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From Cell Biology to Tissue Engineering

Chitooligosaccharide promotes immune organ development in broiler chickens and reduces serum lipid levels

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Summary. This study investigated the effects of chitooligosaccharide on lipid metabolism, immune organ development, and lymphocyte apoptosis in broiler chickens. A total of 480 one-day-old broiler chickens (Arbor Acres) were randomly and evenly assigned to control group and experimental groups I, II, and III. The control group was given a basic diet, while experimental groups I, II, and III were given basic diets for 42 days, supplemented with 50 mg/kg chlortetracycline, 20, and 40 mg/kg chitooligosaccharide, respectively. We found levels of serum triglycerides (TG) and low density lipoprotein cholesterol (LDL-C) to be significantly reduced in experimental group II after 21 days, while the periarterial lymphatic sheath area of the spleens and the average number of bursa of Fabricius nodes were markedly increased. The serum total protein (TP) and high density lipoprotein cholesterol (HDL-C) levels, bursa of Fabricius index, and bursa of Fabricius lobule areas were additionally increased in experimental group III. After 42 days, the serum TP content had also increased and the bursa of Fabricius lobule area was augmented as well in experimental group II. Moreover, the splenic periarterial lymphatic sheath areas and the average numbers of bursa of Fabricius nodes were significantly increased in experimental group III. At both 21 and 42 days, numbers of Caspase-3 positive cells in spleen and bursa of Fabricius were significantly decreased in experimental groups II and III. Our results show that appropriate supplementation of chitooligosaccharide may improve lipid metabolism, promote immune organ development, and inhibit lymphocyte apoptosis in broilers.

Key words: Chitooligosaccharide, Broiler chickens, Lipid metabolism, Immune organ development, Lymphocyte apoptosis

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Introduction

With the development of intensive and large-scale farming and the abuse of additives (e.g. antibiotics and somatotropin), problems such as drug resistance, drug residues in meat, and environmental pollution have increased in importance. These factors pose a direct threat to the health of humans and animals, especially since they compromise the safety of human food. Therefore, we urgently need to find new green feed additives for livestock production in order to ensure the health and safety of humans and animals (Samarasinghe et al., 2003; Huff et al., 2006; Han et al., 2007). Chitosan is the only natural alkaline polysaccharide ever found, there are rich reserves of it in nature, and it has attracted considerable interest due to its extensive biological activities (Knaul et al., 1999). Chitooligosaccharide however, is an oligosaccharide derived from chitosan, with far better pharmacokinetic properties. It features improved water-solubility, it is biodegradable, non-toxic, and is easy to absorb into the body. Since it has an antibacterial effect,

promotes development and function of the immune system, increases radiation damage resistance and reduces blood lipids, it is widely applied in medicine and animal husbandry (Choi et al., 2006; Li et al., 2007). Studies have shown that appropriate supplementation of chitooligosaccharide may improve nutrient digestibility and daily weight gain in broiler chickens, enhance muscle quality, and promote growth and development (Huang et al., 2005; Zhou et al., 2009). Furthermore, nutrient digestibility has also been shown to be augmented after chitooligosaccharide supplementation in layers, while egg production performance and egg quality increased as did leukocyte counts and serum total protein (TP) (Meng et al., 2010). Chitooligosaccharide has also been shown to enhance early weaning feed conversion rate and production performance, and to promote the secretion of serum growth hormones and insulin-like growth factor-I (Tang et al., 2005; Han et al., 2007), to suppress high-fat diet-induced adipocyte differentiation in obese rats, and to improve lipid metabolism (Huang et al., 2015), to enhance macrophage migration activity and phagocytic functions, and to promote cytokine secretion (Yu et al., 2004). At present, there are numerous reports regarding the effects of chitooligosaccharide on serum biomarkers and immune function; however, studies concerning its effect on immune organ development and lymphocyte apoptosis in broiler chickens remain few. Therefore, we investigated the effect of chitooligosaccharide on lipid metabolism, immune organ development, and lymphocyte apoptosis in Arbor Acres (AA) broiler chickens, in order to uncover the mechanism by which chitooligosaccharide modulates immune function. We also provide morphological data and a scientific basis for the application of chitooligosaccharide in broiler chicken production.

Materials and Methods

Housing and care of animals and experimental design

480 one-day-old AA broiler chickens, weighing 48.5±2.6 g, were randomly assigned to control group and experimental groups I, II, and III. Each group included 120 chickens. Broiler chickens were raised in pens sized 1 m^2 until day 21 (d 1 to 21), when they were moved into 2 m^2 pens during the late growth stage (d 22 to 42). There were 20 birds per pen and six pens per group. The control group was fed with a corn-soybean type basic diet, while experimental groups I, II, and III were supplemented with 50 mg/kg chlortetracycline and 20 and 40 mg/kg chitooligosaccharide mixed into the same diet, respectively. The corn-soybean type basic diet was formulated based on the NRC's recommendations regarding broiler's nutrient needs (1994) (Table 1). Both food and water were provided *ad libitum*. Broiler chickens were kept in an environment with controlled temperature and humidity and fed strictly in accordance with the specifications of broiler rearing. The room temperature was maintained at 33±1°C for the first 3 d, after which the temperature was gradually reduced by 3°C per week until reaching 24°C. This temperature was maintained until the end of the 42-day-long study. Artificial light was provided 24 h/d via a light bulb emitting 10 to 20 lux. All animals were vaccinated against Newcastle Disease and Infectious Bursal Disease in a routine immunization procedure. All experimental procedures strictly abided to the Animal Welfare Guidelines of China and were approved by the Animal Welfare committee of the Anhui Science and Technology University.

Chitooligosaccharide and chlortetracycline

The average molecular weight of chitooligosaccharide is 1,500 Dalton, which has a 98% degree of deacetylation and >99% solubility in water. It was obtained from Jiaxing Korui Technical Co., Ltd.

Chlortetracycline has a molecular weight of 478.88 Dalton, and is supplied as brown powder at a concentration of 98%. It was obtained from HeFei Kangdi Feed Co., Ltd.

Collection and processing of samples

At days 21 and 42, 30 broiler chickens per group

Table 1. Composition of basal diets and nutrient contents.

