Effects of Low-Molecular-Weight Chitooligosaccharide on Production Performance, Egg Quality, Serum Biochemical Indices, Cecal Microbial Count, and Spleen Interleukin-2 and Tumor Necrosis Factor-α Gene Expression in Laying Hens: Postprint

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Abstract

This experiment aimed to investigate the effects of dietary supplementation with different levels of low molecular weight (1,000 u) chitosan oligosaccharide on production performance, egg quality, serum biochemical indices, cecal microbial counts, and the expression of interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) genes in the spleen of laying hens. A total of 600 Hy-Line Brown laying hens at 58 weeks of age with similar body weight and laying rate were randomly allocated to 4 groups with 5 replicates of 30 hens each. The control group was fed a basal diet, while the treatment groups were fed experimental diets supplemented with 300, 600, or 900 mg/kg chitosan oligosaccharide. The pre-trial period was 7 d, and the formal trial period was 42 d. The results showed: 1) The laying rates of the groups supplemented with 300, 600, and 900 mg/kg chitosan oligosaccharide increased by 4.52% ($P<0.05$), 2.99% ($P>0.05$), and 4.08% ($P>0.05$), respectively, compared with the control group. 2) At the end of weeks 3 and 6, the Haugh unit of eggs from the groups supplemented with 600 and 900 mg/kg chitosan oligosaccharide increased by 6.87%, 6.69% and 6.47%, 6.60% ($P<0.05$), respectively, compared with the control group. 3) Compared with the control group, dietary supplementation with 600 and 900 mg/kg chitosan oligosaccharide significantly decreased serum glucose and cholesterol contents and aspartate aminotransferase activity ($P<0.05$). 4) Compared with the control group, dietary supplementation with 600 and 900 mg/kg chitosan oligosaccharide significantly increased cecal Bifidobacterium and Lactobacillus counts ($P<0.05$) and significantly decreased cecal Staphylococcus aureus counts

(P<0.05). 5) Compared with the control group, dietary supplementation with 300 and 600 mg/kg chitosan oligosaccharide significantly increased spleen IL-2 mRNA expression level (P<0.05), and dietary supplementation with 600 mg/kg chitosan oligosaccharide significantly increased spleen TNF-α mRNA expression level (P<0.05). These results indicate that dietary supplementation with different levels of chitosan oligosaccharide improved the laying rate and Haugh unit, regulated intestinal microflora, and enhanced the immunity of laying hens, with the optimal supplementation level being 600 mg/kg.

Full Text

Effects of Low Molecular Weight Chitooligosaccharides on Production Performance, Egg Quality, Serum Biochemical Indices, Cecal Microbial Populations, and Spleen Interleukin-2 and Tumor Necrosis Factor-α Gene Expression in Laying Hens

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Abstract: This study investigated the effects of dietary supplementation with different levels of low molecular weight (1,000 u) chitooligosaccharides (COS) on production performance, egg quality, serum biochemical indices, cecal microbial populations, and the expression of interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) genes in the spleen of laying hens. Six hundred 58-week-old Hyline brown laying hens with similar body weight and egg production rate were randomly allocated to 4 groups, each consisting of 5 replicates of 30 hens. The control group received a basal diet, while the experimental groups received the basal diet supplemented with 300, 600, or 900 mg/kg COS. A 7-day preliminary period was followed by a 42-day formal experimental period.

The results demonstrated: (1) Compared with the control group, supplementation with 300, 600, and 900 mg/kg COS increased egg production rate by 4.52% (P<0.05), 2.99% (P>0.05), and 4.08% (P>0.05), respectively. (2) At the end of weeks 3 and 6, the 600 and 900 mg/kg COS groups exhibited Haugh unit improvements of 6.87%, 6.69% and 6.47%, 6.60%, respectively (P<0.05). (3) Dietary supplementation with 600 and 900 mg/kg COS significantly reduced serum glucose and cholesterol concentrations and decreased aspartate aminotransferase activity (P<0.05). (4) Supplementation with 600 and 900 mg/kg COS significantly increased cecal populations of bifidobacteria and lactobacilli (P<0.05) while decreasing *Staphylococcus aureus* populations (P<0.05). (5) Dietary supplementation with 300 and 600 mg/kg COS significantly elevated spleen IL-2 mRNA expression levels (P<0.05), and 600 mg/kg COS significantly increased spleen TNF-α mRNA expression (P<0.05).

