Effects of Grape Proanthocyanidins on Growth Performance and Immune Function in Broiler Chickens Infected with Eimeria tenella (Postprint)

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Abstract

This experiment aimed to investigate the effects of dietary grape proanthocyanidins (GPC) supplementation on growth performance and immune function in broiler chickens infected with Eimeria tenella. A total of 350 one-day-old healthy Cobb-500 broiler chickens (male chicks) were randomly divided into 5 groups with 7 replicates per group and 10 birds per replicate. The blank control group and negative control group were fed the basal diet, while the treatment groups were fed experimental diets supplemented with 7.5, 15.0, and 30.0 mg/kg GPC based on the basal diet. At 14 days of age, each broiler chicken in the negative control group and the three treatment groups was orally challenged with 5$×$10^4 Eimeria tenella oocysts, while each bird in the blank control group was administered the same volume of physiological saline. The experimental period was 42 days, divided into two phases: the starter phase (1–21 days of age) and the grower phase (22–42 days of age). The results showed: 1) Compared with the blank control group, the negative control group exhibited significantly reduced average daily gain (ADG) at all experimental phases ($P<0.05$) and significantly increased feed-to-gain ratio (F/G) at 22–42 and 1–42 days of age ($P<0.05$); dietary supplementation with 7.5 and 15.0 mg/kg GPC significantly ameliorated the Eimeria tenella challenge-induced reductions in ADG and increases in F/G at 22–42 and 1–42 days of age ($P<0.05$); whereas dietary supplementation with 30.0 mg/kg GPC significantly decreased ADG and average daily feed intake (ADFI) at 22–42 days of age and ADG at 1–42 days of age ($P<0.05$). 2) Compared with the negative control group, dietary GPC supplementation significantly reduced cecal lesion scores, bloody fecal droppings count, and oocyst shedding in broiler chickens ($P<0.05$). 3) Dietary GPC supplementation significantly suppressed the Eimeria tenella challenge-induced increase in spleen index in broiler chickens (P<0.05); Eimeria tenella challenge caused a significant increase in T lymphocyte transformation rate (P<0.05), which was further increased by dietary supplementation with 7.5 and 15.0 mg/kg GPC (P>0.05); dietary supplementation with 7.5 and 15.0 mg/kg GPC increased B lymphocyte transformation rate (P>0.05); Eimeria tenella challenge induced a significant increase in peripheral blood $\gamma\delta$ T cell proportion (P<0.05), which was further increased by dietary supplementation with 15.0 mg/kg GPC (P>0.05). 4) Compared with the blank control group, Eimeria tenella challenge significantly increased intraepithelial lymphocyte (IEL) count and cecal mucosal secretory immunoglobulin A (sIgA) level (P<0.05), which were further increased by dietary supplementation with 7.5 and 15.0 mg/kg GPC (P<0.05). In conclusion, dietary GPC supplementation can enhance immune function and alleviate growth suppression caused by Eimeria tenella infection in broiler chickens, with 7.5 and 15.0 mg/kg GPC showing better anticoccidial effects.

Full Text

Effects of Grape Procyanidins on Growth Performance and Immune Function of Broiler Chicks Challenged with *Eimeria Tenella*