Item	Starter phase (1-21 days)	Grower phase (22-42 days)
Ingredient, % as-fed		
Corn	58.00	62.00
Soybean meal (44%, CP)	32.00	27.50
Fish meal (64%, CP)	3.00	2.00
Soybean oil	2.00	3.50
Premix[b]	5.00	5.00
Nutritional content		
CP (%)	21.46	19.08
Lys (%)	1.21	1.04
Met (%)	0.35	0.31
ME(MJ/kg)	12.26	12.73
Ca (%)	1.10	1.02
P (%)	0.59	0.44

Basal diets and treatment diets were identical. Treatment diets were supplemented with 20 mg/kg chitooligosaccharide, 40 mg/kg of chitooligosaccharide, or 50 mg/kg chlortetracycline in addition to the respective basal diet as part of the premix. The premix provided the following in days 0-21 (per kilogram of compound feed): Vitamin A, 240000 IU; Vitamin D3, 54000 IU; Vitamin E, 560 IU; Vitamin K3, 56 mg; Vitamin B1, 16 mg; Vitamin B2, 108 mg; Vitamin B6, 18 mg; Vitamin B12, 0.25 mg; nicotinic acid, 650 mg; pantothenic acid, 240 mg; folic acid, 12 mg; choline chloride, 10 g; copper, 100 mg; iron, 400 mg; zinc 960 mg; manganese, 960 mg; iodine, 10 mg; selenium, 20 mg; calcium, 14-18%; total phosphorus, 2.5%; salt, 4-8%; moisture, 12%. The premix provided the following in days 22-42 (per kilogram of compound feed): Vitamin A, 200000 IU; Vitamin D3, 50000 IU; Vitamin E, 480 IU; Vitamin K3, 50 mg; Vitamin B1, 16 mg; Vitamin B2, 90 mg; Vitamin B6, 18 mg; Vitamin B12, 0.12 mg; nicotinic acid, 600 mg; pantothenic acid, 240 mg; folic acid, 8 mg; choline chloride, 10 g; copper, 100 mg; iron, 400 mg; zinc 960 mg; manganese, 960 mg; iodine, 10 mg; selenium, 20 mg; calcium, 14-18%; total phosphorus, 2.3%; salt, 4-8%; moisture, 12%.

(i.e., 5 birds per pen) were randomly selected and subjected to fasting (water provided) for 12 h. They were weighed and blood was collected from the wing vein via sterile procoagulant vacuum tubes. The chickens were killed via cervical dislocation and immediately dissected to extract the bursa of Fabricius and the spleen. Blood samples were placed at room temperature for 1 h, coagulated, and subsequently centrifuged at 3,000 g for 15 min to separate the serum. We analyzed concentrations of total protein TP, triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) using an automated bioanalyzer (ZY-360, Shanghai Kehua Bio-Engineering Co, Ltd.). Bursa of Fabricius and spleen were weighed using an electronic analytical balance. Immune organ samples of an approximate size of 0.5 cm × 0.5 cm × 0.5 cm were fixed in 4% paraformaldehyde in PBS at room temperature for 72 h, and subsequently subjected to dehydration in graded ethanol series, cleared with xylene, and embedded in paraffin. Afterwards, tissue samples of Bursa of Fabricius and spleen obtained from 30 broiler chickens at days 21 or 42 were cut into successive 6 μm sections with a rotary microtome (RM2235, Leica Biosystems, Germany), and two slices out of every 20 slices were dried on poly-lysine coated glass slides in order to continue with HE and immunohistochemistry staining.

HE staining and microscopic examination

Bursa of Fabricius and spleen sections from each chicken were stained with HE, cleared with xylene, and mounted in neutral gum. At least 15 sections per immune organ and a total of 400 sections per experimental group were utilized for microscopic observation and measurement of histological parameters via upright fluorescence microscopy and a photomicrography system (BX51+DP73, Olympus Co., Ltd., Japan). Five microscopic vision fields in each spleen section were evenly selected to be photographed at 200 × magnification, and then complete splenic periarterial lymphatic sheaths and splenic nodules in each microscopic vision field were manually drawn to calculate the areas via microscopic image analysis software (Image-Pro Plus 6.0). Bursa of Fabricius sections were photographed and merged at 100 × magnification, and then complete bursa of Fabricius nodes in each section were manually drawn to calculate the areas and numbers of bursa of Fabricius nodes using microscopic image analysis software (Image-Pro Plus 6.0). The average number of bursa of Fabricius nodes in the lobules was also calculated.

Immunohistochemistry staining

To prepare bursa of Fabricius and spleen sections from each chicken for immunohistochemistry staining, sections were dewaxed in xylene, dehydrated in graded ethanol in distilled water, washed thrice for 5 min each in 0.01 mol/l PBS (pH 7.4), and immersed in 0.1 mol/l citrate buffer (pH 6.0). Samples were then subjected to microwave antigen retrieval for 15 min, left to cool down to room temperature, were washed thrice for 5 minutes each in 0.01 mol/l PBS, and incubated for 30 min at room temperature in 3% hydrogen peroxide (prepared in methanol) to remove endogenous peroxidase followed by washing thrice for 5 min each in 0.01 mol/l PBS. Sections were then incubated for 30 min at room temperature in 10% normal goat serum (prepared in 0.01 mol/l PBS) to block non-specific secondary binding. The goat serum was removed and sections were incubated with rabbit polyclonal Capase-3 antibody (Cat: 19677-1-AP, 1:200 dilution, Proteintech Group, Inc., USA) at 4°C overnight. The next day, after an equilibration at room temperature for 30 min and washing thrice in 0.01 mol/l PBS for 5 min, sections were incubated with biotinylated goat anti-rabbit secondary antibody (Cat:SA00004-2, 1:200 dilution, Proteintech Group, Inc., USA) at 37°C for 2 h. Subsequently, sections were once more subjected to washing (thrice for 5 min in 0.01 mol/l PBS) and then incubated with streptavidin-labeled tertiary antibody (Cat: SA00001-0, 1:200 dilution, Proteintech Group, Inc., USA) at 37°C for 2 h. They were then washed twice for 5 min in 0.01mol/l PBS and pre-incubated in DAB (3-3 diaminobenzidine) pre-incubation solution at room temperature for 30 min (Cat: D5637-5G, Sigma-Aldrich, Inc., USA). Color was developed with DAB within 5 min and we counterstained cell nuclei with hematoxylin during 3 min, passed sections through a graded ethanol series for dehydration, cleared them with xylene and mounted them in neutral gum. Afterwards, sections were examined and photographed via Olympus BX51 upright fluorescence microscope and DP73 photomicrography system. A total of 15 transverse sections of spleen or bursa of Fabricius of each broiler chicken, corresponding to a total of 300 sections per experimental group, were selected for immunohistostaining. Five regions were evenly selected from the top, bottom, left, right, and center areas in each section, were photographed and the number of Caspase-3 positive cells was counted using the aforementioned microscopic image analysis software.