In conclusion, dietary COS supplementation improved egg production rate and Haugh unit, modulated intestinal microbial flora, and enhanced immune function in laying hens. The optimal supplementation level appears to be 600 mg/kg.

Keywords: laying hens; chitooligosaccharides; production performance; intestinal microbial community; interleukin-2; tumor necrosis factor-α

Introduction

The global trend toward banning antibiotic use and the lack of effective vaccines have compelled animal producers to seek safe disease management alternatives. Probiotics, prebiotics, synbiotics, plant extracts, acidifiers, and immune enhancers have been widely adopted to varying degrees. As prebiotics, various oligosaccharides are being incorporated into livestock diets to improve animal health, production performance, and immunity while modulating intestinal microflora. Certain oligosaccharides are considered prebiotic compounds that resist hydrolysis in the anterior digestive tract, reaching the hindgut where they alter colonic microbial structure.

Chitooligosaccharides are degradation products of chitosan or chitin through enzymatic or chemical hydrolysis. Chitosan and its derivatives have demonstrated diverse functional properties applicable in food, agriculture, and environmental protection. Chitosan with a degree of polymerization below 20 and average molecular weight under 3,900 u is defined as chitooligosaccharide. Unlike chitosan, chitooligosaccharides are water-soluble due to their short chain length and free D-glucosamine units, attracting research interest because of their low viscosity and high solubility at neutral pH.

Recent research has focused on the health benefits of chitooligosaccharides, which exhibit multiple biological effects including cholesterol reduction, blood pressure regulation, anti-infection properties, arthritis control, calcium absorption enhancement, and improved antitumor activity. Pangestuti et al. found that chitooligosaccharides with molecular weight below 1,000 u demonstrated potent anti-inflammatory activity by attenuating pro-inflammatory mediators in the MAPK signaling pathway of LPS-induced BV2 microglial cells. Mei et al. reported that chitooligosaccharide injection in mice effectively resisted cyclophosphamide-induced immunosuppression and enhanced systemic immune responses while modulating immune cell function. Li et al. demonstrated that chitooligosaccharides effectively modulated cecal microflora and improved immune function in broiler chickens. Rozeboom et al. found that dietary chitooligosaccharide supplementation improved nutrient digestibility in pigs. However, research on chitooligosaccharides in laying hens remains limited, particularly regarding low molecular weight formulations. Yan et al. reported that dietary chitooligosaccharide supplementation improved egg production, egg quality, and immunity in laying hens. Building upon previous research on different molecular weights, this study specifically investigated low molecular weight

(1,000 u) chitooligosaccharides and their effects on production performance, egg quality, serum biochemical indices, cecal microbial populations, and spleen IL-2 and TNF-α gene expression.

1.1 Chitooligosaccharides

The experimental chitooligosaccharides were extracted from shrimp and crab shells and purchased from a commercial company, with a deacetylation degree exceeding 90%, molecular weight of 1,000 u, and water solubility of 99%.

1.2 Experimental Design and Diets

Six hundred 58-week-old Hy-line brown laying hens with similar body weight and production performance were randomly divided into 4 groups, each comprising 5 replicates. Each replicate contained 10 cages with 3 hens per cage. Replicates were distributed across upper and lower tiers of battery cages to eliminate cage position effects. Hens were housed in a closed laying house with 16 hours of constant daily lighting, adequate ventilation, and ambient temperature maintained at 20-23°C. The control group received a basal diet, while experimental groups received the basal diet supplemented with 300, 600, or 900 mg/kg chitooligosaccharides. The basal diet composition and nutrient levels are presented in . All diets were fed in mash form. Each cage was equipped with nipple drinkers and feed troughs. Hens had ad libitum access to feed and water throughout the 7-day preliminary period and 42-day formal experimental period, with routine management practices followed.

1.3 Measurement Indices and Methods

1.3.1 Production Performance Daily records were maintained for egg production and egg weight per replicate. Weekly feed intake was recorded to calculate average daily feed intake, egg production rate, average egg weight, and feed-to-egg ratio.

1.3.2 Egg Quality At the end of weeks 3 and 6, 7 eggs were randomly selected from each replicate (35 eggs per group) for quality assessment, including Haugh unit, yolk ratio, albumen height, shell thickness, and shell strength.