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Abstract: This study investigated the effects of dietary grape procyanidins (GPC) on growth performance and immune function in broiler chicks challenged with *Eimeria tenella*. A total of 350 one-day-old healthy male Cobb-500 broiler chicks were randomly allocated to five groups with seven replicates per group and ten chicks per replicate. The blank control group and negative control group were fed a basal diet, while experimental groups received the basal diet supplemented with 7.5, 15.0, or 30.0 mg/kg GPC. At 14 days of age, chicks in the negative control group and three experimental groups were orally challenged with 5$×10^{4}$ *Eimeria tenella* oocysts per bird, while the blank control group received an equivalent volume of saline. The 42-day trial period was divided into two phases: early phase (1–21 days) and late phase (22–42 days). The results showed: (1) Compared with the blank control, the negative control group exhibited significantly reduced average daily gain (ADG) across all stages and significantly increased feed-to-gain ratio (F/G) during 22–42 days and 1–42 days (P<0.05). Dietary supplementation with 7.5 and 15.0 mg/kg GPC significantly ameliorated the reduction in ADG and increase in F/G induced by *Eimeria tenella* challenge during 22–42 days and 1–42 days (P<0.05). However, supplementation with 30.0 mg/kg GPC significantly decreased ADG and average daily feed intake (ADFI) during 22–42 days and ADG during 1–42 days ($P<0.05$). (2) Dietary GPC supplementation significantly reduced cecal lesion scores, bloody stool pile counts, and oocyst excretion numbers compared with the negative control ($P<0.05$). (3) GPC supplementation significantly inhibited the increase in spleen index induced by *Eimeria tenella* challenge ($P<0.05$). The T lymphocyte transformation rate was significantly elevated by coccidial challenge ($P<0.05$), and further increased by 7.5 and 15.0 mg/kg GPC supplementation ($P>0.05$). Supplementation with 7.5 and 15.0 mg/kg GPC increased B lymphocyte transformation rate ($P>0.05$). The proportion of $\gamma\delta$ T cells in peripheral blood was significantly increased by *Eimeria tenella* challenge ($P<0.05$), and further elevated by 15.0 mg/kg GPC supplementation ($P>0.05$). (4) Compared with the blank control, coccidial challenge significantly increased ileal intraepithelial lymphocyte (IEL) numbers and cecal mucosal secretory IgA (sIgA) levels ($P<0.05$), which were further enhanced by 7.5 and 15.0 mg/kg GPC supplementation ($P<0.05$). In conclusion, dietary GPC supplementation improves immune function and alleviates growth inhibition in broiler chicks challenged with *Eimeria tenella*, with 7.5 and 15.0 mg/kg GPC showing the best anticoccidial effects.

Keywords: grape procyanidins; broiler chicks; *Eimeria tenella*; growth performance; immune function

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Coccidia are protozoan parasites that inhabit intestinal cells of poultry. Coccidiosis has become one of the most costly diseases, causing annual global economic losses of up to £2 billion [1], with annual prevention and treatment costs in China reaching 0.6–1.8 billion RMB [2]. Currently, coccidiosis control still relies primarily on anticoccidial drugs, but intensive use over the past 50 years has led to severe drug resistance [3]. Therefore, finding safe and reliable alternatives to anticoccidial drugs is urgently needed.

Grape procyanidins (GPC) are naturally occurring plant polyphenols and the main active components in grape by-products. Numerous studies have demonstrated that GPC possess strong antioxidant activity [4], effectively inhibiting doxorubicin-induced myocardial oxidative damage in rats [5], significantly attenuating acetaminophen-induced hepatotoxicity and hepatocellular DNA damage in mice [6], and alleviating dimethylnitrosamine (DMNA)-induced splenocyte toxicity [7]. Research has also shown that coccidial invasion causes severe oxidative stress in poultry intestines [8], while GPC can inhibit oxidative stress by activating the antioxidant enzyme system, thereby resisting coccidial damage [9].

Studies have indicated that GPC also exerts beneficial effects on immune function [10]. Gessner et al. [11] found that dietary supplementation with grape seed extract and grape marc extract significantly inhibited nuclear factor-kappa B activity in piglet duodenum and reduced enteritis incidence. Hogan et al. [12] reported that grape marc extract (rich in GPC) significantly decreased plasma C-reactive protein concentrations in high-fat diet-induced obese inflammatory rats. Magrone et al. [13] found that wine polyphenols promoted the secretion of interleukin-12 (IL-12), interferon-γ (IFN-γ), interleukin-10 (IL-10), and immunoglobulins, alleviating immune disorders, and also promoted nitric oxide release to protect infected tissues. Whether GPC' s immunomodulatory functions can help poultry combat coccidial infection remains unclear.

