Effects of Dietary Supplementation of Chitosan Oligosaccharide and/or Mycotoxin Adsorbent on Growth Performance, Non-specific Immunity, and Disease Resistance of Litopenaeus vannamei (Postprint)

Authors: Huang Qincheng, Shen Guangrong, Tan Beiping, Zhang Shuanghei, Wen Jing, Dong Xiaohui

Date: 2017-11-08T00:00:00+00:00

Abstract

This experiment aimed to investigate the effects of dietary supplementation of chitosan oligosaccharide (COS) and/or silicoaluminate mycotoxin adsorbent (MA) on growth performance, non-specific immunity, and disease resistance of Pacific white shrimp (Litopenaeus vannamei). A basal diet was formulated (C0 group, as control), and 100 mg/kg COS (C0.1 group), 250 mg/kg COS (C0.25 group), 2,500 mg/kg MA (M2.5 group), 100 mg/kg COS + 2,500 mg/kg MA (C0.1+M2.5 group), and 250 mg/kg COS + 2,500 mg/kg MA (C0.25+M2.5 group) were respectively added to the basal diet to prepare six iso-nitrogenous and iso-lipidic experimental diets, which were fed to Pacific white shrimp with an initial body weight of (0.23$±$0.02) g for 8 weeks. Each group had three replicates, with 40 shrimp per replicate. The results showed: 1) The specific growth rate and weight gain rate of the C0.1+M2.5 group were significantly higher than those of the M2.5 group (P<0.05), the feed conversion ratio of the C0.1+M2.5 group was optimal and significantly lower than those of other groups (P<0.05), and the protein efficiency ratio of the C0.1+M2.5 group was the highest and significantly higher than those of other groups except the C0.25+M2.5 group (P<0.05); the crude protein content of shrimp body in the C0.1+M2.5 group was significantly higher than that in the C0.1 group (P<0.05), while the crude fat content was significantly lower than that in the C0.1 group (P<0.05). 2) Serum AKP activities in the C0.1+M2.5 and C0.25+M2.5 groups were significantly lower than those in the C0.1 and C0.25 groups (P<0.05), serum SOD and PO activities in the C0.1+M2.5 group were significantly higher than those in the M2.5 and C0.1 groups (P<0.05), and serum MDA content in the C0.1+M2.5

group was significantly lower than that in the C0.1 group (P<0.05); hepatopancreas MDA content in the C0.1+M2.5 group was significantly lower than that in the C0.1 group (P<0.05), while hepatopancreas AKP, PO, SOD, and LZM activities in the C0.25+M2.5 group were all decreased compared with the C0.1+M2.5 group, and conversely, hepatopancreas MDA content was increased. 3) After challenge with Vibrio harveyi for 7 days, cumulative mortality of the C0.1+M2.5 and C0.25+M2.5 groups was significantly lower than that of the C0 and M2.5 groups (P<0.05). Based on these results, it can be concluded that: under the conditions of this experiment, dietary supplementation of certain amounts of COS and/or MA could promote the growth of Pacific white shrimp, and the combined supplementation of COS and MA was superior to individual supplementation in terms of growth promotion, immunity enhancement, and disease resistance improvement; overall, the combination of 100 mg/kg COS and 2,500 mg/kg MA showed better effects.

Full Text

Effects of Dietary Chitosan Oligosaccharide and/or Mycotoxin Adsorbent on Growth Performance, Nonspecific Immunity and Disease Resistance of *Litopenaeus vannamei*

HUANG Qincheng[1], SHEN Guangrong[2], TAN Beiping[1], ZHANG Shuang[1], HEI Wenjing[2], DONG Xiaohui[1]*

[1]Laboratory of Aquatic Animal Nutrition and Feed, Guangdong Ocean University, Zhanjiang 524088, China
[2]Shenzhen Yunong Science & Technology Co., Ltd., Shenzhen 518110, China

Abstract: An 8-week feeding trial was conducted to investigate the effects of single or combined supplementation of chitosan oligosaccharide (COS) and silicon aluminate mycotoxin adsorbent (MA) on the growth performance, nonspecific immunity, and disease resistance of *Litopenaeus vannamei*. Six isonitrogenous and isolipidic experimental diets were prepared: a basal diet (C0, control), and five diets supplemented with 100 mg/kg COS (C0.1), 250 mg/kg COS (C0.25), 2,500 mg/kg MA (M2.5), 100 mg/kg COS + 2,500 mg/kg MA (C0.1+M2.5), or 250 mg/kg COS + 2,500 mg/kg MA (C0.25+M2.5). Juvenile shrimp with an initial weight of (0.23$±$0.02) g were fed the experimental diets for 8 weeks. Each treatment had three replicates with 40 shrimp per replicate. The results showed: 1) The specific growth rate (SGR) and weight gain rate (WGR) in the C0.1+M2.5 group were significantly higher than those in the M2.5 group (P<0.05). The C0.1+M2.5 group exhibited the optimal feed conversion ratio (FCR), which was significantly lower than all other groups (P<0.05), and the highest protein efficiency ratio (PER), which was significantly higher than all groups except C0.25+M2.5 (P<0.05). The crude protein content of shrimp body in C0.1+M2.5 was significantly higher than that in C0.1 (P<0.05), while the crude lipid content was significantly lower (P<0.05). 2) Serum alkaline phosphatase (AKP) activity in C0.1+M2.5 and C0.25+M2.5