Calculations: - Yolk ratio (%) = (yolk weight/egg weight) × 100 - Haugh unit: Hu = 100 × log(H - 1.7 × W^0.37 + 7.6), where H represents albumen height and W represents egg weight - Albumen height: Measured using an Egg Multi Tester (EMT-5200), expressed in mm - Shell strength: Determined using an eggshell force gauge (ROBOTMATION, model efg-0503, Japan), expressed in kg/cm^2 - Shell thickness: Average of measurements at the blunt end, pointed end, and equatorial region, precise to 0.01 mm

1.3.3 Serum Biochemical Indices At experiment conclusion, 3 hens per replicate (15 per group) were randomly selected for blood collection from the wing vein after overnight fasting. Blood samples (3 mL) were allowed to clot at a 45° angle for 30 minutes, then centrifuged at 3,000 rpm for 10 minutes. Serum was harvested and stored at -20°C. Serum total protein, glucose, cholesterol, triglyceride concentrations, and alanine aminotransferase and aspartate aminotransferase activities were measured using a 7020 automatic biochemical analyzer with assay kits purchased from Nanjing Jiancheng Bioengineering Institute.

1.3.4 Cecal Microbial Populations At experiment conclusion, 3 hens per replicate were randomly selected, weighed, and slaughtered. Cecal contents were collected and ligated for microbial analysis. One gram of cecal content was suspended in 9 mL phosphate-buffered saline (PBS), vortexed for 5 minutes, and centrifuged at 1,000 rpm for 10 minutes. The supernatant was serially diluted (10-fold). Dilutions of 10^{-2}, 10^{-3}, and 10^{-4} (0.02 mL each) were plated on selective media for *Escherichia coli* and *Staphylococcus aureus* (MacConkey agar, Qingdao Hope Bio-Technology Co., Ltd.). Dilutions of 10^{-5}, 10^{-6}, and 10^{-7} were plated on selective media for bifidobacteria and lactobacilli (bismuth sulfite agar, Qingdao Hope Bio-Technology Co., Ltd.). Three plates were inoculated per dilution. Bifidobacteria were anaerobically cultured at 37°C for 72 hours, lactobacilli for 48 hours, and *E. coli* and *S. aureus* aerobically cultured for 24 hours before colony counting.

Calculation: Microbial count per gram = (average colony count/volume of inoculum) × dilution factor

1.3.5 Spleen IL-2 and TNF-α mRNA Expression Levels At experiment conclusion, 3 hens per replicate were randomly selected, weighed, and slaughtered. Spleens were isolated, weighed, and the spleen index calculated as relative weight to body weight. Spleen samples were stored in liquid nitrogen. Total RNA was extracted from 50 mg spleen tissue using Trizol reagent (Invitrogen) according to manufacturer instructions. RNA pellets were dissolved in diethylpyrocarbonate (DEPC)-treated water, and purity was assessed by measuring OD260/280 ratio (acceptable range: 1.8-2.0). RNA was stored at -80°C. After DNase I treatment to remove genomic DNA, 1 g RNA was reverse transcribed to cDNA using PrimeScript™ RT reagent kit (TaKaRa) and stored at -20°C.

Primers were designed based on chicken β-actin (GenBank accession L08165), TNF-α (AY765397), and IL-2 (AY510091) sequences and synthesized by Shanghai Sangon Biotech. Primer sequences for real-time fluorescent quantitative PCR are shown in .

Real-time PCR protocol: SYBR Green staining method using SYBR® Premix Ex Taq™ kit (TaKaRa) on ABI-7500 system. Reaction mixture (20 L):

SYBR® Premix Ex Taq™ (2×) 10.0 L, forward primer (10 mol/L) 0.4 L, reverse primer (10 mol/L) 0.4 L, ROX Reference Dye II (50×) 0.4 L, cDNA template 2.0 L, dH_2O 6.8 L. Cycling conditions: 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s with fluorescence signal collection. Each sample was run in triplicate. Relative gene expression was calculated using the 2^(-ΔΔCT) method.

1.4 Data Processing and Analysis

Experimental data were analyzed using SAS 9.3 software and expressed as mean ± standard deviation. One-way ANOVA was performed for statistical analysis, with $P<0.05$ considered significant.

