



Effect of Chito-oligosaccharide Supplementation on Immunity in Broiler Chickens*

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ABSTRACT : This study was conducted to determine the effects of dietary supplementation of either 100 mg/kg chito-oligosaccharide (COS) or chlortetracycline (CTC) with corn-soybean-fish meal on immunity in broiler chickens. A total of 147 one-day old male broiler chicks were randomly allocated to 3 treatments with 7 replicate pens per treatment and 7 birds per pen. The experimental diets consisted of a control diet based on corn, soybean and fish meal without COS and any antibiotic supplement and similar diets supplemented with either CTC (80 mg/kg from d 1 to 21 and 50 mg/kg from d 22 to 42) or COS (100 mg/kg from d 1 to 42). During the entire experimental period, all birds had *ad libitum* access to diets and water. The main immune organ indices, T-lymphocyte proliferation, serum cytokine concentrations, serum NO level and serum iNOS activity were measured on d 21 and d 42. On d 21, broilers fed 100 mg/kg COS had improved ($p < 0.01$) indices of spleen, thymus, and bursa of Fabricius compared with the control and CTC birds. Birds receiving 100 mg/kg COS had higher ($p < 0.05$) serum concentrations of IL-1 β , IL-6, IgM, NO and iNOS than birds on the control treatment. Serum Ca²⁺ level of birds fed 100 mg/kg COS tended to be higher ($p = 0.049$) than in birds fed CTC. On d 42, the birds fed 100 mg/kg COS had higher ($p < 0.05$) concentrations of TNF- α and IgM in serum than birds in both the CTC and control treatments. Birds fed 100 mg/kg COS had a higher concentration of IFN- γ than the control group. In conclusion, dietary supplementation of COS appeared to improve the immunity of broilers by promoting the weight of the main immune organs, increasing IgM secretion, stimulating microphages to release TNF- α , IL-1 β , IL-6 and IFN- γ , and activating iNOS to induce NO. (**Key Words :** Chito-oligosaccharide (CTC), Immune Organ Indices, Cytokines, NO, Broiler)

INTRODUCTION

In modern broiler production, intensive genetic selection for fast growth rates and efficient feed conversion of broiler chickens is usually accompanied by greater mortality rates, mainly due to metabolic disorders, and eventually results in susceptibility to infectious diseases (Shapiro et al., 1998).

Antibiotics, as growth promoters and therapeutic medicines to decrease susceptibility to infectious diseases, have been widely used in animal production for many years. However, the existence of several notable problems, such as

antibiotic resistance and residues in animal products, in turn cause environmental pollution and even a hazard to human health (Samarasinghe et al., 2003; Han et al., 2007). With the awareness of serious antibiotic abuse all over the world, scientists have made dedicated attempts to acquire an alternative to the current antibiotics in order to solve, to a certain degree, the existing problems. Antibiotic alternatives should possess either equal or greater growth promoting effect as well as enhancement of immune function compared to feeding antibiotics (Huff et al., 2006).

Chito-oligosaccharides (COS), the degraded products of chitosan, possess non-toxic, biocompatible and biodegradable properties (Choi et al., 2006). Many reports have shown that COS can be a growth promoter for livestock. Our previous studies have proved also that dietary supplementation with COS could improve average daily gain of broilers and increase the apparent digestibility of broiler diets (Li et al., 2007). Meanwhile, the immunity enhancing function of COS has recently aroused great interest. Yu et al. (2004) reported that COS could enhance

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the migratory activity of macrophages which are the key immune competent cells in host defense and regulate immunity by releasing cytokines, increasing the activity of inducible nitric oxide synthase (iNOS), and inducing the synthesis of nitric oxide (NO) *in vitro*.

To optimize macrophage function for the improvement of broiler immunity and thereby decrease economic loss in poultry production due to severe morbidity and mortality, mainly caused by epidemics or plagues in the immunosuppressive state, the use of dietary ingredients such as COS has become potentially one preventive strategy (Qureshi et al., 2000). Therefore, the objective of the present study was to investigate the effects of COS supplementation on immunity by determining the concentration of serum cytokines, NO and calcium and serum iNOS activity in broiler chickens.