Coccidia primarily damage poultry intestines, which are vital organs for digestion, absorption, and immunity; thus, coccidial infection inevitably causes growth suppression and immune disorders. McDougald et al. [14] found that dietary supplementation with 2% muscadine pomace significantly reduced intestinal lesion scores caused by coccidia. Wang et al. [9] also found that GPC could alleviate growth suppression in broilers caused by coccidia, though the mechanism remains unclear. Our previous research showed that GPC could promote lymphocyte proliferation and activation in vitro [15] and improve immune function in broilers when added to wheat-soybean meal-based diets [16], suggesting that GPC may resist coccidial infection by enhancing immune function. Additionally, our studies have demonstrated that cecal tonsil sIgA and intraepithelial lymphocytes (IEL) play important roles in poultry resistance to coccidia [17], but whether GPC can resist coccidia by activating intestinal immunity remains poorly understood.

This study aimed to investigate the effects of GPC on growth performance, intestinal lesion severity, immune organ indices, lymphocyte proliferation and activation, cecal mucosal sIgA levels, and ileal IEL proliferation in broiler chicks challenged with *Eimeria tenella*, to preliminarily reveal the mechanisms underlying GPC's anticoccidial effects and provide a basis for its application in broiler production.

1.1 Experimental Materials

GPC was provided by Tianjin Jianfeng Natural Product R&D Co., Ltd., with a procyanidin content of 99.47%, comprising oligomeric procyanidins (OPC) at 65.19%, monomeric procyanidins at 9.88%, and polymeric procyanidins at 24.93%.

1.2 Experimental Design and Diets

A total of 350 one-day-old healthy male Cobb-500 broiler chicks were randomly divided into five groups with seven replicates per group and ten chicks per replicate. The blank control group and negative control group were fed the basal diet, while experimental groups received the basal diet supplemented with 7.5, 15.0, or 30.0 mg/kg GPC. At 14 days of age, chicks in the negative control group and three experimental groups were orally challenged with 5$\times$10^{4}$ *Eimeria tenella* oocysts per bird, while the blank control group received an equivalent volume of saline. The 42-day experimental period was divided into two phases: early phase (1–21 days) and late phase (22–42 days).

Based on NRC (1994) and NY/T 33-2004, a wheat-soybean meal basal diet was formulated. The composition and nutrient levels of the basal diet are shown in Table 1 .

Table 1 Composition and nutrient levels of basal diets (air-dry basis) %

Items	Content
Ingredients	
Wheat	21.00 (21.13)
Soybean meal	19.00 (19.18)
Soybean oil	1.00 (1.03)
$CaHPO_4$	0.68 (0.65)
$CaHCO_3$	0.90 (0.88)
NaCl	0.62 (0.60)
DL-Met	0.21
L-Lys · HCl	0.19
Thr	0.18
Vitamin premix[1]	0.03
Mineral premix[2]	0.15
Choline chloride	0.10
Phytase	0.01
NSP enzyme	0.01
Carrier	0.02
Total	100.00
Nutrient levels[3]	
ME (MJ/kg)	12.50
CP	21.00
Ca	0.90
TP	0.68
AP	0.45
Lys	1.20
Met	0.50
Met+Cys	0.85
Thr	0.80
Trp	0.22

[1] Vitamin premix provided per kg of diet: VA 12,500 IU, VD_3 2,500 IU, VE 15 IU, VK_3 2.65 mg, VB_1 2 mg, VB_2 6 mg, VB_{12} 0.025 mg, biotin 0.35 mg, folic acid 1.25 mg, calcium pantothenate 12 mg, niacin 50 mg.
[2] Mineral premix provided per kg of diet: Cu 8 mg, Zn 75 mg, Fe 80 mg, Mn 100 mg, Se 0.15 mg, I 0.35 mg.
[3] Nutrient levels in parentheses were measured values, while others were calculated values.

1.3 Management

Chicks were raised in single-tier wire cages under natural light supplemented with artificial lighting at 30 lx intensity: 24 h light during days 1–7, and 23 h light with 1 h dark after day 8. House temperature was maintained at 33–35°C during week 1, then decreased by 2°C weekly until reaching 21°C. Chicks had ad libitum access to feed and water and received routine vaccinations.