Results

2.1 Effects of Low Molecular Weight Chitooligosaccharides on Production Performance

The effects of low molecular weight chitooligosaccharides on feed-to-egg ratio, egg production rate, average daily feed intake, and average egg weight are presented in . Throughout the experimental period, egg production rates in the 300, 600, and 900 mg/kg COS groups increased by 4.52% ($P<0.05$), 2.99% ($P>0.05$), and 4.08% ($P>0.05$) compared with the control group, respectively. No significant differences were observed among groups in feed-to-egg ratio, average daily feed intake, or average egg weight ($P>0.05$).

2.2 Effects of Low Molecular Weight Chitooligosaccharides on Egg Quality

The effects on Haugh unit, yolk ratio, albumen height, shell thickness, and shell strength are shown in . At the end of weeks 3 and 6, Haugh units in the 600 and 900 mg/kg COS groups increased by 6.87%, 6.69% and 6.47%, 6.60%, respectively ($P<0.05$). The 900 mg/kg COS group exhibited reduced yolk ratio by 7.10% and 7.26% at weeks 3 and 6, respectively ($P<0.05$). No significant differences were detected in albumen height, shell thickness, or shell strength among groups ($P>0.05$).

2.3 Effects of Low Molecular Weight Chitooligosaccharides on Serum Biochemical Indices

Serum biochemical indices are presented in . Compared with the control group, serum glucose concentrations decreased by 17.44% and 21.36% in the 600 and 900 mg/kg COS groups ($P<0.05$). Serum cholesterol concentrations decreased by 10.28%, 10.83%, and 12.92% in the 300, 600, and 900 mg/kg COS groups ($P<0.05$). Aspartate aminotransferase activity decreased by 10.66% and 8.29% in the 600 and 900 mg/kg COS groups ($P<0.05$). No significant differences

were observed in serum total protein, triglyceride concentrations, or alanine aminotransferase activity (P>0.05).

2.4 Effects of Low Molecular Weight Chitooligosaccharides on Spleen Index and Cecal Microbial Populations

Results for spleen index and cecal microbial populations are shown in . No significant differences were found in spleen index among groups (P>0.05). Compared with the control group, cecal bifidobacteria populations increased by 11.68%, 8.09%, and 8.86% in the 300, 600, and 900 mg/kg COS groups (P<0.05). Cecal lactobacilli populations increased by 9.97% and 13.59% in the 600 and 900 mg/kg groups (P<0.05). Cecal *S. aureus* populations decreased by 10.49%, 12.08%, and 15.67% in the 300, 600, and 900 mg/kg groups (P<0.05). No significant differences were observed in cecal *E. coli* populations (P>0.05).

2.5 Effects of Low Molecular Weight Chitooligosaccharides on Spleen TNF-α and IL-2 mRNA Expression Levels

The effects on spleen TNF-α and IL-2 mRNA expression are presented in . Compared with the control group, IL-2 mRNA expression levels increased significantly in the 300 and 600 mg/kg COS groups (P<0.05), while TNF-α mRNA expression increased significantly in the 600 mg/kg group (P<0.05).

Discussion

3.1 Effects on Production Performance and Egg Quality

Various oligosaccharides have been incorporated into livestock diets as prebiotics to enhance growth performance, immune function, and intestinal microflora modulation. Previous studies have demonstrated that chitooligosaccharides possess antifungal and antimicrobial activities, improve intestinal health in male broilers, and enhance nutrient digestibility and growth performance. The improved production performance observed in this study may be attributed to enhanced dry matter and protein digestibility. These findings align with Meng et al.'s research in laying hens but differ from Yan et al.'s results. Chen et al. similarly reported improved dry matter and nitrogen digestibility in weaned pigs fed chitooligosaccharides. Additionally, mannan oligosaccharide supplementation significantly improved reproductive performance in broiler breeders. Given the structural similarity between chitooligosaccharides and mannan oligosaccharides, similar benefits for poultry production can be inferred. Performance improvements have been attributed to mannan oligosaccharides' ability to maintain intestinal health and inhibit pathogen adhesion. We therefore hypothesize that chitooligosaccharides exert comparable effects. However, research on chitooligosaccharides in laying hens remains limited, necessitating further investigation.

The Haugh unit is commonly used to measure albumen quality and assess egg freshness. This study demonstrated that dietary supplementation with 600 or 900 mg/kg low molecular weight chitooligosaccharides significantly improved Haugh units and albumen height, indicating enhanced egg freshness.