groups was significantly lower than in C0.1 and C0.25 groups (P<0.05). Serum superoxide dismutase (SOD) and phenoloxidase (PO) activities in C0.1+M2.5 were significantly higher than those in M2.5 and C0.1 groups (P<0.05), while serum malondialdehyde (MDA) content was significantly lower than in C0.1 (P<0.05). Hepatopancreatic MDA content in C0.1+M2.5 was significantly lower than in C0.1 (P<0.05). Activities of AKP, PO, SOD, and lysozyme (LZM) in hepatopancreas of C0.25+M2.5 were lower than those in C0.1+M2.5, whereas hepatopancreatic MDA content was conversely higher. 3) Following challenge with *Vibrio harveyi* for 7 days, cumulative mortality rates in C0.1+M2.5 and C0.25+M2.5 groups were significantly lower than those in C0 and M2.5 groups (P<0.05). These results indicate that dietary supplementation with COS and/or MA can promote growth, enhance nonspecific immunity, and improve disease resistance in *L. vannamei*. Combined supplementation of COS and MA was more effective than single supplementation, with the combination of 100 mg/kg COS and 2,500 mg/kg MA showing the best overall effects under the present experimental conditions.

Keywords: chitosan oligosaccharide; mycotoxin adsorbent; *Litopenaeus vannamei*; growth performance; nonspecific immunity; disease resistance

Introduction

Litopenaeus vannamei is one of the three major cultured shrimp species worldwide, accounting for two-thirds of global shrimp production. In recent years, frequent disease outbreaks have severely impacted the healthy and sustainable development of the shrimp aquaculture industry. Consequently, developing new green feed additives to enhance the immunity and disease resistance of cultured species while improving economic benefits has become a research priority in the feed industry. Although antibiotics can effectively prevent and treat various diseases, concerns about residues and environmental pollution have made the search for safe, efficient, and green alternatives to antibiotics increasingly urgent.

Chitosan oligosaccharide (COS), also known as chitosan oligosaccharide or low-molecular-weight chitosan, is the only naturally occurring alkaline amino oligosaccharide with a positive charge. It is produced by enzymatic hydrolysis of chitosan and has a degree of polymerization between 2 and 20. COS has been shown to promote animal growth, improve intestinal environment, inhibit harmful bacteria, and promote the establishment of intestinal microflora. Studies have demonstrated that chitosan-montmorillonite polymers are excellent novel antimicrobial agents. Dietary COS supplementation has been found to improve the immune capacity, growth performance, and survival rate of *L. vannamei*. Similar beneficial effects have been reported in tilapia (*Oreochromis*), golden pompano (*Trachinotus ovatus*), red swamp crayfish (*Procambarus clarkii*), gibel carp (*Carassius auratus gibelio*), and sea cucumber (*Apostichopus japonicus*

Selenka*). COS also exhibits growth-promoting and quality-improving effects in terrestrial animals.

Feed mold contamination is a critical factor affecting feed quality. Animals consuming mycotoxin-contaminated feed may experience acute or chronic mycotoxin poisoning, leading to decreased immune function, reduced feed utilization, and impaired production performance. Mycotoxin adsorbents (MA) mainly include modified montmorillonite and other silicon aluminates, composite minerals, and specific yeast extracts. Silicon aluminates are clay-type minerals containing aluminum oxide and silicon dioxide, such as zeolite, bentonite, and kaolin, which possess strong adsorption capacity due to their large specific surface area and ion exchange capacity. Research has shown that dietary MA supplementation can improve laying performance, enhance serum antioxidant function, and improve health status in laying hens, while reducing serum malondialdehyde (MDA) content and reversing oxidative damage and immunotoxicity caused by moldy feed in broilers. Combined supplementation of silicon aluminate products with β-glucan or mannan oligosaccharide has been reported to improve growth performance, enhance nonspecific immunity, improve digestive enzyme activity, and increase resistance to *Vibrio alginolyticus* and hypoxia tolerance in *L. vannamei*. Yeast cell wall polysaccharides combined with silicon aluminate complexes have also been shown to improve immune function in growing pigs.

However, any single adsorbent has limitations and cannot completely adsorb all harmful substances or toxins. Combining different types of adsorbents can achieve better results. COS and silicon aluminate MA share similar functions in improving growth performance and immunity in cultured animals, but no studies have been reported on their simultaneous use in aquatic animals. Therefore, this experiment evaluated the rational use of COS and MA by individually or jointly adding them to diets and measuring growth indices, body composition, serum and hepatopancreatic nonspecific immune enzyme activities, and disease resistance in *L. vannamei*.

1.1 Experimental Materials

The COS (degree of polymerization 2–8, oligosaccharide content $ $85%) and MA (100% hydrated silicon aluminate) used in this experiment were provided by Shenzhen Yunong Science & Technology Co., Ltd.

1.2 Experimental Diets and Design

A basal diet was formulated as the control (C0 group). Five additional diets were prepared by supplementing the basal diet with 100 mg/kg COS (C0.1), 250 mg/kg COS (C0.25), 2,500 mg/kg MA (M2.5), 100 mg/kg COS + 2,500 mg/kg MA (C0.1+M2.5), or 250 mg/kg COS + 2,500 mg/kg MA (C0.25+M2.5), resulting in six isonitrogenous and isolipidic experimental diets. The composition

and nutrient levels are shown in Table 1 . All ingredients were ground to pass through an 80-mesh sieve, weighed accurately according to formulation requirements, and mixed thoroughly using the stepwise dilution method for micro-ingredients. The diets were processed into 1.0 mm and 1.5 mm pellets using an F-26 twin-screw extruder (South China University of Technology, Guangzhou), cooked at 60 °C for 30 minutes, air-dried in a ventilated area, sealed in self-sealing bags, and stored at -20 °C until use.