MATERIALS AND METHODS

This experiment was conducted under protocols approved by the China Agriculture University Animal Care and Use Committee. The COS, birds and basal diets used in the present experiment were the same as those used in the experiment of Li et al. (2007).

Preparation of COS

The average molecular weight of COS was 1,500 Da and the major components of COS were chitobiose, chitotriose, chitotetraose, chitopentaose and chitohexaose (Li et al., 2007).

Animals, diets and experimental design

A total of 147 male, 1 d old, Arbor Acres broiler chicks (43.5 ± 0.36 g of body weight) were randomly allocated to 3 dietary treatment groups with 7 replicate pens per treatment and 7 birds per pen. The Control (Group 1) was fed the corn-soybean-fish basal diet without COS and any antibiotic supplement. CTC (Group 2) was supplemented with 80 mg/kg CTC for the starter phase (d 1 to 21) and 50 mg/kg CTC for the grower phase (d 22 to 42) of the broilers. COS (Group 3) was supplemented with 100 mg/kg COS to the basal diet throughout the whole experimental period. All diets were fed in mash form and the essential nutrients were formulated to meet the requirement suggested by NRC (1994). All birds were raised in an environmentally controlled room and had *ad libitum* access to diets and water. The birds were inoculated with ND vaccines on d 7 and d 28 and with IBD vaccines on d 14 and d 21 during the study.

Sampling and analyses

On d 21 and d 42, one bird per pen (seven birds per treatment group) was randomly selected, weighed and

euthanized for sampling.

Immune organ indices

Seven euthanized birds per group were dissected to collect the spleen, bursa of Fabricius and thymus, which were then weighed immediately. The indices for spleen, bursa of Fabricius and thymus were formulated as: immune organ weight (g)/body weight (kg).

T-lymphocyte proliferation assay

A whole blood sample (3 ml) was collected by cardiac puncture into an anticoagulant vacuum tube to determine peripheral blood T-lymphocyte proliferation according to the method of Yuan et al. (2005) with some modifications.

Briefly, a total of 3 ml blood was poured slowly into 4 ml Ficoll-Hypaque solution (Tianjin Blood Research Center, China) in a 10 ml test tube and then centrifuged at $2,000 \times g$ for 10 min at room temperature. The white lymphocytes layered in the middle of the test tube were collected and washed three times with RPMI-1640 incomplete culture medium (Gibco, UK) and centrifuged at $2,500 \times g$ for 10 min at room temperature, and then re-suspended in 2 ml of RPMI-1640 complete culture medium which was supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% (v/v) foetal calf serum. Live lymphocytes were counted to adjust the density to 2×10^6 cells per mL through trypan blue dye exclusion. An aliquot of 180 μ l of cell suspension and 20 μ l concanavalin A (Con A; Sigma, USA; 5 μ g/ml of final concentration) solution was added into each well of a 96-well microtitre plate. Meanwhile, another 180 μ l of cell suspension and 20 μ l of RPMI-1640 complete culture medium without Con A, as a blank control, were added into each well of plates under the same procedures. All cells were incubated at 37°C under 5% CO₂ for 66 h, and then 10 ml of 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium (MTT; Sigma, USA; 10 mg/ml) was added to the plates which were then incubated for another 6-8 h. MTT crystals were then dissolved in 100 ml of 10% SDS-0.04 mol/L HCl solution. Eventually, the plates were read using an automated ELISA reader (Sunrise, Tecan, Austria) at 570 nm. The results of lymphocyte proliferation were expressed as stimulation index (SI) calculated by the formula: SI = Absorbance of wells incubated with Con A/Absorbance of wells incubated without Con A.