Data processing

Data were statistically analyzed with one-way ANOVA using the SPSS20.0 software package. Variance uniformity and normal distribution of data were confirmed via Levene's test and Kolmogorov-Smirnov test, and significant differences between experimental groups were detected via Least Significant Difference test. $P<0.05$ was considered to be statistically significant. All data are represented in the form of mean ± SD.

Results

Serum total protein and lipid metabolism-related biomarkers

Table 2 shows the effects of chitooligosaccharide on serum levels of lipid metabolism-related biomarkers in broiler chickens (Table 2). Low-dose chitooligosaccharide supplementation for 21 days provoked a significant reduction of TG (-19.05%; P<0.05), an effect also mediated by supplementation of 40 mg/kg chitooligosaccharide since TG decreased by 17.46% (P<0.05) in the respective groups. Furthermore, high-dose chitooligosaccharide supplementation reduced TC (11.21%; P<0.05) and provoked an increase in TP (10.56%; P<0.05). These values did not significantly change over time in experimental group I. When compared to experimental group I, serum TP was markedly elevated by 13.55% (P<0.05), while serum TC content was significantly reduced by 12.25% (P<0.05) in experimental group III; lipid metabolism-related serum indicators in experimental group II experienced no significant changes.

When compared to the control group, serum levels of TP in experimental groups II and III were significantly increased after 42 days (15.72% and 21.29%, respectively; P<0.05), while those of LDL-C were markedly decreased (-15.33% and -12.67%, respectively; P<0.05); serum TG and TC were significantly reduced by 30.88% and 9.09% (P<0.05), respectively, while the serum HDL-C content was significantly elevated by 21.89% (P<0.05) in experimental group III; the serum LDL-C content was markedly decreased by 12.00% (P<0.05) in experimental group I. When compared to experimental group I, serum TC content markedly decreased by 12.18% (P<0.05) in experimental group III, while there were no significant changes of lipid metabolism-related serum indicators in

Table 2. Effect of dietary chitooligosaccharide supplementation on serum indices in the serum of broiler chickens.

Item	Control group	Experimental group I	Experimental group II	Experimental group III
d 21				
TP (Total protein, g/dL)	2.74±0.06^{b}	2.66±0.10^{b}	2.97±0.12^{ab}	3.03±0.10^{a}
TG (Triglyceride, mmol/L)	0.63±0.01^{a}	0.61±0.03^{ab}	0.51±0.02^{b}	0.52±0.02^{b}
TC (Total cholesterol, mmol/L)	3.47±0.12^{a}	3.51±0.11^{a}	3.49±0.24^{a}	3.08±0.12^{b}
HDL (HDL cholesterol, mmol/L)	2.20±0.08	2.30±0.06	2.23±0.13	2.31±0.08
LDL (LDL cholesterol, mmol/L)	1.51±0.05	1.38±0.04	1.49±0.09	1.46±0.05
d 42				
TP (Total protein, g/dL)	3.30±0.11^{b}	3.55±0.13^{ab}	3.81±0.17^{a}	4.00±0.18^{a}
TG (Triglyceride, mmol/L)	0.68±0.04^{a}	0.61±0.06^{ab}	0.57±0.05^{ab}	0.47±0.03^{b}
TC (Total cholesterol, mmol/L)	3.41±0.22^{a}	3.53±0.08^{a}	3.27±0.11^{ab}	3.10±0.09^{b}
HDL-C (HDL cholesterol, mmol/L)	2.01±0.14^{b}	2.20±0.05^{ab}	2.11±0.06^{ab}	2.45±0.06^{a}
LDL-C (LDL cholesterol, mmol/L)	1.50±0.06^{a}	1.32±0.04^{b}	1.27±0.09^{b}	1.31±0.03^{b}

Serum of 21- and 42-day-old broiler chickens was collected and subsequently analyzed for the concentrations of TP, TG, TC, HDL, and LDL. Mean values listed in the same row that are indexed with distinct superscripts denote significant differences among each other (P<0.05).

Table 3. Effect of dietary chitooligosaccharide supplementation on weight and organ index of spleen and bursa of Fabricius of broiler chickens.

Item	Control group	Experimental group I	Experimental group II	Experimental group III
d 21				
Spleen weight (g)	0.79±0.05	0.64±0.06	0.85±0.08	0.86±0.05
Bursa of Fabricius weight (g)	1.70±0.25^{ab}	1.43±0.15^{b}	1.78±0.33^{ab}	2.37±0.29^{a}
Spleen index (%)	0.11±0.01^{a}	0.09±0.01^{b}	0.12±0.01^{a}	0.13±0.02^{a}
Bursa of Fabricius index (%)	0.19±0.03^{b}	0.17±0.02^{b}	0.21±0.05^{b}	0.32±0.04^{a}
d 42				
Spleen weight (g)	3.72±0.20^{a}	2.60±0.25^{b}	4.07±0.32^{a}	4.09±0.47^{a}
Bursa of Fabricius weight (g)	1.36±0.18^{b}	1.63±0.92^{ab}	1.96±0.20^{ab}	2.27±0.31^{a}
Spleen index (%)	0.17±0.02^{a}	0.12±0.01^{b}	0.19±0.02^{a}	0.17±0.02^{a}
Bursa of Fabricius index (%)	0.08±0.01^{b}	0.07±0.01^{b}	0.09±0.01^{b}	0.12±0.02^{a}

The weight and organ index of spleen and bursa of Fabricius in 21- and 42-day-old broiler chickens were assessed and calculated to analyze the effect of chitooligosaccharide on the development of immune organs. Mean values listed in the same row that are indexed with distinct superscripts differ significantly from each other (P<0.05).

experimental group II.