1.4.1 Growth Performance

Initial chick body weight was recorded at the start of the experiment. Fasted body weight was measured by replicate before 09:00 on days 14, 21, and 42. Daily feed consumption was recorded to calculate feed consumption during 1-14, 15-21, and 22–42 days, from which average daily gain (ADG), average daily feed intake (ADFI), and feed-to-gain ratio (F/G) were calculated.

1.4.2 Bloody Stool Piles, Cecal Lesion Scores, and Oocyst Excretion

Bloody stool piles were counted on day 19 (5 days post-challenge). On day 21 (7 days post-challenge), one chick per replicate was randomly selected and euthanized for cecal lesion scoring according to Johnson and Reid [18]. The scoring criteria were as follows:

- **0 point:** No lesions
- **1 point:** Thin scattered petechiae; normal cecal contents
- **2 points:** Numerous petechiae; cecal contents slightly normal; cecal wall slightly thickened
- **3 points:** Large amounts of blood or cecal cores in ceca; thickened cecal wall; obvious cecal deformation and atrophy
- **4–5 points:** Severe cecal atrophy; lesions extending to rectum; extremely thickened cecal wall; blood clots or cecal cores in ceca

When lesions differed between the two ceca, the more severe side was used for scoring.

Oocyst excretion was measured according to Lillehoj [19]. From days 7–10 post-challenge, total fecal collection was used to harvest oocysts, which were counted using a McMaster's chamber slide to determine oocysts per gram of feces (OPG) per bird per day. The average value over 4 days was used for statistical analysis.

1.4.3 Immune Organ Indices, Lymphocyte Transformation Rate, and $\gamma\delta$ T Cell Proportion

Immune organ indices, lymphocyte transformation rates, and peripheral blood $\gamma\delta$ T cell proportions were determined on day 21 (7 days post-challenge).

The lymphocyte transformation test followed Mosmann [20] with minor modifications. Wing vein blood was collected with heparin sodium anticoagulation and immediately diluted 1:1 with D-Hanks solution. One milliliter of lymphocyte separation medium (purchased from Academy of Military Medical Sciences) was added to a centrifuge tube, followed by slowly adding 2 volumes of diluted blood to form a layer over the separation medium. After centrifugation at 1,800×g for 30 min at 4°C, the liquid separated into three layers: plasma (top), separation medium (middle), and red blood cells/granulocytes (bottom). A thin turbid band at the interface between the top and middle layers contained lymphocytes and monocytes. Approximately 1 mL of this intermediate leukocyte layer was collected, washed 3 times with 3–5 volumes of RPMI-1640 medium (purchased from Gibco, USA) at 2,500 r/min for 10 min at 4°C, and the supernatant was discarded. Cells were resuspended in complete RPMI-1640 medium, stained with trypan blue, and counted (viability >95%), then adjusted to 1$×10ˆ{7}$ cells/mL. Cell suspension (190 L) was plated in 96-well plates, followed by addition of 10 L phosphate-buffered saline (PBS), concanavalin A (ConA) (Sigma, USA, final concentration 45 g/mL), or lipopolysaccharide (LPS) (Sigma, USA, final concentration 25 g/mL). PBS served as the control group, ConA as the T lymphocyte transformation group, and LPS as the B lymphocyte transformation group. The 200 L culture system was incubated at 40°C in a 5% CO_2 incubator for 56 h. After incubation, 100 L of supernatant was gently removed, 100 L of RPMI-1640 medium without fetal bovine serum was added along with 10 L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma, USA) at 5 mg/mL initial concentration, and incubation continued for 4 h. After incubation, 100 L of dimethyl sulfoxide (DMSO) (Sigma, USA) was added to each well, and optical density (OD) values were measured at 570 nm using an ELISA reader after complete dissolution of purple crystals.

The proportion of $\gamma\delta$ T cells in peripheral blood lymphocytes was determined according to Zhang et al. [21].