Determination of the relevant criteria in serum

Another 5 ml blood sample was collected by cardiac puncture into an anticoagulant-free vacuum tube and then centrifuged at $3,000 \times g$ for 10 min at room temperature for the determination of the serum concentrations of calcium ion (Ca²⁺), NO, iNOS, tumor necrosis factor- α (TNF- α),

Table 1. Effect of dietary COS supplementation on indices of main immune organs (g/kg BW) and stimulation index (SI) of T-lymphocyte proliferation in broilers

Item	Diet			SEM	p-value
	Control	CTC	COS		
d 21					
Spleen index	0.67 ^b	0.65 ^b	0.96 ^a	0.04	<0.01
Thymus index	2.49 ^b	2.44 ^b	3.14 ^a	0.11	<0.01
Bursa index	2.09 ^b	2.02 ^b	2.87 ^a	0.10	<0.01
Stimulation index (SI)	0.99	0.90	1.00	0.03	0.38
d 42					
Spleen index	1.10	1.17	1.24	0.06	0.67
Thymus index	2.09	2.10	2.58	0.18	0.50
Bursa index	1.00	1.32	1.35	0.12	0.46
Stimulation index	1.09	1.02	1.05	0.03	0.71

^{a,b} Means within a row with the same or no letter do not differ ($p > 0.05$).

interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interferon- γ (IFN- γ), immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin M (IgM). All determination procedures followed the manufacturer's instructions for the commercial kits.

The concentration of serum Ca²⁺ was analyzed by automatic biochemical analyzer (RA-1000, Bayer Corp., Tarrytown, NY) using a commercially available kit (Zhongsheng Biochemical Co., Ltd., Beijing, China).

The concentration of NO was measured by the Greiss reaction assay through determining production of the reactive nitrogen intermediate, nitrite, which was the stable product of NO (Green et al., 1982). The absorbance at 570 nm was read by an automatic ELISA reader and nitrite concentration was determined by using sodium nitrite as the standard (Ding et al., 1988).

The activity of iNOS was determined using an iNOS assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum TNF- α , IL-1 β and IL-6 concentrations were analyzed using the commercially available ¹²⁵I RIA kits (SINO-UK Huaying Institute of Biological Technology, Beijing, China). Serum IFN- γ concentration was determined by using EIASA kits (R&D Systems, Inc., Minn. USA). Serum IgA, IgG and IgM were measured according to the method of Mast and Goddeeris (1999).

Statistical analysis

Data were subjected to ANOVA using the GLM procedure of SAS (SAS Institute, 1996) with pen as the experiment unit. The mean differences among treatments were separated by Duncan's multiple range tests. Results were expressed as least-square means and SEM. A p-value of less than 0.05 was considered significant.

RESULTS

Immune organ indices and T-lymphocyte proliferation

On d 21, the SI of T-lymphocyte proliferation was not affected by COS supplement (Table 1). However, the indices of spleen, thymus and bursa of Fabricius were significantly improved by supplementation with 100 mg/kg COS compared with the control and CTC treatments ($p < 0.05$).

On d 42, no significant differences in the immune organ indices and SI of T-lymphocyte proliferation were noted in any treatment.

Serum Ca²⁺, NO and iNOS

On d 21, serum Ca²⁺ level of birds fed 100 mg/kg COS tended to be higher ($p = 0.049$) than those of birds in the CTC treatment, whereas there was no difference in serum Ca²⁺ level between Control and COS groups (Table 2). The

Table 2. Effect of dietary COS supplementation on serum Ca²⁺ and NO concentrations and iNOS activity in broilers

Items	Diet			SEM	p-value
	Control	CTC	COS		
d 21					
Serum Ca ²⁺ (mmol/L)	6.5 ^{ab}	6.0 ^b	7.1 ^a	0.19	0.049
Nitric oxide (μ mol/L)	39.5 ^b	50.0 ^a	51.3 ^a	1.83	<0.01
iNOS activity (U/mg)	8.6 ^b	11.6 ^a	11.9 ^a	0.58	0.03
d 42					
Serum Ca ²⁺ (mmol/L)	5.7	6.0	5.8	0.16	0.09
Nitric oxide (μ mol/L)	47.4	58.5	51.8	3.56	0.44
iNOS activity (U/mg)	9.9	10.6	10.7	0.28	0.50

^{a,b} Means within a row with the same or no letter do not differ ($p > 0.05$).