Weight and organ indices of spleen and bursa of Fabricius

Table 3 illustrates the effects of chitooligosaccharide on weights and organ indices of bursa of Fabricius and spleen in broiler chickens (Table 3). After 21 days, the bursa of Fabricius index had significantly increased (+68.42%; P<0.05) in experimental group III, remained essentially unchanged in experimental group II, while chlortetracycline supplementation provoked a marked reduction of that index (-18.18%; P<0.05). Weights of bursa of Fabricius and spleen did not significantly change throughout the first half of the experiment in either experimental group compared to the control group. Compared to experimental group I, the bursa of Fabricius weight and indices of the spleen and bursa of Fabricius were significantly elevated by 65.73%, 44.44%, and 88.24%, respectively (P<0.05) in experimental group III, and the spleen index was also markedly increased by 33.33% (P<0.05) in experimental group II. No significant changes were observed in spleen weights of experimental groups II, III, or in the bursa of Fabricius weight or organ index of experimental group II when compared to experimental group I.

After 42 days and compared to the control group, bursa of Fabricius weight and organ index were significantly increased by 66.91% and 50.00% (P<0.05), respectively, in experimental group III, while spleen

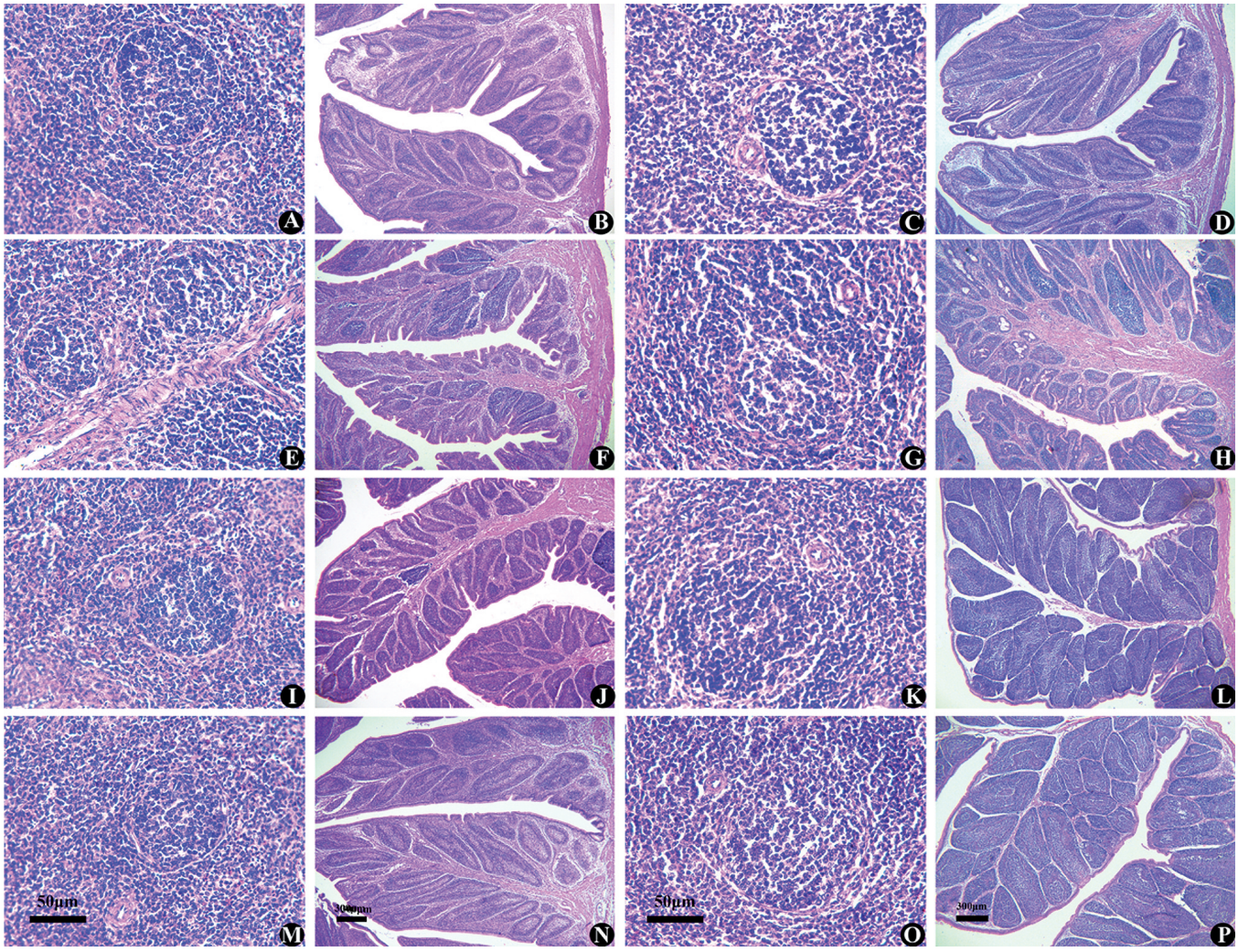

Fig. 1. Microstructure of spleen and bursa of Fabricius in broiler chickens. Tissue sections of spleen and bursa of Fabricius were subjected to hematoxylin and eosin (H&E) staining in order to analyze the effect of dietary chitooligosaccharide supplementation on immune organ microstructures in 21- and 42-day-old broiler chickens. **A-D.** Control group. **E-H.** Experimental group I. **I-L.** Experimental group II. **M-P.** Experimental group III. **A, E, I, M.** Spleen of 21-day-old broiler chickens. **B, F, J, N.** Bursa of Fabricius of 21-day-old broiler chickens. **C, G, K, O.** Spleen of 42-day-old broiler chickens. **D, H, L, P.** Bursa of Fabricius of 42-day-old broiler chickens. Scale bars: A, C, E, G, I, K, M, O, 50 μm; B, D, F, H, J, L, N, P, 300 μm.

weight and organ index in experimental group I were markedly reduced by 30.11% and 29.41% (P<0.05), respectively. There were no significant differences between spleen weight and organ index of experimental groups II and III, or between the bursa of Fabricius weight and organ index of experimental groups I or II when compared to the control group. Spleen weights and organ indices in experimental groups II and III were significantly elevated by 56.54%, 57.31%, and 58.33%, 41.66% (P<0.05), respectively, and the bursa of Fabricius index was also markedly increased (+71.43%; P<0.05) in experimental group III when compared to experimental group I. Bursa of Fabricius weights of experimental groups II and III as well as the bursa of Fabricius index in experimental group II experienced no significant changes.