1.4.4 Ileal IEL Numbers and Cecal Mucosal sIgA Levels

Ileal IEL numbers and cecal mucosal sIgA levels were measured on day 21 (7 days post-challenge). sIgA levels were expressed as the ratio of positive area covered by sIgA to total field area, following the method reported by Gao [17].

1.5 Statistical Analysis

Data were initially processed using Excel 2010 and analyzed by one-way ANOVA using SPSS 16.0 software, with Duncan' s multiple comparison test. Non-continuous observational data including intestinal lesion scores, bloody stool pile counts, and oocyst excretion numbers, which did not follow normal distribution, were analyzed by the Kruskal-Wallis non-parametric test. Significance was set at $P<0.05$. Results are expressed as mean±SD.

2.1 Effects of GPC on Growth Performance of Broiler Chicks Challenged with *Eimeria tenella*

As shown in Table 2 , dietary GPC supplementation had no significant effect on growth performance of broiler chicks during 1-14 days (P>0.05). During 15-21 days, ADG in the negative control group was significantly lower than in the blank control group (P<0.05), while GPC supplementation alleviated this ADG reduction, with growth performance showing no significant difference from the blank control (P>0.05). During 22-42 days, the negative control group showed significantly reduced ADG and increased F/G compared with the blank control (P<0.05). Supplementation with 7.5 and 15.0 mg/kg GPC significantly improved the ADG reduction and F/G increase induced by coccidial challenge (P<0.05). However, supplementation with 30.0 mg/kg GPC significantly decreased ADG and ADFI during 22-42 days and ADG during 1-42 days compared with the blank control (P<0.05). During 1-42 days, ADG in the negative control and 30.0 mg/kg GPC groups was significantly lower than in other groups (P<0.05), while F/G in the negative control group was significantly higher than in other groups (P<0.05). In summary, coccidial challenge reduced growth performance, and supplementation with 7.5 and 15.0 mg/kg GPC alleviated this growth inhibition.

Table 2 Effects of GPC on growth performance of broiler chicks challenged with *Eimeria tenella*

Items	Blank control	Negative control	7.5 mg/kg GPC	15.0 mg/kg GPC	30.0 mg/kg GPC
1-14 days					
ADG (g)	32.23±4.10	32.11±4.11	34.26±3.54	32.71±4.44	33.04±2.95
ADFI (g)	43.44±2.52	42.41±2.34	43.39±2		
15–21 days					
ADG (g)	42.79±5.43^a	36.23±4.64^b	40.94±4.23^{ab}	39.08±5.31^{ab}	39.48±3.53^{ab}
ADFI (g)	69.49±4.7				
22–42 days					
ADG (g)	81.58±2.16^a	72.17±3.51^b	77.47±1.85^a	78.86±2.13^a	72.98±4.60^b
ADFI (g)	159.10±10.3				
1–42 days					
ADG (g)	58.05±1.08^a	52.23±1.76^b	56.14±0.92^a	56.18±1.06^a	53.38±2.30^b
ADFI (g)	105.61±2.88				

In the same row, values with no letter or the same letter superscripts indicate no significant difference (P>0.05), while different lowercase letters indicate significant difference (P<0.05). The same as below.

2.2 Effects of GPC on Cecal Lesion Scores, Bloody Stool Piles, and Oocyst Excretion

As shown in Table 3 , dietary GPC supplementation significantly reduced cecal lesion scores, bloody stool pile counts, and oocyst excretion numbers compared with the negative control group (P<0.05). Oocyst excretion numbers were reduced by 66.9%, 80.8%, and 45.4% in the 7.5, 15.0, and 30.0 mg/kg GPC groups, respectively, with the 15.0 mg/kg GPC group showing the best anticoccidial effect.