3.2 Effects on Serum Biochemical Indices

Serum biochemical parameters are frequently used to evaluate animal responses to probiotics. This study revealed that 600 and 900 mg/kg chitooligosaccharide supplementation significantly reduced serum glucose concentrations and aspartate aminotransferase activity while decreasing cholesterol and triglyceride levels. These results are consistent with Sugano et al.' s findings. Previous studies have confirmed the lipid-lowering effects of chitosan, though the cholesterol-reduction mechanism remains controversial. The primary mechanism may involve bile acid binding, which reduces lipid absorption in the intestine, consequently lowering cholesterol and stimulating hepatic bile acid synthesis.

3.3 Effects on Cecal Microbial Populations

Intestinal microflora is crucial for immune system maturation and normal intestinal morphological development. Microbial communities enhance intestinal barrier function, reduce pathogen adhesion to mucosa, and decrease allergen entry. Oligosaccharides are typically defined as prebiotics that selectively stimulate beneficial bacterial growth. Liu et al. demonstrated that chitooligosaccharide supplementation increased lactobacilli and decreased fecal *E. coli* in weaned pigs. This study showed that 600 or 900 mg/kg chitooligosaccharide supplementation in laying hens increased cecal bifidobacteria and lactobacilli while reducing *S. aureus* populations. No et al. reported that chitooligosaccharides generally exhibit stronger bactericidal effects against Gram-positive bacteria (e.g., *S. aureus*) than Gram-negative bacteria (*E. coli*), easily inhibiting *S. aureus* growth while having limited effects on *E. coli*. These findings align with previous research, though the mechanisms underlying chitooligosaccharides' antimicrobial activity remain unclear. One proposed mechanism involves electrostatic interaction between the positively charged glucosamine monomers in chitooligosaccharides and negatively charged microbial cell membranes, causing intracellular component leakage. Another potential mechanism involves competitive exclusion of *S. aureus* through increased bifidobacteria and lactobacilli populations.

3.4 Effects on Spleen TNF-α and IL-2 mRNA Expression

Chitooligosaccharides as immunopotentiators are defined as compounds that bind specifically to cell surface receptor proteins on phagocytes or lymphocytes, activating the animal' s non-specific immune system through cytokine synergy and stimulating effective immune responses. Activated macrophages release cytokines including TNF-α, IL-1β, IL-6, and IFN-γ, which inhibit tumor cell and microbial growth with the assistance of nitric oxide and nitric oxide synthase.

IL-2, secreted by activated T cells, possesses potent T cell growth factor activity, including lymphocyte proliferation, maturation, and differentiation, and is widely recognized as a key cytokine in T cell-dependent immune responses. TNF-α, secreted by activated T cells, increases intestinal permeability.

The antitumor activity of chitooligosaccharides was first reported in the early 1970s, primarily attributed to the cationic properties of amino groups. Subsequent research indicated that molecular weight significantly influences antitumor activity, with antitumor effects resulting from enhanced natural killer cell activity. This study demonstrated that 600 mg/kg low molecular weight chitooligosaccharide supplementation significantly increased spleen IL-2 and TNF-α mRNA expression, suggesting enhanced cytokine secretion. The mutual interaction between IL-2 and T-lymphocytes increases mature T lymphocytes, enabling greater cytokine secretion, while elevated IL-2 and TNF-α levels further promote T lymphocyte proliferation. Deng et al. reported that chitooligosaccharide supplementation (average molecular weight 1,500 u) promoted immune organ weight gain, increased IgM secretion, and stimulated macrophage release of IL-1β, IL-6, and TNF-α, thereby improving broiler immunity. Conversely, Walsh et al. found that high molecular weight chitooligosaccharides (5-10 ku, 10-50 ku, 50-100 ku) did not significantly affect gastrointestinal cytokine expression in weaned pigs.

Conclusion

Dietary supplementation with low molecular weight chitooligosaccharides improved egg production rate and Haugh unit, increased cecal bifidobacteria and lactobacilli populations, decreased cecal *S. aureus* populations, and enhanced spleen IL-2 and TNF-α mRNA expression. Based on comprehensive evaluation of all measured parameters, the recommended supplementation level is 600 mg/kg.

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