The microstructure of spleen and bursa of Fabricius

Figure 1 and Table 4 show the effects of chitooligosaccharide on the structure of bursa of Fabricius and spleen in broiler chickens (Fig. 1 and Table 4). Microscopic observation of tissue samples obtained after 21 days indicated that the microstructure of the spleen of the control group was characterized by relatively large spleen nodes, thick periarterial lymphatic sheaths, and tightly packed lymphocytes; the bursa of Fabricius structure of the control group was organized, and the bursa of Fabricius lobule was relatively large, with many, tightly packed bursa of Fabricius nodes. These findings correspond to expectations for healthy broiler chickens. In contrast, splenic lymphocytes were loosely organized in experimental group I, and splenic node and periarterial lymphatic sheath areas were significantly decreased by 24.81% and 25.66% (P<0.05), respectively; the bursa of Fabricius lobule was smaller, with a reduced number of nodes and a decreased lobule volume; proportions of connective tissue were increased, and the bursa of Fabricius lobule area and the average number of bursa of Fabricius nodes were markedly reduced by 22.92% and 38.56% (P<0.05), respectively. Splenic lymphocytes in experimental group II were packed relatively tightly, the mean periarterial lymphatic sheath area was significantly increased by 52.86% (P<0.05), and we observed a trend (albeit non-significant) towards a decrease of the splenic node area (P=0.12); no significant changes were observed in bursa of Fabricius lobule area or bursa of Fabricius node volume, while the average number of bursa of Fabricius nodes in the lobule markedly increased by 23.65% (P<0.05). In experimental group III, splenic lymphocyte counts were increased by 18.62% as well as the periarterial lymphatic sheath area (P<0.05) with tightly packed lymphocytes, while we found a marked reduction of the splenic node area (14.59%; P<0.05); the bursa of Fabricius lobule area was markedly increased by 17.08% (P<0.05), while no significant changes were detected in the average number of bursa of Fabricius nodes. The areas of splenic nodes, periarterial lymphatic sheath, and bursa of Fabricius nodes, as well as the average number of bursa of Fabricius nodes in the lobules were all markedly elevated (P<0.05) in experimental groups II and III when compared to experimental group I.

Tissue samples of bursa of Fabricius and spleen obtained from chickens pertaining to the control group after 42 days displayed abundant, large splenic nodes and thick periarterial lymphatic sheaths, while the number of bursa of Fabricius nodes was increased, and nodes contained tightly packed lymphocytes. Only a trend towards a decrease in splenic nodes and periarterial lymphatic sheath areas could be observed in experimental group I when compared to the control group (P>0.05); the volume of bursa of Fabricius nodes was reduced, the proportion of connective tissue was increased, but neither the lobule area nor the average number of bursa of Fabricius nodes were significantly altered. The development of splenic and bursa of Fabricius tissues in experimental groups II and III was improved, the number of splenic nodes was increased, lymphocytes were tightly packed, splenic node areas

Table 4. Effect of dietary chitooligosaccharide supplementation on immune organ parameters in broiler chickens.

Item	Control group	Experimental group I	Experimental group II	Experimental group III
d 21				
Splenic node area ($10^3\mu m^2$)	9.39±1.46^{a}	7.06±1.12^{c}	8.64±0.92^{ab}	8.02±0.94^{b}
Periarterial lymphatic sheath area ($10^3\mu m^2$)	14.34±2.17^{c}	10.66±1.18^{d}	21.92±2.51^{a}	17.01±3.25^{b}
Bursa of Fabricius lobule aera (mm^2)	2.40±0.22^{b}	1.85±0.23^{c}	2.56±0.24^{b}	2.81±0.24^{a}
The average numbers of bursa of Fabricius nodes in the lobules (pcs)	25.88±2.36^{b}	15.90±2.11^{c}	32.00±1.87^{a}	28.67±4.39^{b}
d 42				
Splenic node area ($10^3\mu m^2$)	9.68±1.16^{b}	8.37±1.09^{b}	12.35±2.36^{a}	11.09±1.53^{a}
Periarterial lymphatic sheath area ($10^3\mu m^2$)	14.74±1.26^{b}	13.22±1.47^{b}	21.29±2.00^{a}	21.41±3.04^{a}
Bursa of Fabricius lobule aera (mm^2)	2.35±0.12^{bc}	2.22±0.20^{c}	3.18±0.16^{a}	2.95±0.29^{ab}
The average numbers of bursa of Fabricius nodes in the lobules (pcs)	23.38±3.07^{c}	20.75±1.98^{c}	27.89±4.73^{b}	32.88±3.91^{a}

The areas of splenic node, periarterial lymphatic sheath, and bursa of Fabricius lobule as well as the average numbers of bursa of Fabricius nodes in the bursa of Fabricius lobule were measured to ascertain the effect of chitooligosaccharide on the microstructure of immune organs in 21- and 42-day-old broiler chickens. Mean values listed in the same row that are indexed with distinct superscripts differ significantly from each other (P<0.05).