Table 3 Effects of GPC on cecal lesion score, bloody stool piles, and coccidial oocyst excretion of broiler chicks challenged with *Eimeria tenella*

Items	Negative control	7.5 mg/kg GPC	15.0 mg/kg GPC	30.0 mg/kg GPC
Cecal lesion score	3.07 ± 0.31^a	1.13 ± 0.21^c	1.20 ± 0.11^c	2.22 ± 0.40^b
Bloody stool piles	4.40 ± 0.51^a	1.60 ± 0.40^b	1.20 ± 0.20^b	[illegible]

2.3 Effects of GPC on Immune Organ Indices and Peripheral Blood Lymphocytes

As shown in Table 4 , the spleen index in the negative control group was significantly higher than in the blank control group (P<0.05), while no significant differences were observed between GPC-supplemented groups and the blank control (P>0.05). Peripheral blood T lymphocyte transformation rate was significantly increased by coccidial challenge compared with the blank control (P<0.05), and further increased by 16.1% and 5.4% in the 7.5 and 15.0 mg/kg GPC groups, respectively (P>0.05). The B lymphocyte transformation rate in the 7.5 and 15.0 mg/kg GPC groups was significantly higher than in the 30.0 mg/kg GPC group (P<0.05). The proportion of $\gamma\delta$ T cells in peripheral blood was significantly increased by coccidial challenge (P<0.05), and the 15.0 mg/kg GPC group showed higher $\gamma\delta$ T cell proportion than the negative control, though the difference was not significant (P>0.05). No significant differences were observed in thymus index or bursa of Fabricius index among groups (P>0.05).

Table 4 Effects of GPC on immune organ indexes and peripheral lymphocytes of broiler chicks challenged with *Eimeria tenella*

Items	Blank control	Negative control	7.5 mg/kg GPC	15.0 mg/kg GPC	30.0 mg/kg GPC
Spleen index	1.16 ± 0.08^b	1.47 ± 0.20^a	1.06 ± 0.15^b	0.99 ± 0.17^b	1.05 ± 0.16^b
Thymus index	2.20 ± 0.17	1.94 ± 0.21	[illegible]	[illegible]	[illegible]
$\gamma\delta$ T cell proportion	0.15 ± 0.04^b	0.21 ± 0.03^a	0.21 ± 0.04^a	0.23 ± 0.01^a	0.21 ± 0.02

2.4 Effects of GPC on Intestinal Immune Function

As shown in Table 5 , ileal IEL numbers in the negative control group increased by 41.9% compared with the blank control (P<0.05). The 7.5 and 15.0 mg/kg GPC groups showed further increases in IEL numbers, rising by 69.3% and 116.7% compared with the blank control, respectively (P<0.05), while the 30.0 mg/kg GPC group showed no significant difference from the negative control (P>0.05). Cecal mucosal sIgA levels in the negative control group increased by 36.3% compared with the blank control (P<0.05). GPC supplementation further elevated sIgA levels, with the 7.5, 15.0, and 30.0 mg/kg GPC groups showing increases of 107.6%, 149.6%, and 94.3% compared with the blank control, respectively (P<0.05). In summary, dietary supplementation with 7.5 and 15.0 mg/kg GPC effectively regulated intestinal IEL numbers and sIgA levels, with the 15.0 mg/kg GPC group showing the best effect.

Table 5 Effects of GPC on intestinal immune function of broiler chicks challenged with *Eimeria tenella*

<table>
<tr><th>Items</th><th>Blank control</th><th>Negative control</th><th>7.5 mg/kg GPC</th><th>15.0 mg/kg GPC</th><th>30.0 mg/kg GPC</th></tr>
<tr><td>IEL number</td><td colspan="5">11.00$ ±1.04^{d}|15.61±2.01^{c}|18.62±2.05^{b}|23.84±2.22^{a}|15.25±1.81^{c}||$sIgAlevel$(±0.72^{d}|11.65±1.34^{c}|17.75±</td></tr>
</table>