Table 3. Effect of dietary COS supplementation on serum IL-1 β (pg/ml), IL-6 (pg/ml), TNF- α (ng/ml) and IFN- γ (pg/ml) levels in broilers

Items	Diet			SEM	p-value
	Control	CTC	COS		
d 21					
Interleukin-1 β	0.20 ^{ab}	0.17 ^b	0.27 ^a	0.02	0.03
Interleukin-6	186.8 ^b	223.5 ^{ab}	295.3 ^a	17.81	0.02
Tumor necrosis factor- α	2.4	2.6	3.8	0.54	0.54
Interferon- γ	44.0	44.7	51.6	3.72	0.68
d 42					
Interleukin-1 β	0.19	0.24	0.28	0.02	0.10
Interleukin-6	182.1	213.6	221.0	11.61	0.37
Tumor necrosis factor- α	1.27 ^b	1.27 ^b	1.80 ^a	0.09	0.01
Interferon- γ	41.8 ^b	58.0 ^{ab}	62.1 ^a	3.67	0.04

^{a, b} Means within a row with the same or no letter do not differ ($p > 0.05$).

serum NO concentration and serum iNOS activity of birds fed 100 mg/kg COS and CTC were significant higher ($p < 0.05$) than those of birds in the Control group.

On d 42, there was no difference in serum Ca²⁺ and NO concentrations as well as the activity of serum iNOS among treatments.

Serum cytokine production

On d 21, the serum IL-1 β level of birds fed 100 mg/kg COS was higher ($p < 0.05$) than in birds fed CTC, although there was no difference between the COS and Control treatments (Table 3). The level of IL-6 in birds on the COS treatment was significant higher ($p < 0.05$) than the Control treatment, but did not differ between COS and CTC groups. However, no significant differences in the levels of TNF- α and IFN- γ were observed among treatments.

On d 42, the levels of serum IL-1 β and IL-6 were not affected by COS and CTC supplementation. However, the level of IFN- γ was significantly improved ($p < 0.05$) by supplementation with 100 mg/kg COS compared with the Control treatment. Birds on COS treatment had a significantly decreased ($p < 0.05$) concentration of serum TNF- α compared with the other treatments.

Serum immunoglobulin (Ig)

On d 21, serum IgM concentration of birds fed CTC and

COS was significantly higher ($p < 0.05$) than in the Control group, although the serum IgA and IgG levels did not differ (Table 4).

On d 42, no significant differences in levels of serum IgA and IgG were observed among treatments. however, the level of serum IgM in the birds of the COS group was significantly higher ($p < 0.05$) than in the CTC and Control groups.

DISCUSSION

Roura et al. (1992) reported that the growth promoting effect of antibiotics resulted from affecting the ability of the immune system to react to infection in broilers. Dietary supplementation of CTC in this experiment as negative control treatment would result in increased shedding of salmonellae and improve the severity of disease, as reported in Royal et al. (1970). Enhancing the ability of immunity to resist the diseases of livestock without antibiotics would not only benefit the animal's health, welfare and production efficiency but is also a crucial strategy in efforts to improve the microbiological safety of poultry products (Huff et al., 2006).

Chito-oligosaccharide is a positively charged oligosaccharide which is easily obtained by hydrolysis of chitosan. Compared to chitosan, COS has good water

Table 4. Effects of dietary COS supplementation on serum IgA, IgG and IgM concentrations ($\mu\text{g/mL}$) in broilers

Items	Diet			SEM	p-value
	Control	Chlortetracycline	COS		
d 21					
Immunoglobulin A	65.8	52.1	44.4	3.82	0.07
Immunoglobulin G	335.4	350.1	351.8	5.23	0.41
Immunoglobulin M	117.1 ^b	153.3 ^a	167.1 ^a	8.04	0.02
d 42					
Immunoglobulin A	48.3	47.9	51.4	3.95	0.93
Immunoglobulin G	335.8	347.8	327.6	5.40	0.31
Immunoglobulin M	155.6 ^b	192.1 ^b	383.3 ^a	24.87	<0.01

^{a, b} Means within a row with the same or no letter do not differ ($p > 0.05$).

solubility and low viscosity that enables COS to be easily absorbed through the intestine and to quickly enter the bloodstream (Jeon et al., 2000; Chae et al., 2005), and subsequently it has been shown to possess multiple biological activities, such as antifungal (Zhang et al., 2003), antibacterial (Jeon et al., 2001), antitumor (Jeon and Kim, 2002) and immune enhancing effects (Mori et al., 1998). In the present study, we observed the effects of dietary supplementation of COS on immunity in broiler chickens from facets of immune organs, immune cells and immune cytokines.

