet used in this experiment was purchased from Xinjiang Tiankang Feed Technology Co., Ltd. (China). Antimicrobial peptides used in the study were supplied from a commercial company (Guangzhou Yingbao Biotechnology Co., Ltd., China). 15% bacitracin zinc premix was purchased from Lukang Biotechnology Co., Ltd. (China).

### Immune Biochemical Indicators

At the end of the experiment, blood samples were taken from the wing vein of the animals to the vacuum blood collection tube, allowed to stand for 24 h, and then centrifuged at 3000 rpm/min for 30 min to collect serum

Items	Content, %
<b>Ingredients</b>	
Corn	62.85
Soymeal bean	31.50
Soyoil bean	1.30
Limestone	1.50
CaHPO <sub>4</sub>	1.50
NaCl	0.35
Met	0.08
Premix	0.92
Total	100
<b>Nutrient levels</b>	
ME/(MJ/Kg)	12.08
CP	19.00
Ca	0.95
AP	0.41
Lys	0.923
Met	0.393
<i>The premix provided the following per kg of diet (without antibiotics): Cu: 5 mg, Fe: 75 mg, Mn: 56 mg, I: 0.35 mg, Se: 0.14 mg, Zn: 38 mg, Vit. A: 1420 IU, Vit D<sub>2</sub>: 190 IU, Vit. E: 9.5 IU, Vit. K: 0.38 mg, Riboflavin: 3.4 mg, Pantothenic acid: 9.4 mg, Nicotinic acid: 26 mg, Vit. B<sub>12</sub>: 0.009 mg, Choline: 1225 mg, Biotin: 0.14 mg, Folic acid: 0.52 mg, Thioflavin: 1.0 mg, Pyridoxic acid: 2.8 mg</i>	

for determination of total protein and albumin. Total protein and albumin contents were determined using commercial kits (Nanjing Jiancheng Technology Co., Ltd., China). After the chickens were euthanized, the livers were separated, washed with normal saline, 2 g of the analytical balance was weighed, cut with scissors, placed into a beaker to add 10 mL of normal saline, placed into a homogenization mechanism to prepare the homogenate, centrifuged at 2500 r/min for 10 min, and the supernatant was taken and stored at  $-20^{\circ}\text{C}$  for the determination of serum immunoglobulin, that is, IgG, IgM, and IgA contents using ELISA kits (Shanghai Yanchun Biotechnology Co., Ltd., China).

### Immune Organ Index

On the 42<sup>nd</sup> day of the feeding, the yellow-feathered broilers were weighed and slaughtered. The thymus, spleen, and bursa of Fabricius of the animals were harvested, and the adipose tissue on each organ was removed. Surface water was cleaned with filter paper, and each immune organ was weighed and calculated as follows.

Immune Organ Index = Immune Organ Weight/Pre-Slaughter Live Weight

### Number of Intestinal Bacteria

After the euthanasia, 1 g of duodenal, jejunal and ileal contents was quickly collected into a microcentrifuge tube and suspended with physiological saline to obtain a  $1 \times 10^{-7}$  sub-dilution. According to the dilution selected for the preliminary experiment, 40  $\mu\text{L}$  of the sub-dilution was inoculated onto the Eosin Methylene Blue (EMB) and Salmonella Shigella (SS) medium for *Escherichia coli* and *Salmonella* spp. culture, respectively. For the cultivation of the *Lactobacillus* spp., the sub-dilution was inoculated onto the Man-Rogosa-Sharpe (MRS) agar plates and incubated at microaerophilic conditions at  $37^{\circ}\text{C}$  for 48 h.

### Small Intestinal Mucosal Cell Count

The left and right intestinal tissues of the 5 cm group were respectively taken from the duodenum, jejunum and ileum, which were soaked and fixed in 4% paraformaldehyde. The fixed samples were dehydrated with 70%, 85%, 95%,

100% and 100% ethanol, respectively. The samples were cleared with xylene and embedded with an embedding machine after wax transmission. The samples were sectioned with a microtome and stained with hematoxylin and eosin. Five sections were randomly selected, and images were collected using an HMIAS-200 optical microscope color image analysis system under a (10 x 40) x microscope field of view. The changes in the number of lymphocytes and cup-shaped cells were counted.