1.3 Experimental Animals and Management

The feeding trial was conducted at the Donghai Island Marine Biological Research Base of Guangdong Ocean University. Postlarvae were purchased from Zhanjiang Zhonglian Shrimp Hatchery and acclimated in outdoor cement tanks (4.9 m × 4.5 m × 1.8 m) prior to the experiment. At the start of the trial, shrimp were fasted for 24 hours before being randomly assigned to six groups according to the experimental design. Healthy, active shrimp with an initial average weight of $(0.23\$\pm0.02)gwereselectedandstockedintoanindoorseawaterculturesystem.Eachgrouphadthreereplicates,wi$ fiberglass tanks. Shrimp were fed four times daily at 08:00, 12:00, 16:00, and 20:00. Feeding amount was adjusted based on consumption and weather conditions to ensure complete consumption with minimal residual feed. During the first two weeks, water was exchanged every two days, and thereafter daily. Water temperature was maintained at 25.5–30.0 °C, salinity at 26.5–28.0, with continuous aeration ensuring dissolved oxygen >6.8 mg/L, pH 7.8–8.2, and ammonia nitrogen <0.03 mg/L. The experimental period lasted 8 weeks.

1.4 Sample Collection

At the end of the feeding trial, shrimp were fasted for 24 hours before sampling. All shrimp in each tank were weighed and counted for growth performance calculation. Five shrimp per tank were randomly selected, individually bagged, and stored at -20 °C for body composition analysis. Another ten shrimp per tank were randomly selected for hemolymph collection using a 1 mL sterile syringe from the sinus at the base of the fifth pereiopod. Hemolymph was immediately transferred to 1.5 mL centrifuge tubes, placed in an icebox with crushed ice, and allowed to stand overnight at 4 °C before centrifugation at 8,000 r/min for 10 minutes at 4 °C. The supernatant was collected and stored at -80 °C for subsequent analysis. After blood collection, hepatopancreas was rapidly dissected, immediately frozen in liquid nitrogen, and then transferred to -80 °C storage for analysis.

1.4.1 Conventional Analysis of Feed and Whole Shrimp Samples

Conventional nutrient analysis was performed on feed and whole shrimp samples. Moisture content was determined by drying at 105 °C to constant weight. Crude protein content was measured using the Kjeldahl method (Kjeltec™ 8400, Sweden). Crude lipid content was determined by Soxhlet extraction using petroleum

ether as the solvent. Crude ash content was measured by incineration in a muffle furnace at 550 °C.

1.4.2 Detection of Immune Enzyme Activities and MDA Content in Serum and Hepatopancreas

Hepatopancreas crude enzyme extract was prepared by weighing appropriate amounts of tissue and adding 9 volumes of ice-cold physiological saline (w/v = 1:9), followed by homogenization in an ice bath. The homogenate was centrifuged at 2,500 r/min for 10 minutes at 4 °C, and the supernatant was carefully collected, aliquoted, and stored at -80 °C until analysis. Activities of alkaline phosphatase (AKP), superoxide dismutase (SOD), phenoloxidase (PO), lysozyme (LZM), and malondialdehyde (MDA) content in serum and hepatopancreas were determined using assay kits from Nanjing Jiancheng Bioengineering Institute.

1.4.3 Calculation Formulas for Growth Performance Indices

Weight gain rate (WGR, %) = 100 × (final mean weight - initial mean weight) / initial mean weight
Specific growth rate (SGR, %/d) = 100 × (ln final mean weight - ln initial mean weight) / feeding days
Protein efficiency ratio (PER) = (final body weight - initial body weight) / (feed intake × dietary crude protein content)
Feed conversion ratio (FCR) = dry weight of feed consumed / (final body weight - initial body weight)
Survival rate (SR, %) = 100 × number of shrimp at experiment end / number of shrimp at experiment start

1.5 Challenge Test

After the feeding trial, ten shrimp from each tank were selected for the challenge test while continuing to receive the corresponding experimental diets and water exchange. *Vibrio harveyi* used for challenge was provided by the Guangdong Provincial Key Laboratory of Aquatic Animal Pathogen Biology and Epidemiology. A pre-test determined the 7-day median lethal dose (LD_{50}) for *L. vannamei* as 3.89$×10^{8}$ CFU/mL. During challenge, 30 μL of bacterial suspension at this concentration was injected into the dorsal muscle of the second and third abdominal segments. Mortality was recorded for 7 days, and cumulative mortality rate (CMR) was calculated as: CMR (%) = 100 × cumulative number of dead shrimp / initial number of shrimp.

1.6 Data Processing

All data are expressed as mean ± standard deviation (mean±SD). Statistical analysis was performed using SPSS 17.0 software with one-way ANOVA. When

significant differences were detected among groups, Duncan' s multiple comparison test was applied. The significance level was set at $P<0.05$.