The main immune organs such as thymus, spleen and bursa of Fabricius in avian species perform greatly significant roles in immunity of animals, including the main local sites for the maturation, differentiation and proliferation of lymphocytes. The bursa of Fabricius and thymus, both very important central immunity organs, are the humoral immunity centre in poultry. The spleen, a crucial non-specific peripheral lymphoid organ, has a dominant role in the generation of immune responses because of the absence of well-developed lymph nodes in most avian species, including the chicken (Mast and Goddeeris, 1999). In our study, we found that dietary supplementation of COS significantly increased the relative weights of spleen, thymus and bursa of Fabricius as reflected by indices of these main immune organs on d 21. Our results were similar to those of Wang et al. (2003), who reported that dietary supplementation of 0.1% COS improved the relative weight of thymus and bursa of Fabricius in broilers. Chen et al. (2006) also found that dietary supplementation of COS not only promoted the growth performance but also significantly enhanced the relative weight of thymus, spleen, and bursa of Fabricius in the partridge. From the above observations, we could elementarily postulate that COS had immune enhancing effects.

The T-lymphocyte is the central regulatory cell of the immune system, whereas lymphocyte proliferation response to Con A is a significant criterion reflecting the function of the T-lymphocyte and cellular immunity (Mao et al., 2005). Although the stimulation index of T-lymphocyte proliferation was not affected in the current study, we found that dietary supplementation of 100 mg/kg COS not only increased the concentrations of serum IL-1 β and IL-6 on d 21 but also enhanced the concentrations of IFN- γ and TNF- α on d 42 in broiler chickens. Macrophages, the first line of immunological defense against pathogens and the important part of the innate disease resistance mechanism, perform significant roles such as phagocytic, microbiocidal and tumoricidal functions (Qureshi et al., 2000). Immune promoters such as COS were generally identified as compounds that bound specifically with the cell surface receptor proteins of phagocytes or lymphocytes to stimulate

the effective generation of an immune response by the cooperation of cytokines to activate the non-specific immune system of animals (Lambrecht et al., 1999). During this process, the activated macrophages would release cytokines such as TNF- α , IL-1 β , IL-6 and IFN- γ to inhibit the growth of a wide variety of tumor cells and microorganisms with the help of NO cytolytic function and iNOS (Higuchi et al., 1990). There are many reports which suggest that COS can enhance the migratory activity of macrophage to produce IL-1, IL-6 and TNF- α , as this study reflected. Fraifeld et al. (1995) elucidated that COS induced the release of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α , which would cause fever or the hepatic secretion of acute phase response to enhance specific and non-specific immunity and thereby decreased the mortality of birds. Maeda and Kimura (2004) found that COS induced the activation of macrophages through the production of IFN- γ from the intestinal intraepithelial lymphocytes to perform an antitumor function.