3.1 Effects of GPC on Growth Performance of Broiler Chicks Challenged with *Eimeria tenella*

Coccidia are protozoan parasites that inhabit intestinal epithelium, primarily destroying intestinal epithelial cells and affecting normal digestion and absorption, resulting in slow growth and reduced feed efficiency. The present results showed no significant differences in growth performance among groups during 1–14 days, consistent with previous findings [16], likely because the feeding period was too short for GPC to exert effects. After challenge, coccidial damage to the intestine caused digestive and absorptive dysfunction and nutrient waste, while dietary supplementation with 7.5 and 15.0 mg/kg GPC alleviated this damage, consistent with Wang et al. [9] and McDougald et al. [14]. This suggests that supplementation with 7.5 and 15.0 mg/kg GPC can maintain healthy growth and resist growth inhibition caused by coccidial infection. Coccidial infection induces oxidative stress and immunosuppression, and our previous research revealed that GPC possesses strong antioxidant and immune-promoting effects [20-21], which may be important mechanisms for GPC' s improvement of broiler growth performance.

Notably, although the 30.0 mg/kg GPC group showed significantly lower F/G than the negative control throughout the experimental period, ADG did not differ significantly from the negative control due to lower ADFI, suggesting that

30.0 mg/kg GPC had limited anticoccidial effect, possibly because high-dose GPC negatively affected feed palatability.

3.2 Effects of GPC on Cecal Lesion Scores, Bloody Stool Piles, and Oocyst Excretion

The intestine is the most important digestive and absorptive organ, and its health directly affects growth rate. After invading intestinal epithelial cells, coccidia destroy intestinal integrity, cause bleeding and bloody stools, parasitize and proliferate massively in intestinal epithelial cells, and are excreted in feces. This study demonstrated that dietary GPC supplementation significantly reduced cecal lesion scores, bloody stool pile counts, and oocyst excretion numbers in coccidia-challenged broilers, consistent with Wang et al. [9].

Our previous studies showed that GPC has no significant direct killing effect on coccidia (unpublished data), suggesting that GPC may resist coccidial infection by activating the host' s immune system.

3.3 Effects of GPC on Immune Organ Development and Peripheral Blood Lymphocytes

Similar to mammalian lymph nodes, the spleen is the largest peripheral immune organ in chickens and an important site for immune responses and antibody production. Its developmental status is closely related to immune status. Additionally, the spleen is directly connected to blood circulation and vulnerable to oxidative stress from free radicals. Studies have shown that coccidial infection disrupts the antioxidant system and damages spleen tissue structure in broilers [22-23]. Other reports indicate that coccidial infection causes swelling of splenic parenchymal cells, increased blood cells in red pulp, and massive inflammatory cell infiltration [24]. This study found that the spleen index was significantly increased in the coccidia-infected negative control group, possibly related to antioxidant imbalance and inflammatory responses induced by coccidial infection. Our previous research showed that dietary supplementation with 7.5 and 15.0 mg/kg GPC had no significant effect on spleen index in broilers under normal conditions [16], suggesting GPC may not significantly affect spleen index under healthy conditions. However, this study showed that GPC supplementation restored the spleen index of coccidia-infected broilers to normal levels, indicating that GPC significantly improves spleen index in coccidia-infected broilers, possibly related to its anti-inflammatory [11-12] and oxidative stress-relieving effects [9]. The mechanism by which GPC inhibits coccidia-induced spleen index increase requires further investigation.

T and B lymphocyte transformation rates reflect the reactivity and proliferative activity of T and B lymphocytes. Cellular immunity plays a dominant role after coccidial infection. This study found that T lymphocyte transformation rate was significantly higher in coccidia-infected broilers than in the blank control, indicating an immune response to coccidial invasion. Dietary GPC supplementation increased T lymphocyte transformation rate, consistent with previous studies [15-16]. GPC may activate cellular immunity, promote cytokine secretion by T lymphocytes, and resist coccidial invasion, representing one potential anticoccidial mechanism. Humoral immunity plays a relatively weaker role after coccidial infection, and this study found no significant differences in thymus index or bursa of Fabricius index between the negative and blank control groups. However, GPC supplementation increased B lymphocyte transformation rate, possibly because coccidial invasion destroyed intestinal mucosal barriers, allowing massive invasion of pathogenic microorganisms that activated humoral immunity and promoted antibody secretion by B lymphocytes, helping prevent secondary diseases caused by coccidial infection.