2.1 Effects of Dietary COS and/or MA on Growth Performance of *L. vannamei*

As shown in Table 2 , no significant differences in specific growth rate (SGR) were observed among C0.25, C0.1+M2.5, and C0.25+M2.5 groups ($P>0.05$), but these three groups were significantly higher than C0 and M2.5 groups ($P<0.05$). The C0.1 group showed no significant difference from other groups ($P>0.05$). Weight gain rate (WGR) in C0.1, C0.25, C0.1+M2.5, and C0.25+M2.5 groups did not differ significantly ($P>0.05$), but all were significantly higher than C0 and M2.5 groups ($P<0.05$). The feed conversion ratio (FCR) in C0.1+M2.5 was significantly lower than all other groups ($P<0.05$). The C0.25+M2.5 group had significantly higher FCR than C0.1+M2.5 ($P<0.05$) but significantly lower than remaining groups ($P<0.05$). The C0 group showed no significant difference from C0.25 ($P>0.05$) but was significantly higher than C0.1+M2.5 and C0.25+M2.5 ($P<0.05$) and significantly lower than C0.1 and M2.5 ($P<0.05$). The C0.1 group did not differ significantly from C0.25 ($P>0.05$) but was significantly lower than M2.5 ($P<0.05$). Protein efficiency ratio (PER) in C0.1+M2.5 showed no significant difference from C0.25+M2.5 ($P>0.05$) but was significantly higher than all other groups ($P<0.05$). No significant differences in PER were found among C0, C0.1, and M2.5 groups ($P>0.05$), all of which were significantly lower than C0.1+M2.5 and C0.25+M2.5 ($P<0.05$). The PER in C0.25 was significantly lower than C0.1+M2.5 ($P<0.05$) but not different from other groups ($P>0.05$). No significant differences in survival rate were observed among all groups ($P>0.05$).

2.2 Effects of Dietary COS and/or MA on Body Composition of *L. vannamei*

As presented in Table 3 , no significant differences were detected in moisture or crude ash content among all groups ($P>0.05$). The crude protein content in C0.1+M2.5 showed no significant difference from C0.25 ($P>0.05$) but was significantly higher than all other groups ($P<0.05$). C0, C0.1, and M2.5 groups did not differ significantly in crude protein content ($P>0.05$), all being significantly lower than C0.1+M2.5 and significantly higher than C0.25+M2.5 ($P<0.05$). The C0.25+M2.5 group exhibited significantly lower crude protein content than all other groups ($P<0.05$). All supplemented groups showed significantly lower crude lipid content than C0 ($P<0.05$). M2.5, C0.1+M2.5, and C0.25+M2.5 groups did not differ significantly from C0.25 ($P>0.05$) but were significantly lower than C0 and C0.1 ($P<0.05$). The C0.1 group showed no significant difference from C0.25 ($P>0.05$) but was significantly lower than C0 and significantly higher than other groups ($P<0.05$).

2.3 Effects of Dietary COS and/or MA on Serum Nonspecific Immune Enzyme Activities and MDA Content

As shown in Table 4 , serum AKP activity in C0.25+M2.5 showed no significant difference from M2.5 ($P>0.05$) but was significantly lower than all other groups ($P<0.05$). Serum AKP activity in C0.1+M2.5 did not differ significantly from M2.5 ($P>0.05$) but was significantly higher than C0.25+M2.5 ($P<0.05$) and significantly lower than remaining groups ($P<0.05$). Serum AKP activity in C0.1 showed no significant difference from C0 ($P>0.05$) but was significantly lower than C0.25 ($P<0.05$) and significantly higher than other groups ($P<0.05$). No significant differences in serum SOD activity were observed among C0, C0.1, and C0.25 groups ($P>0.05$), all of which were significantly lower than other groups ($P<0.05$). Serum SOD activity in M2.5 showed no significant difference from C0.25+M2.5 ($P>0.05$) but was significantly lower than C0.1+M2.5 ($P<0.05$) and significantly higher than C0, C0.1, and C0.25 ($P<0.05$). Serum SOD activity in C0.1+M2.5 did not differ significantly from C0.25+M2.5 ($P>0.05$) but was significantly higher than all other groups ($P<0.05$). No significant differences in serum PO activity were found among C0, C0.1, and M2.5 groups ($P>0.05$), all being significantly lower than C0.25 and C0.1+M2.5 ($P<0.05$). The highest serum PO activity was observed in C0.25, which showed no significant difference from C0.1+M2.5 ($P>0.05$) but was significantly higher than all other groups ($P<0.05$). Serum LZM activity in C0.1 was significantly higher than M2.5 and C0.25+M2.5 ($P<0.05$) but not different from other groups ($P>0.05$). The lowest serum LZM activity was observed in C0.25+M2.5, which was significantly lower than C0.1 and C0.1+M2.5 ($P<0.05$) but not different from other groups ($P>0.05$).

Serum MDA content in M2.5 and C0.1+M2.5 showed no significant difference from C0.25+M2.5 ($P>0.05$) but was significantly lower than all other groups ($P<0.05$). Serum MDA content in C0.1 did not differ significantly from C0 and C0.25 ($P>0.05$) but was significantly higher than other groups ($P<0.05$).

2.4 Effects of Dietary COS and/or MA on Hepatopancreatic Nonspecific Immune Enzyme Activities and MDA Content

As shown in Table 5 , hepatopancreatic AKP activity in C0.1+M2.5 showed no significant difference from C0.1 and M2.5 ($P>0.05$) but was significantly higher than other groups ($P<0.05$). The lowest hepatopancreatic AKP activity was observed in C0.25, which did not differ significantly from C0.25+M2.5 and C0 ($P>0.05$) but was significantly lower than other groups ($P<0.05$). Hepatopancreatic SOD activity was significantly higher only in C0.1 compared to C0 ($P<0.05$), with no significant differences among other groups ($P>0.05$). Hepatopancreatic LZM activity in C0.25 showed no significant difference from C0.1 and M2.5 ($P>0.05$) but was significantly higher than other groups ($P<0.05$). Hepatopancreatic LZM activity in C0 did not differ significantly

from C0.1+M2.5 and C0.25+M2.5 (P>0.05) but was significantly lower than other groups (P<0.05). No significant differences in hepatopancreatic LZM activity were observed among C0.1, M2.5, C0.1+M2.5, and C0.25+M2.5 groups (P>0.05).