### Statistical Analysis

The one-way analysis of variance (ANOVA) method was used for the statistical analysis of the groups. Statistical differences and trend analysis were considered significant at  $P \leq 0.05$ . The statistical analysis was done with the SPSS software package.

## RESULTS

The effect of antimicrobial peptides from fly maggots on immune biochemical parameters in the yellow-feathered broilers was investigated. It can be seen from *Table 2* that there was no significant difference in the serum total protein and IgA contents among the study groups ( $P > 0.05$ ). The contents of albumin, IgG and IgM in the AMPs group and the bacitracin zinc group were higher than those in the control group ( $P > 0.05$ ). The contents of IgM in the AMPs group were higher than those in the bacitracin zinc group and the control group ( $P > 0.05$ ). There was no significant difference ( $P > 0.05$ ) in the contents of albumin and IgG in the AMPs group and bacitracin zinc group.

The changes in the immune organ index of the fly maggot antimicrobial peptide of yellow-feathered broilers were shown in *Table 3*. The spleen index and thymus index in the AMPs group were higher than those in the control group and the bacitracin zinc group ( $P < 0.05$ ). Compared with the control group, there was no significant difference in bursa of Fabricius index in the bacitracin zinc group and the AMPs group ( $P > 0.05$ ).

The changes in the number of *E. coli* and *Lactobacilli* in different parts of the chicken small intestine were shown in *Fig. 1* and *Fig. 2*. The number of *E. coli* in the duodenum,

**Table 2.** Effect of maggot antimicrobial peptides on the biochemical immune indexes of the yellow-feathered broilers

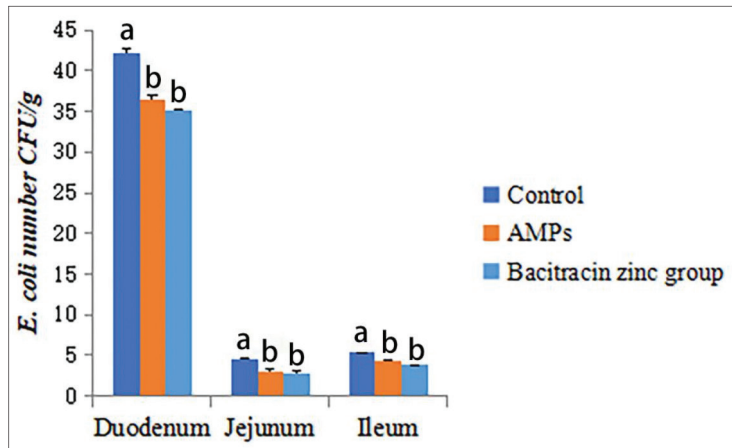
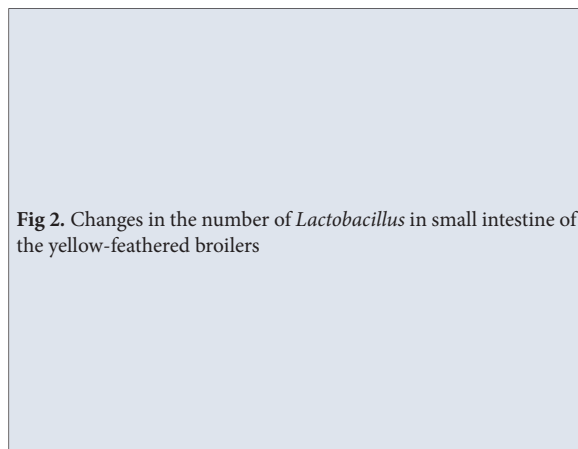
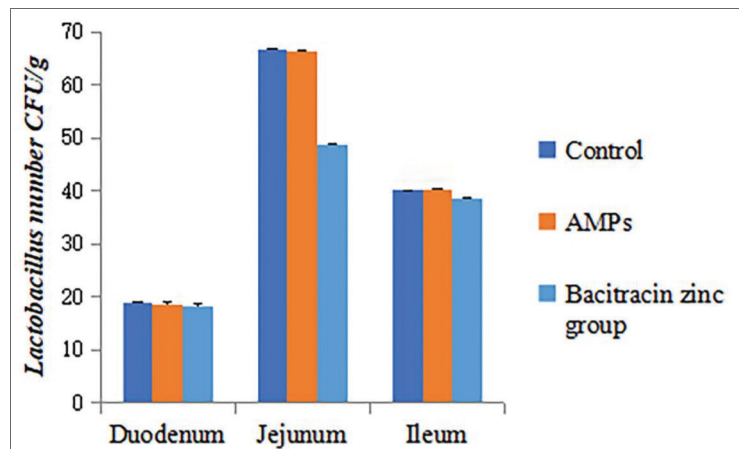
Parameters	Groups		
	Control	AMPs	Bacitracin Zinc Group
Total Protein (mg/mL)	38.24±0.66	38.89±0.10	38.31±0.49
Albumin (mg/mL)	21.99±0.87 <sup>b</sup>	23.10±0.23 <sup>a</sup>	22.35±0.15 <sup>a</sup>
IgG (mg/mL)	3.39±0.11 <sup>b</sup>	4.35±0.21 <sup>a</sup>	4.12±0.07 <sup>a</sup>
IgM (mg/mL)	0.76±0.03 <sup>c</sup>	1.13±0.06 <sup>a</sup>	0.85±0.06 <sup>b</sup>
IgA (mg/mL)	0.69±0.04	0.76±0.02	0.73±0.03