Bessay et al. [25] and Swinkels et al. [26] reported that $\gamma\delta$ T cell numbers increased rapidly in chicken intestines after coccidial infection. Yun et al. [27] compared changes in intestinal lymphocyte subsets between two chicken lines after coccidial infection and found that high-tolerance chickens had significantly higher intestinal $\gamma\delta$ T cell numbers than low-tolerance chickens after infection. This study found that the proportion of $\gamma\delta$ T cells in peripheral blood was significantly higher in the coccidia-infected negative control group than in the blank control, consistent with previous research. $\gamma\delta$ T cells regulate epithelial cell proliferation and differentiation by promoting production of keratinocyte growth factor and epidermal growth factor (EGF) [28-30], and coccidial infection can activate $\gamma\delta$ T cells to exert autoimmune protection. This study did not find that GPC supplementation significantly increased $\gamma\delta$ T cell proliferation in peripheral blood, possibly because although intestinal $\gamma\delta$ T cell proportion increased significantly after coccidial infection, circulating $\gamma\delta$ T cells in blood did not reach significant levels.

3.4 Effects of GPC on Intestinal Immune Function of Broiler Chicks Challenged with *Eimeria tenella*

Mucosal immune cells account for 80% of all immune cells in the body, and mucosal immunity plays a crucial role in immune protection. The mucosal immune system comprises organized mucosa-associated lymphoid tissue and diffuse mucosa-associated lymphoid tissue. Organized mucosa-associated lymphoid tissue is the primary site for antigen capture and generation of immune effector and memory cells. Cecal tonsils are part of organized mucosa-associated lymphoid tissue, while IEL are part of diffuse mucosa-associated lymphoid tissue. IEL are a unique lymphocyte population that plays important roles in cell-mediated mucosal immunity and maintenance of epithelial integrity. They contain intracellular granules (such as perforin, granzymes, and serine esterases) with cytotoxic activity and can secrete cytokines (such as TNF-α, IFN-γ, and IL-2), playing important roles in defending against intestinal pathogen invasion [31-32]. This study showed that dietary GPC supplementation significantly increased ileal IEL numbers in coccidia-challenged broilers, suggesting that GPC may directly or indirectly kill coccidia by promoting IEL proliferation and activation and stimulating secretion of cytokines and intracellular granules.

B cells in mucosa-associated lymphoid tissue are primarily sIgA-producing cells that secrete sIgA directly into adjacent mucosa upon antigen stimulation to exert local immune effects. Zheng et al. [23] found that *Eimeria tenella* infection caused significant proliferation of local lymphocytes in cecal mucosa, and locally sensitized B lymphocytes in cecal mucosa produced sIgA that retained coccidia in the intestinal lumen for elimination by digestive fluids and other substances, suggesting that local secretory antibodies and cellular immunity coordinate to provide comprehensive protection. Liu [33] proposed that sIgA may protect against coccidial infection through several mechanisms: sIgA adheres to parasite surfaces, reducing parasite vitality through neutralization, opsonization, and steric hindrance; induces conformational changes in host cell receptor molecules; exerts antibody-dependent cell-mediated cytotoxicity directly on sporozoites and merozoites; and prevents invasion and intracellular development. This study showed that dietary GPC supplementation significantly increased cecal mucosal sIgA secretion levels in coccidia-challenged broilers, thereby reducing coccidial contact damage to intestinal cells through steric hindrance.

In conclusion: (1) Dietary supplementation with appropriate GPC levels can inhibit growth performance decline caused by coccidial infection; (2) Dietary GPC supplementation can increase peripheral blood lymphocyte transformation rates after coccidial infection; (3) Dietary GPC supplementation can increase ileal IEL numbers and cecal mucosal sIgA levels after coccidial infection, maintaining intestinal health; (4) Under the coccidial infection model conditions of this study, the appropriate dietary GPC supplementation level for broiler chicks is 7.5–15.0 mg/kg.

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Note: Figure translations are in progress. See original paper for figures.