Hepatopancreatic MDA content showed no significant differences among C0.25, M2.5, C0.1+M2.5, and C0.25+M2.5 groups (P>0.05), all being significantly lower than C0 and C0.1 (P<0.05), while no significant difference was found between C0 and C0.1 (P>0.05).

2.5 Effects of Dietary COS and/or MA on Cumulative Mortality after *Vibrio harveyi* Challenge

As illustrated in Figure 1 [Figure 1: see original paper], C0 showed the highest cumulative mortality rate, significantly higher than all supplemented groups (P<0.05). M2.5 exhibited significantly lower mortality than C0 (P<0.05) but significantly higher than other supplemented groups (P<0.05). No significant differences in cumulative mortality were observed among C0.1, C0.25, C0.1+M2.5, and C0.25+M2.5 groups (P>0.05), all of which were significantly lower than C0 and M2.5 (P<0.05).

3.1 Effects of Dietary COS and/or MA on Growth Performance of *L. vannamei*

The present results indicate that compared with the control group, single supplementation of COS or MA improved the specific growth rate, weight gain rate, and feed conversion ratio of *L. vannamei*. These findings are consistent with previous studies showing that COS can significantly improve weight gain, specific growth rate, and feed conversion ratio in *L. vannamei*, enhance transcription of growth factors in tilapia, improve growth rate in golden pompano, and increase weight gain and feed utilization in broilers. Similarly, MA has been reported to improve growth performance in various animals, with zeolite supplementation increasing growth rate and feed conversion in broilers and improving production performance in laying hens.

Oligosaccharides primarily affect animal growth performance through several mechanisms: promoting intestinal villus growth, maintaining intestinal mucosal integrity, enhancing nutrient absorption, optimizing intestinal microflora structure, increasing beneficial bacteria proportion, inhibiting *Escherichia* proliferation, and improving intestinal environment to promote nutrient absorption. Silicon aluminate adsorbents may contain various mineral elements; for instance, Azomite (mainly hydrated silicon aluminate) contains over 70 trace and macro elements that supplement dietary mineral deficiencies. Trace elements are closely related to animal growth and development, and rare earth elements in silicon aluminate adsorbents can activate growth factors, promote

enzyme conversion, improve feed conversion efficiency, and accelerate growth. Additionally, their large surface area and adsorption capacity can adsorb harmful bacteria in the digestive tract, optimize intestinal environment, improve digestive enzyme activity, and promote nutrient absorption.

In this study, combined supplementation of COS and MA improved specific growth rate, weight gain rate, and protein efficiency ratio while significantly reducing feed conversion ratio compared with the control or single supplementation groups. Furthermore, C0.1+M2.5 showed significantly higher specific growth rate and weight gain rate than M2.5 but no significant difference from C0.1, while C0.25+M2.5 was significantly higher than M2.5 but not different from C0.25, suggesting that COS may play a dominant role in the combined supplementation. As non-digestible carbohydrates, oligosaccharides are poorly absorbed by animals, and excessive supplementation may cause adverse diarrhea and negative effects. Although C0.25+M2.5 significantly improved growth performance compared with C0 and showed a slight but non-significant decrease compared with C0.1+M2.5, whether COS was excessive in this combination requires further investigation.

3.2 Effects of Dietary COS and/or MA on Body Composition of *L. vannamei*

Previous studies have shown that single COS supplementation had no significant effects on whole-body moisture or muscle crude ash content in turbot (*Scophthalmus maximus*) or on moisture and crude ash content in *L. vannamei*, which aligns with our findings that single or combined COS and MA supplementation did not significantly affect moisture or crude ash content in shrimp.

Several studies have reported that COS supplementation reduces body lipid content. Dietary COS or probiotics have been shown to decrease body fat content in pufferfish (*Fugu obscurus*), and chitosan supplementation at 1 g/kg or higher reduced crude lipid content in *L. vannamei*. COS has also been found to regulate lipid metabolism and reduce abdominal fat content in broilers and ducks. Our results are consistent with these findings, showing significantly lower crude lipid content in all supplemented groups compared with the control. The mechanism may involve the positively charged alkaline amino groups of chitosan forming a barrier around negatively charged lipid droplets, preventing their digestion and absorption and facilitating excretion. COS, with superior properties to chitosan, likely exerts similar effects. However, some studies reported increased muscle crude lipid content in red swamp crayfish fed 1% or 3% chitosan, possibly due to differences in tested tissues (whole shrimp in our study), experimental species, or polymerization degrees between COS and chitosan.

The M2.5 group showed significantly reduced crude lipid content compared with the control, and further reduction was observed in C0.1+M2.5 and C0.25+M2.5 groups compared with C0.1 and C0.25 groups, indicating that combined COS and MA supplementation had a greater effect on reducing lipid deposition. This

may be attributed to the formation of stable complexes between COS and rare earth elements in the adsorbent, which regulate lipid metabolism.