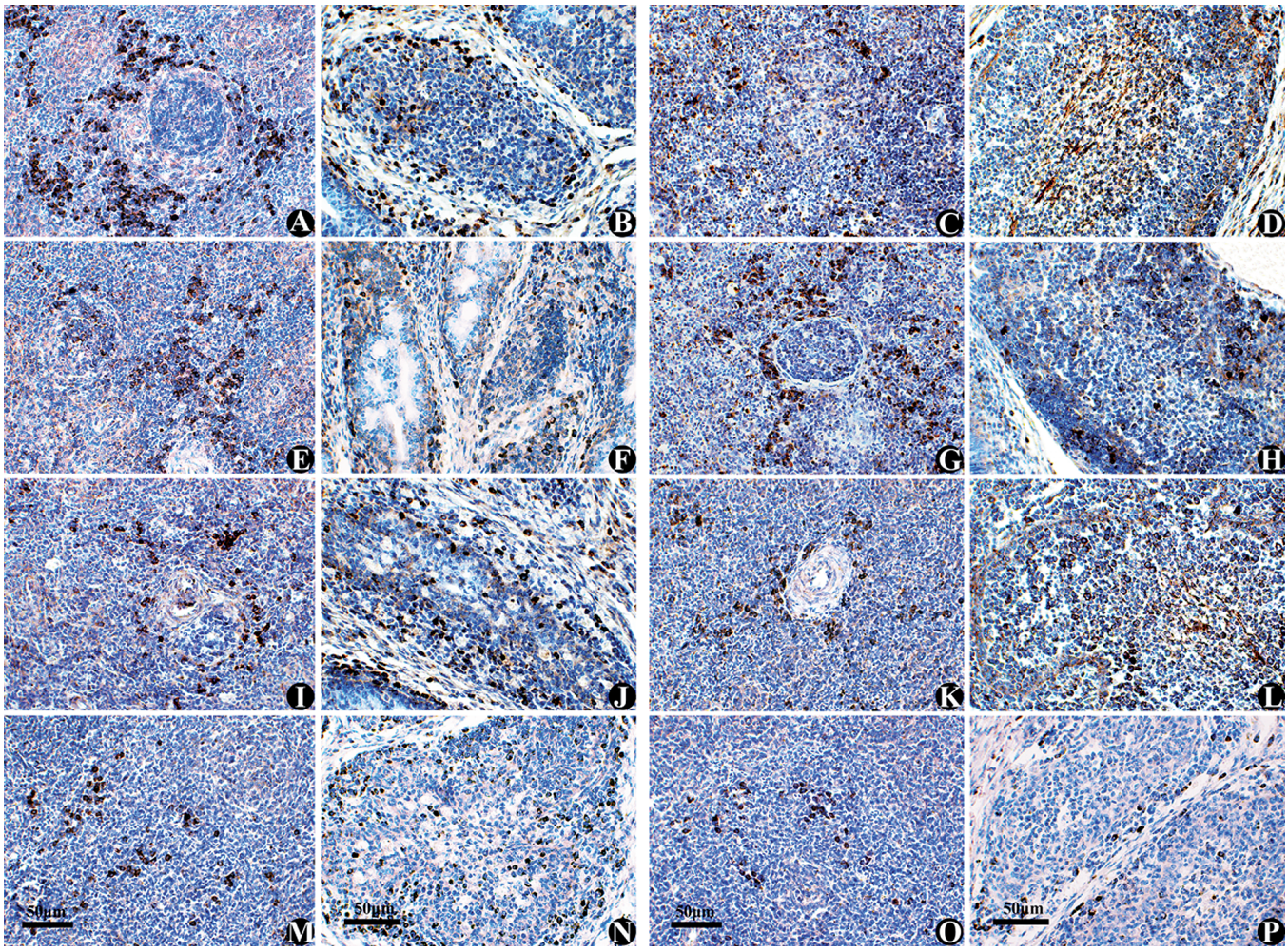

Fig. 2. Distribution of Caspase-3 positive cells in spleen and bursa of Fabricius of broiler chickens. Tissue sections of spleen and bursa of Fabricius were subjected to immunohistochemical staining for the expression of Caspase-3, which can mainly be found in lymphocytes. Caspase-3 positive cells were stained with a brown-black color. **A-D.** Control group. **E-H.** Experimental group I. **I-L.** Experimental group II. **M-P.** Experimental group III. **A, E, I, M.** Spleen of 21-day-old broiler chickens. **B, F, J, N.** Bursa of Fabricius of 21-day-old broiler chickens. **C, G, K, O.** Spleen of 42-day-old broiler chickens. **D, H, L, P.** Bursa of Fabricius of 42-day-old broiler chickens. Scale bar: 50 μm.

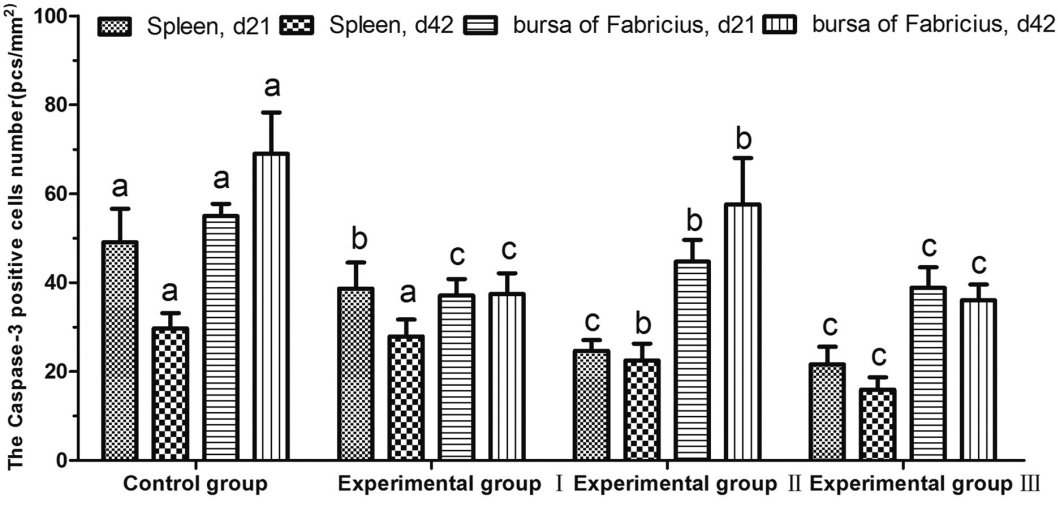

Fig. 3. Ratio of Caspase-3 positive cells in the spleen and bursa of Fabricius of broiler chickens. The number of Caspase-3 positive cells per unit area was counted and analyzed in the spleen and bursa of Fabricius. Different lowercase letters per column indicate significant differences ($P<0.05$).

were significantly increased by 27.58% and 14.57% (P<0.05), respectively, periarterial lymphatic sheath areas were markedly elevated by 44.44% and 45.25% (P<0.05), respectively, bursa of Fabricius lobules were enlarged in both groups and were significantly enlarged in experimental group II (+35.32%, P<0.05), and the average numbers of bursa of Fabricius nodes in the lobules were markedly increased by 19.29% and 40.63% (P<0.05), respectively. The areas of splenic nodes, periarterial lymphatic sheath and bursa of Fabricius lobule as well as the average number of bursa of Fabricius nodes were all significantly increased in groups II and III (P<0.05) when compared to experimental group I.