In the present study, we also found that dietary supplementation improved the activity of iNOS and increased the concentration of NO on d 21 in broiler chickens. Nitric oxide possesses many kinds of biological effects such as vascular homeostatis, neurotransmission and host defense (Moncada et al., 1991). As a small reactive molecule, NO is an end product of the metabolism of the L-arginine-NO pathway synthesized by a family of NOS enzymes (Lancaster, 1992; Nathan, 1992), which exists in two types of NOS. Two constitutive isoforms, neuronal (nNOS) and endothelial (eNOS) synthase, have been proved to exist in neurons or endothelial cells as signaling molecules (John et al., 1997), whereas the inducible isoform, iNOS, was first isolated from activated macrophages and was expressed after the stimulation of macrophages by endotoxins such as LPS, cytokines and microbial pathogens (Nathan, 1992; Xie et al., 1992). Several researchers have reported the effect of COS on the production of NO. Tokoro et al. (1989) and Kobayashi et al. (1990) found that COS had a potential immune therapeutic function in mice through an effect on NO production. Seo et al. (2000) reported that the synergism between the effects of IFN- γ and water soluble chitosan on NO synthesis depended mainly on the increased secretion of TNF- α induced by COS. Wu and Tsai (2007) also found that COS in combination with IFN- γ enhanced NO production and iNOS expression in murine macrophages RAW 264.7. The results of the present study, together with the above reports, indicated that oral intake of COS had beneficial effects on macrophage-mediated immunity by stimulating the activity of iNOS, inducing the synthesis of NO and releasing TNF- α , IL and IFN- γ (Yu et al., 2004; Kim et al., 2006; Dou et al., 2007).

As discussed above, macrophages could act as the key

immune-competent and immune-regulator cells in host defense by affecting immune parameters via numerous cytokines such as IL-1 β , IL-6, IFN- γ and TNF- α to promote the proliferation and differentiation of immune cells, as well as releasing moderate production of NO from the expression of iNOS in activated macrophages to kill pathogens or tumor cells under the appropriate immune stimulant from the environment (Karupiah et al., 1993; Qureshi et al., 2000). However, excessive production of NO has been described in many pathophysiological conditions such as inflammation status (Kolios et al., 2004; Naseem, 2005). In a previous study, our laboratory showed that dietary supplement of 100 mg/kg COS improved the average daily gain and feed conversion rate of broiler chickens in the normal state (Li et al., 2007), which indicated that NO production in the present study was not excessive but moderate to enhance immunity and thereby promote the growth of broiler chickens.

Moreover, we found that a dietary supplement of COS tended to improve the level of serum Ca²⁺ on d 21 and increased the concentration of IgM during the entire experimental period. Calcium was an important second messenger that played a key role in signaling T-lymphocyte activation. In this study, COS did not affect T-lymphocyte proliferation but Han et al. (2005) postulated Ca²⁺ acted as an important signal transmitter between the mannose receptor, the major receptor responsible for COS uptake, and cytokine production in RAW 264.7 cells. Chito-oligosaccharide is a unique, positively charged oligosaccharide, which could strongly bind to a negatively charged surface, such as the cation Ca²⁺. The complex between COS and the divalent metal cation was due to electrostatic interaction between carboxyl groups or amino groups (electron donor) and Ca²⁺ (electron withdrawer) (Gotoh et al., 2004). Zafar et al. (2004) found that oligosaccharide intake could increase Ca²⁺ bioavailability and improve Ca²⁺ absorption and retention as well as inhibit bone resorption in ovariectomized rats. Jung et al. (2006) also found that the intake of COS had the beneficial function of preventing negative mineral balance through improving Ca²⁺ bioavailability in ovariectomized rats. Because COS could form the soluble complex of Ca-COS to inhibit the formation of insoluble Ca²⁺-phosphate salt, COS could be used as a potent calcium fortifier with a high Ca²⁺ bioavailability (Jung et al., 2006). On the other hand, the level of IgM in serum was improved by dietary supplement of COS, which was similar to the findings of Wu and Tsai (2004) who reported that COS improved IgM secretion of human hybridoma HB4C5 cells. Wu et al. (2002) showed that COS could improve the proliferation of Con A and LPS challenged lymphocytes in rats and found that dietary supplementation of COS increased the levels of serum IgG and IgM.

In conclusion, dietary supplementation of 100 mg/kg of COS improved the immunity of broilers. This increase was likely mediated through the effects of COS on promoting the development of the main immune organs, increasing IgM secretion, stimulating macrophages to release TNF- α , IL-1 β , IL-6 and IFN- γ , and activating iNOS to induce NO. Thus, COS is a potential alternative to the use of antibiotics in broiler production. However, further study is needed to elucidate the mechanism of the interaction between COS and macrophages by which COS improves immune function in broilers.

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