In this study, C0.1+M2.5 showed significantly higher crude protein content than C0.1 and M2.5 groups, indicating that combined supplementation promoted protein synthesis and accumulation. Previous research demonstrated that chitosan supplementation significantly increased RNA/DNA ratio in fish, reflecting accelerated muscle protein synthesis. Similar results have been reported in broilers, where chitosan increased muscle crude protein content. The mechanisms may involve enhanced nutrient absorption, improved protein utilization efficiency, and increased RNA translation efficiency, thereby accelerating polypeptide chain synthesis. Silicon aluminate MA contains various trace elements that promote metabolism after absorption, and their large surface area and adsorption capacity can prolong feed retention time in the digestive tract, improving digestion and absorption efficiency. The synergistic effect of COS and MA thus increased crude protein content in *L. vannamei*.

3.3 Effects of Dietary COS and/or MA on Nonspecific Immunity and Disease Resistance of *L. vannamei*

COS can promote macrophage phagocytosis, increase hydrolase activity, and stimulate cytokine release. Its immunoenhancing mechanisms include: 1) acting as a positive chemotactic agent to promote monocyte differentiation into macrophages, increasing reactive oxygen species content for oxidative bactericidal defense; and 2) containing abundant free amino groups that can adsorb hydrogen ions (H^+), activate T lymphocytes, induce type IV hypersensitivity, and activate macrophages to enhance bactericidal activity. Dietary MA primarily adsorbs harmful substances such as bacteria and heavy metals, protects the gastrointestinal tract, supplements mineral elements, and activates enzymes to improve animal health.

Crustaceans rely primarily on nonspecific immunity, which includes cellular and humoral immunity, with the latter mediated by factors such as PO, SOD, LZM, and AKP. PO exists as prophenoloxidase in hemocytes and can be activated by proteins or polysaccharides to produce active PO, which oxidizes phenols to quinones that are converted to melanin for encapsulating invading pathogens. In this study, PO activity in serum and hepatopancreas increased to varying degrees in all supplemented groups, with C0.1+M2.5 showing significantly higher serum PO activity than C0.1 and M2.5 groups, indicating that combined supplementation better enhanced nonspecific immunity.

SOD eliminates excess free radicals, enhances phagocytic defense, and plays crucial roles in preventing aging, resisting biomolecular damage, and improving immune function. MDA, a terminal product of membrane lipid peroxidation, is highly toxic and can damage biomembrane structure and permeability, serving as an indicator of oxidative stress. In this study, C0.1+M2.5 showed the highest serum SOD activity, significantly higher than C0, C0.1, and M2.5 groups, with

increased hepatopancreatic SOD activity in all supplemented groups. These results align with previous studies demonstrating that COS can increase serum total SOD activity in *L. vannamei* and golden pompano. MA has also been shown to improve antioxidant capacity, with montmorillonite significantly increasing serum T-SOD activity and total antioxidant capacity in laying hens, and Azomite significantly increasing serum SOD activity in *L. vannamei*. The significantly lower MDA content in serum and hepatopancreas of C0.1+M2.5 compared with C0 and C0.1 groups is consistent with findings in tiger shrimp (*Penaeus monodon*).

Serum AKP in shrimp originates primarily from the liver and plays important roles in phosphate group transfer and calcium-phosphorus metabolism as a phosphoprotein phosphatase and component of phagolysosomes. While some studies reported no significant effects of low COS doses on serum AKP activity, our results showed significantly lower serum AKP activity in C0.1+M2.5 and C0.25+M2.5 groups compared with the control. Elevated serum AKP activity is associated with obstructive jaundice, liver cancer, and cholestatic hepatitis, where excessive AKP production and bile reflux increase serum levels. Diseased shrimp have shown significantly higher serum AKP activity than healthy individuals. Therefore, the lower serum AKP activity in supplemented groups, coupled with higher hepatopancreatic AKP activity, may indicate a healthier physiological state.

Serum LZM in shrimp originates from blood and is an alkaline protein that hydrolyzes acetylaminopolysaccharides in Gram-positive bacterial cell walls, forming a hydrolytic enzyme system to eliminate foreign invaders. COS has been shown to enhance IL-2 receptor expression on T cells, accelerating T cell maturation. In this study, LZM activity in serum and hepatopancreas increased to varying degrees in C0.1 and C0.1+M2.5 groups compared with the control, consistent with previous reports that COS can improve LZM activity and immunity in *L. vannamei* and red swamp crayfish. Thus, combined COS and MA supplementation enhanced LZM activity and nonspecific immunity.

The *Vibrio harveyi* challenge test demonstrated that single or combined COS and MA supplementation significantly improved disease resistance. Cumulative mortality results indicated that single MA supplementation was less effective than single COS or combined supplementation, with the high-dose combination (250 mg/kg COS + 2,500 mg/kg MA) showing the strongest disease resistance. These findings are consistent with reports that COS can improve resistance to *Aeromonas hydrophila* in hybrid tilapia and *Vibrio harveyi* in golden pompano, and that Azomite can reduce mortality after *Vibrio alginolyticus* challenge in shrimp. The antimicrobial mechanism of COS involves its protonated ammonium groups interacting with negatively charged bacterial membranes, disrupting membrane function and causing cytoplasmic leakage, while water-soluble COS with low molecular weight can enter bacterial cells and disturb normal physiological metabolism.

Interestingly, C0.25+M2.5 showed decreased activities of AKP, PO, SOD, and

LZM in serum and hepatopancreas compared with C0.1+M2.5, along with increased MDA content. Combined with growth performance and challenge test results, this suggests that while 250 mg/kg COS + 2,500 mg/kg MA provided the strongest disease resistance, its growth-promoting and immunoenhancing effects were diminished compared with the lower dose combination. Further systematic research is needed to optimize the ratio of COS to MA.