Lymphocyte apoptosis in spleen and bursa of Fabricius

Figure 2 shows the effect of chitooligosaccharide on the number of caspase-3 positive cells in spleen and bursa of Fabricius (Fig. 2). Figure 3 shows the effect of chitooligosaccharide on the distribution of lymphocyte apoptosis in those organs (Fig. 3). The cytoplasm of caspase-3 positive cells in bursa of Fabricius and spleen appeared brownish black under the microscope. These cells were mainly distributed in the marginal zones of the spleen, within periarterial lymphatic sheaths and splenic cords, as well as in the cortex of bursa of Fabricius nodes and connective tissue between nodes. After 21 days, numbers of caspase-3 positive cells in the marginal zones of the spleen and within the cortex of bursa of Fabricius nodes had increased in the control group. When compared to the control group, numbers of caspase-3 positive cells in the bursa of Fabricius and spleen of animals pertaining to experimental group I were markedly decreased by 21.23% and 32.53% (P<0.05), respectively. In experimental groups II and III and in comparison with the control group, numbers of splenic caspase-3 positive cells were significantly reduced by 49.80% and 55.97% (P<0.05), respectively, and those in the bursa of Fabricius were also markedly decreased by 18.60% and 29.41% (P<0.05), respectively. When compared experimental groups II and III with experimental group I. Numbers of splenic caspase-3 positive cells were significantly decreased by 36.26% and 44.10% (P<0.05) in experimental groups II and III, respectively, but the number of bursa of Fabricius caspase-3 positive cells in experimental group II was significantly increased by 20.65% (P<0.05).

Until the end of the experiment after 42 days, numbers of caspase-3 positive cells in the splenic cords and bursa of Fabricius nodes in the control group further increased. When compared to the control group, the number of bursa of Fabricius caspase-3 positive cells in experimental group I was markedly decreased by 45.71% (P<0.05), while no significant changes could be detected regarding the number of splenic caspse-3 positive cells. When comparing experimental groups II and III to the control group, we found numbers of splenic caspase-3 positive cells to be significantly reduced by 24.33% and 46.53% (P<0.05), respectively, and numbers of bursa of Fabricius caspase-3 positive cells to be markedly decreased by 16.48% and 47.74% (P<0.05), respectively. Numbers of splenic caspase-3 positive cells were markedly reduced by 19.42% and 43.07% (P<0.05) in experimental groups II and III, respectively, when compared to experimental group I. Only experimental group II displayed a significant increase by 53.84% (P<0.05) in bursa of Fabricius capase-3 positive cell counts when compared to experimental group I.

Discussion

Chitooligosaccharide supplementation may promote serum protein synthesis and improve lipid metabolism in broiler chickens

As a new prebiotic, chitooligosaccharide is not only able to support development and maintenance of the intestinal flora, to improve the chickens' overall health and to increase production performance, it may also enhance immune function and regulate lipid metabolism (Knaul et al., 1999; Berry and Lui, 2000). Studies have shown that a dietary supplementation with appropriate amounts of chitooligosaccharide significantly impacts serum levels of various biomarkers in poultry, inducing a marked decrease in serum TG levels and an increase in HDL-C concentration in broiler chickens (Zhou et al., 2009). It has also been shown to markedly reduce levels of serum total cholesterol and LDL-C in diabetic rats receiving a high-fat diet (Huang et al., 2015). In this study, we present evidence that the addition of 20 mg/kg chitooligosaccharide to the diet may significantly decrease serum TG levels within 21 days and LDL-C levels within 42 days. Dietary supplementation with 40 mg/kg chitooligosaccharide furthermore significantly decreased serum TG and TC levels within 21 and 42 days, while it significantly increased serum HDL-C levels within 42 days. The chitooligosaccharide-related reduction of lipid absorption in the intestine, caused by binding of bile acids, provides a possible explanation since it stimulates both the elimination of cholesterol and the synthesis of bile acids in the liver (Zhen et al., 2003). In addition, previous studies reported that chitooligosaccharide significantly increased serum TP, total cholesterol and HDL-C in broiler chickens; however, it is able to diminish serum TG concentrations (Li et al., 2007). Furthermore, dietary supplementation with oligochitosan has been demonstrated to increase serum IgG, IgM, and IgA levels in broiler chickens and to improve humoral immune functions (Huang et al., 2007). In the present study, we show that the addition of 20 and 40 mg/kg chitooligosaccharide to the diet may significantly increase serum TP levels within 42 days, while we observed a marked reduction of serum LDL-C levels after 42 days, suggesting that moderate chitooligosaccharide supplementation may promote the synthesis of liver proteins, improve lipid metabolism and

lower blood lipid concentrations. In the context of the physiological balance between protein anabolism and proteolysis, the serum protein level usually reflects the state of protein metabolism and immunity function in vivo. Serum total protein comprises albumin and globulin fractions, both of which were shown to reflect the hepatic protein metabolic status in response to dietary treatments in early-weaned piglets (Stoll et al., 1998).