<sup>a,b,c</sup> Means within a row followed by the different superscripts differ significantly ( $P < 0.05$ )

Table 3. Effect of maggot antimicrobial peptides on the immune organ index of the yellow-feathered broilers

Parameters	Groups		
	Control	AMPs	Bacitracin Zinc Group
Thymus index (g/kg)	3.53±0.38 <sup>b</sup>	3.91±0.35 <sup>a</sup>	3.50±0.22 <sup>b</sup>
Spleen index (g/kg)	1.58±0.66 <sup>b</sup>	1.69±0.29 <sup>a</sup>	1.55±0.58 <sup>b</sup>
Bursa index (g/kg)	1.31±0.11	1.61±0.43	1.31±0.25

<sup>a,b,c</sup> Means within a row followed by the different superscripts differ significantly ( $P < 0.05$ )

Fig 1. Changes in the number of *E. coli* in the small intestine of the yellow-feathered broilers. Differences in <sup>a,b,c</sup> means significant difference between the groups after different superscripts ( $P < 0.05$ )Fig 2. Changes in the number of *Lactobacillus* in small intestine of the yellow-feathered broilers

jejunum, and ileum of broilers in the AMPs and bacitracin zinc groups were lower than that in the control group ( $P < 0.05$ ). However, there was no significant difference in the number of *Lactobacillus* in the small intestine in each segment ( $P > 0.05$ ).

The number of lymphocytes in the small intestine in the AMPs group was higher than that in the bacitracin zinc group and the control group. Moreover, the number of lymphocytes in the duodenum in the bacitracin zinc peptide group was higher than that in the control group. However, there was no difference between the bacitracin zinc group and the control group in the ileum of the small intestine ( $P > 0.05$ ). The influence of the antimicrobial peptides on the number of lymphocytes was given in Table 4.

The changes in the distribution of goblet cells in different parts of the small intestine of broilers were shown in Table 5. The number of goblet cells in the duodenum and jejunum of the small intestine was higher in the AMPs group than in the bacitracin zinc group and the control group ( $P < 0.05$ ). However, there was no difference in the number of *Lactobacillus* in the small intestine in each segment ( $P > 0.05$ ).