Under the present experimental conditions, dietary supplementation with COS and/or MA promoted growth, enhanced nonspecific immunity, and improved disease resistance in *L. vannamei*. Combined supplementation was superior to single supplementation, with the combination of 100 mg/kg COS and 2,500 mg/kg MA showing the best overall effects.

References

[1] WANG Z S, YAN C Z, YAN Y J, et al. Integrated assessment of biomarker responses in caged shrimps (*Litopenaeus vannamei*) exposed to complex contaminants from the Maluan Bay of China[J]. *Ecotoxicology*, 2012, 21(3): 869-881.

[2] LOTZ J M, SOTO M A. Model of white spot syndrome virus (WSSV) epidemics in *Litopenaeus vannamei*[J]. *Diseases of Aquatic Organisms*, 2002, 50(3): 199-209.

[3] LI A K, LIN Y, ZHAO Y X. Research progress on antibiotic alternatives in feed[J]. *Feed Industry*, 2013(20): 1-6.

[4] GAO W, CHEN S, DING Z K, et al. Effects of dietary chitosan oligosaccharide supplementation on animal organisms[J]. *Chinese Journal of Animal Nutrition*, 2014, 26(2): 322-326.

[5] CHEN M L. Study on antimicrobial activity and mechanism of chitosan-montmorillonite composites[D]. Master's thesis. Zhengzhou: Henan Agricultural University, 2012.

[6] LI Z D, CHEN X E, LIAO Z, et al. Effects of chitosan oligosaccharide on growth and immunity of *Litopenaeus vannamei*[J]. *South China Fisheries Science*, 2011, 7(4): 36-42.

[7] QIN C B, ZHANG Y T, LIU W S, et al. Effects of chito-oligosaccharides supplementation on growth performance, intestinal cytokine expression, autochthonous gut bacteria and disease resistance in hybrid tilapia *Oreochromis niloticus* ×*Oreochromis aureus* [J]. *Fish & Shellfish Immunology*, 2014, 40(1): 267-274.

[8] LIN S M, MAO S H, GUAN Y, et al. Dietary administration of chitooligosaccharides to enhance growth, innate immune response and disease resistance of *Trachinotus ovatus*[J]. *Fish & Shellfish Immunology*, 2012, 32(5): 909-913.

[9] REN X F, ZHOU X, ZHAO C Y, et al. Effects of chitosan on growth, serum immune factors, muscle composition and digestive enzymes of red swamp crayfish (*Procambarus clarkii*)[J]. *Journal of Dalian Ocean University*, 2013, 28(5): 468–474.

[10] CAO D, ZHOU H Q. Effects of chitosan on growth, protein synthesis and muscle nutrient composition of gibel carp (*Carassius auratus gibelio*)[J]. *Freshwater Fisheries*, 2004, 34(1): 6–9.

[11] BAI Y. Effects of chitosan, chitosan oligosaccharide and their complexes on growth and quality-related indices of sea cucumber (*Apostichopus japonicus* Selenka)[D]. Master' s thesis. Qingdao: Ocean University of China, 2015.

[12] ZHOU T X, CHEN Y J, YOO J S, et al. Effects of chitooligosaccharide supplementation on performance, blood characteristics, relative organ weight, and meat quality in broiler chickens[J]. *Poultry Science*, 2009, 88(3): 593–600.

[13] XIE X P, YI W, ZHUANG Z M, et al. Mycotoxins in feed and their prevention measures[J]. *China Animal Husbandry & Veterinary Medicine*, 2013, 40(5): 101–106.

[14] CHEN J F, LUO L, QU X Y, et al. Effects of mycotoxin adsorbent on laying performance, trace element content in egg yolk, serum antioxidant and biochemical indices of laying hens[J]. *Chinese Journal of Animal Nutrition*, 2016, 28(10): 3183–3191.

[15] WANG F. Effects of montmorillonite on performance and health of broilers fed diets containing moldy peanut meal[D]. Master' s thesis. Lanzhou: Gansu Agricultural University, 2016.

[16] ZHANG R X, HUANG K, SONG M M, et al. Effects of compound mycotoxin adsorbent on antioxidant function and immunity of broilers fed moldy feed[J]. *Feed Industry*, 2015, 36(9): 32–35.

[17] TAN C G. Effects of several additives on growth, serum nonspecific immunity and disease resistance of *Litopenaeus vannamei*[D]. Master' s thesis. Shanghai: Shanghai Ocean University, 2012.

[18] ZHANG Y, ZHENG L L, ZHU Y J, et al. Effects of yeast cell wall polysaccharides and aluminosilicate complex on growth performance, immune indices and nutrient digestibility in pigs[J]. *Chinese Journal of Animal Nutrition*, 2012, 24(9): 1799–1804.

[19] KARAMANLIS X, FORTOMARIS P, ARSENOS G, et al. The effect of a natural zeolite (Clinoptilolite) on the performance of broiler chickens and the quality of their litter[J]. *Asian-Australasian Journal of Animal Sciences*, 2008, 21(11): 1642–1650.

[20] BILAL T, KAYGISIZ F, ESECELI H, et al. Effect of vitamin D_3 and/or zeolite supplementation to laying hen rations added microbial phytase on performance and enterprise income[J]. *Journal of Animal and Veterinary Advances*,

2012, 8(5): 1013-1020.

[21] KOTANI T, WAKIYAMA Y, IMOTO T, et al. Improved larviculture of ocellate puffer *Takifugu rubripes* through control of stocking density[J]. *Aquaculture*, 2011, 312(1/2/3/4): 95-101.