Moderate chitooligosaccharide supplementation may promote immune organ development in broiler chickens

The spleen and bursa of Fabricius are both important immune organs in birds. Maturation, differentiation, and proliferation of B-lymphocytes mainly take place in the bursa of Fabricius and this organ also participates in humoral immunity. The spleen is a peripheral lymphoid organ and plays a dominant role in the generation of immune responses. Previous studies have shown that dietary supplementation with different doses of chitooligosaccharide may promote the development of immune organs and enhance immune functions (Yin et al., 2008). Wang et al. reported that a supplementation of the diet of broiler chickens with 1 g/kg chitooligosaccharide increased the relative weights of thymus and bursa of Fabricius and may also augment serum concentrations of anti-NDV antibodies (Wang et al., 2003). Another study revealed that dietary chitooligosaccharide supplementation enhanced growth performance and increased the relative weight of the spleen in partridges (Chen et al., 2006). We complement these data by showing that the addition of as little as 40 mg/kg chitooligosaccharide may increase spleen weight and bursa of Fabricius organ index in broiler chickens during the production cycle. This difference may be explained by the lower purity and water-solubility of the compounds used in those former studies. Our results suggest that chitooligosaccharide plays a significant role in promoting the development of the spleen and bursa of Fabricius. In addition, we found that the supplementation of the diet of chickens with both 20 and 40 mg/kg chitooligosaccharide significantly increased the splenic node area and the average number of bursa of Fabricius nodes in broiler chickens within 42 days. This treatment also markedly increased the areas of splenic periarterial lymphatic sheaths in 21-day-old and 42-day-old broiler chickens. Our histological results indicate that moderate dietary supplementation with chitooligosaccharide may promote lymphocyte proliferation in the spleen and bursa of Fabricius, improve immune organ structure, and enhance both cellular and humoral immune functions. There are two possible explanations for the effect mediated by chitooligosaccharide. Firstly, chitooligosaccharide may promote the secretion of growth hormone (GH) and the development of the intestinal structure, thus enhancing digestion and absorption of nutrients in the digestive tract (Xu et al., 2013). Secondly, chitooligosaccharide may regulate the activities of various enzymes by affecting the metabolism of proteins, carbohydrates, lipids, and inorganic salts (Huang et al., 2007; Liao et al., 2007; Kim et al., 2014), thus providing the necessary nutrients for the development of immune organs and the proliferation of lymphocytes.

Chitooligosaccharide supplementation may inhibit lymphocyte apoptosis in immune organs of broiler chickens

Apoptosis is an important (intracellular) event in the regulation of normal cellular activities. Proliferation and apoptosis of lymphocytes are kept in a dynamic balance, which is critical for maintaining normal immune functions of the body. It has been reported that chitooligosaccharide may induce apoptosis of oral tumor cells, induce cell cycle arrest, and thus inhibit tumor cell growth (Wimardhani et al., 2014). According to other studies, chitosan may furthermore prevent p53 activation induced by serum starvation in human astrocytes in vitro, diminish the oxidative stress in Plasmodium-infected hepatocytes, and inhibit the apoptosis of hepatocytes and astrocytes *in vitro* (Koo et al., 2002; Tripathy et al., 2015). Chitooligosaccharide is an oligosaccharide resulting from the degradation of chitosan. Chitooligosaccharide has a greater biological activity than chitosan and significantly inhibits tumor cell growth and formation of metastases. It may also diminish the expression of proliferating cell nuclear antigen (PCNA) and the synthesis of DNA in human hepatocellular carcinoma cells, reduce the percentage of cells in the S phase, and inhibit the proliferation of those tumor cells (Shen et al., 2009). Chitooligosaccharide may furthermore suppress the LPS-induced expression of the apoptotic protein caspase-3 in vascular endothelial cells and inhibit LPS-induced cell apoptosis by increasing the ratio of Bcl-2/Bax (Li et al., 2012). Our results suggest that a dietary supplementation of 20 and 40 mg/kg chitooligosaccharide may markedly inhibit caspase-3 expression in splenic and bursa of Fabricius lymphocytes in broiler chickens aged 21 or 42 days. We also provide evidence that the number of apoptotic cells per unit area is reduced when diets are supplemented with chitooligosaccharide, suggesting that appropriate chitooligosaccharide supplementation may inhibit lymphocyte apoptosis in the spleen and bursa of Fabricius of broiler chickens, improve splenic and bursa of Fabricius microstructures, slow bursa of Fabricius degeneration, and enhance immune function. It is not yet completely clear how these effects are mediated by chitooligosaccharide. On one hand, it has to be considered that cell apoptosis is affected by many factors, particularly by oxidative stress. Chitooligosaccharide may decrease lipid peroxidation by enhancing the activity of antioxidant enzymes and improving the capacity of free radical clearance of the immune organs, and thus inhibiting pro-apoptotic pathways. On the other hand, chitooligosaccharide may regulate cytokine secretion and improve the level of serum Ca^{2+}, promoting the proliferation of T and B lymphocytes and the development of immune organs (Han et al., 2005;

Deng et al., 2008). Changes in immune organ growth, development and structure directly affect the body's immunity and resistance.

Previous studies have reported that the quality of life of broilers before slaughter directly affects their immune function and the results of experiments substantiate this result. Environmental factors such as feeding density, temperature, humidity, and transport conditions may lead to stress, immunosuppression, and decrease of muscle quality (Gade and Christensen, 1998; Zulkifli et al., 2009; Dadgar et al., 2012). This may be due to the environmental factor damaging the antioxidant system of the body, as well as the induction of oxidative stress and apoptosis. In this study, all chickens were raised in a comfortable environment before slaughter, at a feeding temperature of 24°C. Density met the standards of broiler breeding, and broiler chickens were not transported over long distances or for extended durations, which guarantees the reliability of the present study. In addition, relevant studies have found that chitooligosaccharide may augment the activity of antioxidant enzymes, reduce malonaldehyde (MDA) content, and inhibit the expression of inducible nitric oxide synthase. All these processes contribute to an attenuation of oxidative stress (Nam et al., 2007; Liu et al., 2009), implying that supplementation of chitooligosaccharide may effectively suppress the oxidative stress caused by environmental factors, reduce cell apoptosis, improve the life quality of broilers before slaughter, and ensure the animals' health.

Conclusions

Dietary supplementation with 20 mg/kg chitooligosaccharide may improve lipid metabolism and immunity of broiler chickens; however, supplementation with 40 mg/kg chitooligosaccharide achieves even stronger effects. They are likely mediated via reduction of serum TG, TC, and LDL-C levels, the promotion of immune organ development, and the improvement of their microstructures, the inhibition of lymphocyte apoptosis, and the overall enhancement of the animals' immune functions. Thus, chitooligosaccharide is a potential alternative to the use of antibiotics in broiler production. However, further research is needed to elucidate the precise mechanisms with which chitooligosaccharide modulates the immune function in broilers.

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