## DISCUSSION

Humoral immunity plays an indispensable and important role in animal immunity, and the health status of livestock and poultry is closely related to serum protein. The increase of serum total protein and albumin contents is the embodiment of vigorous protein metabolism, indicating



**Table 4.** Effects of each experimental group on the number of intestinal mucosal lymphocytes

Parameters	Groups		
	Control	AMPs	Bacitracin Zinc Group
Duodenum	101.00±0.02 <sup>c</sup>	108.00±0.14 <sup>a</sup>	104.00±0.44 <sup>b</sup>
Jejunum	89.00±0.02 <sup>b</sup>	99.00±0.37 <sup>a</sup>	89.00±0.29 <sup>b</sup>
Ileum	85.00±0.06 <sup>b</sup>	94.00±0.16 <sup>a</sup>	86.00±0.43 <sup>b</sup>

<sup>a,b,c</sup> Means within a row followed by the different superscripts differ significantly ( $P<0.05$ )

**Table 5.** Effects of each experimental group on the number of goblet cells in the chicken small intestinal mucosa

Parameters	Groups		
	Control	AMPs	Bacitracin Zinc Group
Duodenum	98.00±0.21 <sup>b</sup>	100.00±0.11 <sup>a</sup>	99.00±0.71 <sup>b</sup>
Jejunum	105.00±0.34 <sup>b</sup>	108.00±0.63 <sup>a</sup>	106.00±0.71 <sup>b</sup>
Ileum	124.00±0.83	127.00±0.11	125.00±0.53

<sup>a,b,c</sup> Means within a row followed by the different superscripts differ significantly ( $P<0.05$ )

that the absorption and utilization rate of amino acids and proteins in the body is improved. Immunoglobulins are the most important molecules in the immune response and play an important role in the process of anti-infection. Studies have shown<sup>[9,10]</sup> that the AMPs participate in the first immune defense and regulate body immunity through different mechanisms, which has attracted much attention. Wang et al.<sup>[11]</sup> showed that the addition of the antimicrobial peptides decreased the concentration of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 and effectively improved the performance, systematic inflammation, and improved fecal microbiota composition of the broilers. Jozefiak et al.<sup>[12]</sup> reported that dietary supplementation of fly maggot powder significantly enhanced the immune capacity of broilers, enhanced the body's immune response, and played a role in preventing livestock and poultry diseases. In this experiment, the contents of albumin, IgG and IgM in the AMPs group were significantly higher than those in the control group ( $P<0.05$ ), indicating that the antimicrobial peptide of the fly maggots can induce the activation of immune function in the yellow-feathered broilers, promote the synthesis of immunoglobulin, and maintain the active immune ability of the body, which is basically consistent with the above reports.

The immune organ index is an important reflection of the growth and development of the immune organs. The development status and function of the immune organs directly affect the immune level of poultry. At present, relevant studies have shown that the changes in the volume of immune organs are actually affected by the rate of apoptosis and cell proliferation of the lymphocytes<sup>[13]</sup>, and antimicrobial peptides can play an indispensable role in immune regulation and immune homeostasis<sup>[14]</sup>. The spleen index and the thymus index in the AMPs

group were higher than those in the control group and the bacitracin zinc group ( $P<0.05$ ). This indicates that the antimicrobial peptide of fly maggots could promote the development and maturation of the immune system organs and improve the immune activity of the yellow-feathered broilers. In addition, the antimicrobial peptides and bacitracin zinc showed different results possibly due to their different mechanisms of action. Yang et al.<sup>[15]</sup> showed that the antimicrobial peptides are capable of promoting systemic humoral immune responses of chickens at an early age. The increased content of the immunoglobulins in serum and the antibody-forming cells in the bursa of Fabricius strengthen the viability of chicken. In addition to the direct eradication of microorganisms, antimicrobial peptides may be used as a signal to modulate or amplify adaptive immune responses. Antimicrobial peptides serve as 'alarm' signals in mobilizing the immune system and activating innate and adaptive immune systems.