[22] DONG X H, YANG Y Z, ZHENG S X, et al. Effects of different forms of cobalt on growth and tissue cobalt content of *Litopenaeus vannamei*[J]. *Journal of Guangdong Ocean University*, 2006, 26(6): 8-12.

[23] YANG Z Q, MAO S Y, DONG M X, et al. Study on effects of rare earth elements on trace element content in blood of broilers[J]. *Chinese Rare Earths*, 1992(4): 20-23.

[24] JING S H, HU Z Y, HUANG W. Research and application status of rare earth elements[J]. *Swine Industry Science*, 2010, 27(4): 58-60.

[25] JIANG Z Y. Study on effects of rare earth elements on animal growth and disease resistance[J]. *Acta Agriculturae Boreali-Sinica*, 2004, 19(S1): 64-68.

[26] OUHIDA I, PÉREZ J F, PIEDRAFITA J, et al. The effects of sepiolite in broiler chicken diets of high, medium and low viscosity. Productive performance and nutritive value[J]. *Animal Feed Science and Technology*, 2000, 85(3/4): 183-194.

[27] SUN Z B, YAN H, MA Y J. Application of oligosaccharides in animal production[J]. *Agricultural Science Research*, 2004, 25(4): 80-84.

[28] CAI S C, ZHANG L M, ZHANG D R, et al. Effects of chitosan oligosaccharide and xylo-oligosaccharide on growth, body composition and blood biochemical indices of juvenile turbot (*Scophthalmus maximus*)[J]. *Progress in Fishery Sciences*, 2015, 36(6): 29-36.

[29] NIU J, LIU L X, LIN Y J, et al. Effect of dietary astaxanthin on growth, survival, and stress tolerance of postlarval shrimp, *Litopenaeus vannamei*[J]. *Journal of the World Aquaculture Society*, 2011, 42(6): 795-802.

[30] HUA X M, ZHOU H Q, ZHANG Y F, et al. Effects of dietary chitosan and probiotics on growth and some digestive enzyme activities of juvenile pufferfish (*Takifugu obscurus*)[J]. *Acta Hydrobiologica Sinica*, 2005, 29(3): 299-305.

[31] SONG T. Effects of different dietary levels of chitosan oligosaccharide on growth performance, fat deposition and meat quality of Beijing ducks[D]. Master' s thesis. Wuhan: Huazhong Agricultural University, 2005.

[32] KANAUCHI O, DEUCHI K, IMASATO Y, et al. Mechanism for the inhibition of fat digestion by chitosan and for the synergistic effect of ascorbate[J]. *Bioscience Biotechnology & Biochemistry*, 1995, 59(5): 786-790.

[33] RAZDAN A, PETTERSSON D. Hypolipidaemic, gastrointestinal and related responses of broiler chickens to chitosans of different viscosity[J]. *The British Journal of Nutrition*, 1996, 76(3): 387-397.

[34] HAN L R. Effects of chitosan oligosaccharide, rare earth elements and their complex on growth, immune response and disease resistance of sea cucumber (*Apostichopus japonicus* Selenka)[D]. Master' s thesis. Qingdao: Ocean University of China, 2014.

[35] LIU M. Effects of chitosan on meat quality of broiler chickens[J]. *Journal of Northeast Agricultural University*, 2011, 42(3): 25-28.

[36] WU Y N, WU Q Y, ZHOU Y M, et al. Zeolite improves animal health and its mechanism[J]. *Journal of the Chinese Cereals and Oils Association*, 2013, 28(5): 105-111.

[37] WANG B X, YE J A. Research progress on immune-related enzymes in shrimp serum[J]. *China Feed*, 2009(3): 27-28, 36.

[38] AMPARYUP P, CHAROENSAPSRI W, TASSANAKAJON A. Two prophenoloxidases are important for the survival of *Vibrio harveyi* challenged shrimp *Penaeus monodon*[J]. *Developmental & Comparative Immunology*, 2009, 33(2): 247-256.

[39] DEL MAESTRO R F. An approach to free radicals in medicine and biology[J]. *Acta Physiologica Scandinavica. Supplementum*, 1980, 492: 153-168.

[40] NIU J, LIN H Z, JIANG S G, et al. Comparison of effect of chitin, chitosan, chitosan oligosaccharide and N-acetyl-D-glucosamine on growth performance, antioxidant defenses and oxidative stress status of *Penaeus monodon*[J]. *Aquaculture*, 2013, 372-375: 1-8.

[41] FERNANDEZ N J, KIDNEY B A. Alkaline phosphatase: beyond the liver[J]. *Veterinary Clinical Pathology*, 2007, 36(3): 223-233.

[42] WU Y, XING D L, ZHU G Q, et al. Study on blood pathology of explosive epidemic disease in Chinese shrimp (*Penaeus chinensis*)[J]. *Journal of Fishery Sciences of China*, 1998(3): 53-57.

[43] OMKAR, SHUKLA G S. Nature of dichlorvos intoxication in a freshwater prawn, *Macrobrachium lamarrei* (H. Milne Edwards)[J]. *Environmental Research*, 1985, 37(2): 349-354.

[44] SUZUKI K, OKAWA Y, HASHIMOTO K, et al. Protecting effect of chitin and chitosan on experimentally induced murine candidiasis[J]. *Microbiology and Immunology*, 1984, 28(8): 903-912.

[45] YAN Q, SHEN Y X, WANG Z. Study on preparation and antibacterial properties of chitosan oligosaccharide[J]. *Food Research and Development*, 2003, 24(2): 26-29.

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