The homeostasis of the intestinal bacteria in poultry plays an important role in nutrition, immunity and metabolism<sup>[16]</sup>. The gastrointestinal tract of poultry consists of the esophagus, crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, colon, and cloaca. The poultry gastrointestinal tract is much shorter as compared to other mammals relative to their body length. Thus, microbiota that grows in such a small gastrointestinal tract with a relatively short transit time requires unique adaptations to adhere to the mucosal wall and proliferation. The ceca has a lower passage rate and is favorable to diverse groups of bacteria, which affect nutrient utilization and the overall health of poultry<sup>[17]</sup>. According to Hirsch et al.<sup>[18]</sup>, antimicrobial peptides from rat-tailed maggots of the drone fly *Eristalis tenax* show potent activity against multidrug-resistant Gram-negative bacteria. And

novel AMPs highlight the potent and broad spectrum of antibacterial activity, a safe and stable tryptophan-rich amphiphilic peptide, called WRK-12, has a broad spectrum of antimicrobial activity against a variety of multidrug-resistant bacteria, including Methicillin-resistant *Staphylococcus aureus* (MRSA), colistin-resistant and tigecycline-resistant *E. coli* [19]. At present, although no exact mechanism has been found for the AMPs to affect intestinal bacteria, in explaining their antibacterial activity it is attributed to the different surface charges of peptides and pathogens. Specifically, the AMPs are positively charged and can attach to negatively charged cell membranes by electrostatic interaction. Thereby, they physically disrupt the phospholipid bilayer of the bacteria by blocking enzyme activity or inhibiting the synthesis of proteins and nucleic acids [20,21]. At the same time, the AMPs can selectively inhibit the growth of bacteria in the intestine, which may indicate a significant competitive advantage of the AMPs compared with the antibiotics [22,23]. Furthermore, antimicrobial peptides are critical components of host defense limiting bacterial infections at the gastrointestinal mucosal surface. Rowan et al. [24] found through computer model studies that the synthetic form of a chicken novel beta-defensin identified is active against predominantly intestinal pathogens. The mucosal barrier formed by cationic antimicrobial peptides (CAMPs) is believed to be crucial for host protection from pathogenic gut infection [25]. Daneshmand et al. [26] showed that the AMPs increased the population of *Lactobacillus* spp. and harmful bacteria challenged in the ileum of *E. coli*-chickens.

Immune-related cells in the intestinal mucosa are the first cells in contact with the body by pathogenic microorganisms and play a protective role during the infection, forming an epithelial mechanical cleaning barrier and mucosal immune barrier to resist the bacterial invasion. These cells are mainly composed of mast cells, intraepithelial lymphocytes, lamina propria lymphocytes and goblet cells. Lymphocytes have a role in protecting the intestinal mucosal immune system, and many autoimmune diseases and intestinal diseases in animals are associated with decreased lymphocyte numbers and dysfunction [27-29]. Goblet cells are glandular-type cells that can secrete glycoproteins and play an important role in intestinal immunity before passive immunity is established in neonatal animals [30,31]. The results showed that dietary supplementation of the antimicrobial peptides was able to significantly increase the number of intestinal intraepithelial lymphocytes and villus height in the duodenum and jejunum of chickens at 28, 42 and 56 days [32].

The results of this experiment revealed that dietary supplementation of the fly maggot antimicrobial peptide could significantly reduce the number of *E. coli* in the duodenum, jejunum, and ileum of the yellow-feathered

broilers, while effectively increased the number of *Lactobacilli* spp. in each segment of the small intestine. The number of the lymphocytes and goblet cells in each segment of the small intestine in the AMPs group was significantly higher than that in the control group, which was basically consistent with the above reports, indicating that the fly maggot antimicrobial peptide could effectively regulate the number of beneficial and harmful bacteria in the intestine, improve the intestinal microecological environment, and facilitate the rapid establishment and maintenance of intestinal microbial balance in the yellow-feathered broilers, which is of great significance for body homeostasis.

In conclusion, the addition of the antimicrobial peptides of the fly maggots in the basal diet could significantly increase the serum globulin content, promotes the development of immune organs, reduces the number of intestinal harmful bacteria such as *E. coli*, increases the number of beneficial bacteria such as *Lactobacilli* spp., improves the intestinal epithelial cells, and improves the body's immunity in the yellow-feathered broilers. Therefore, future work and research should be tailored to a better understanding of the mechanisms of action of antimicrobial peptides to investigate their full potential in the poultry farming industry.

#### AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

#### CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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#### AUTHOR CONTRIBUTIONS

HS and JW conceived and supervised this study. ZW completed the main experimental content. JY and SG collected and analyzed data. LD and XX wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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