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Effects of discontinuous administration of β-glucan and glycyrrhizin on the growth and immunity of white shrimp *Litopenaeus vannamei*

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ABSTRACT

A 6-week growth trial was conducted to compare the effects of different feeding strategies of dietary immunostimulants on the growth and immunity of white shrimp *Litopenaeus vannamei* (4.70 ± 0.20 g). Six feeding strategies were set, including feeding immunostimulants-free diet continuously (control), feeding dietary β-glucan or glycyrrhizin continuously, feeding dietary β-glucan discontinuously, feeding dietary β-glucan and glycyrrhizin alternately. The results showed that compared with glycyrrhizin, β-glucan could maintain the immunity of shrimps at a higher level during the experimental period. However, continuously applying β-glucan or glycyrrhizin into the diet caused immunity fatigue in *L. vannamei*. On the 27th day, the total haemocyte count (THC), superoxide anion and superoxide dismutase (SOD) activity of the shrimps fed with β-glucan continuously were no longer significantly higher than those in the control group. Meanwhile, phenoloxidase (PO) activity was no longer significantly higher on the 35th day. THC, PO activity and SOD activity of the shrimps fed with glycyrrhizin were no longer significantly higher than those in the control group on the 25th, 37th, 29th day, respectively. Discontinuous administration of β-glucan or glycyrrhizin could eliminate the immunity fatigue. Shrimps fed with dietary β-glucan 2 days followed by the basal diet for 5 days showed the highest specific growth rate (SGR). It was concluded that this feeding strategy is most suitable for *L. vannamei*.

1. Introduction

In the past few years, shrimp industry has suffered from diseases due to vibrios such as *Vibrio harvey*, *V. damsela* and *V. alginolyticus* (Saulnier et al., 2000), and viruses such as monodon baculovirus virus (MBV), white spot syndrome virus (WSSV) and taura syndrome virus (TSV) (Walker and Mohan, 2009). To solve this problem, several antibiotics were applied (Roque et al., 2001). However, it arose many other problems, such as spread of drug resistant pathogens, negative impact on the environment and risk for human health (Holmström et al., 2003). Under these conditions, immunostimulants were used to boost immune system and improve resistance to infections for shrimp. Several immunostimulants, such as β-glucan, peptidoglycan, lipopolysaccharide, live bacteria and killed bacteria were widely used around the world (Smith et al., 2003). In our previous studies, it has been proved that β-glucan isolated from brewer's yeast slurry (Tan et al., 2004), soybean isoflavones (Chen et al., in press), probiotics isolated from intestine (Li et al., 2009) or culture water (Zhang et al., 2009) and glycyrrhizin (unpublished data) have immuno-enhancement effects on white shrimp *Litopenaeus vannamei*. Moreover, the optimal supplement levels of these immunostimulants in diet were also determined.

Shrimps need several months to grow from larva to adult. Therefore, long-term using of immunostimulants is needed. However, some researches have proved that continuous administration of β-glucan may cause immunity fatigue. For example, Chang et al. (2000) found that continuous administration of β-glucan at the dose of 2000 mg/kg for 40 days to *Penaeus monodon* could elevate the respiratory burst (RB) to the highest level (O.D. 630 nm 0.229) on the 24th day. After the 24th day, however, the RB decreased to the level (O.D.630 nm 0.040) as that in treatment with β-glucan-free basal diet.

Research on the immune system of drosophila revealed that different non-self particles would react with different receptors and stimulate the generation of different immune factors (Hultmark, 2003). Because of the conservation of immune system, it is suggested that alternate administration of different immunostimulants may activate different parts of immune system of shrimps and take advantage of different immunostimulants to solve the problem of immunity fatigue and enhance the immunity continuously. However, there is no report on the discontinuous administration of immunostimulants for shrimps.

In the present work, a six-week feeding trial was conducted to compare the effects of continuous and discontinuous administration of β-glucan or glycyrrhizin, and the alternate administration of these

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two immunostimulants on the growth and immunity of *L. vannamei*, furthermore, to screen the optimal administration strategy. The aim of the present study was to investigate if the discontinuous administration of immunostimulants could eliminate the potential immunity fatigue.

2. Materials and methods

2.1. Experimental design and diets

Based on the nutritional requirements of *L. vannamei* recommended previously (Shiau, 1998), a basal diet was formulated (Table 1). 0.2% β-glucan (Angel Company, Hubei, China; extracted from yeast *Saccharomyces cerevisiae*) and 0.06% glycyrrhizin (Sigma, USA; extracted from glycyrrhiza root) were added into the basal diet, respectively, to prepare the two experimental diets. The dose of β-glucan was according to concentration recommended previously (Liao et al., 1996); the dose of glycyrrhizin was according to our previous work (unpublished data). There were six treatments with different feeding strategies using the basal diet and the two experimental diets, respectively (Table 2). The six feeding strategies were as follows: (1) shrimps were fed the immunostimulant-free basal diet (Control); (2) fed with dietary β-glucan (2000 mg/kg) continuously (Treatment 1); (3) fed with dietary glycyrrhizin (600 mg/kg) continuously (Treatment 2); (4) fed with dietary β-glucan (2000 mg/kg) for 7 days, then with basal diet for 7 days, and so on (Treatment 3); (5) fed with dietary β-glucan (2000 mg/kg) for 2 days, then with basal diet for 5 days, and so on (Treatment 4); (6) fed with dietary β-glucan (2000 mg/kg) for 7 days, then with dietary glycyrrhizin (600 mg/kg) for 7 days, and so on (Treatment 5).

The diets were formulated by thoroughly mixing the dry ingredients with fish oil and then adding cold water until a stiff dough resulted. The stiff dough was then passed through an extruder with a diameter of 1.5 mm. After that, the string-like diets were broken up and sieved into proper pellet size (about 3.0 mm in length). Diets were then stored in plastic bags at −20 °C until use.

2.2. Experimental animals and culture condition

L. vannamei juveniles were bought from a commercial farm in Jiaonan, Qingdao, China and acclimated in a re-circulated seawater system for 2 weeks prior to the feeding trial.

Table 1
Composition of the basal diets (% dry weight).

Ingredients	Percentage
Fish meal[1]	25.00
Shrimp head meal	5.00
Peanut meal	14.00
Squid visceral meal	5.00
Soybean meal	18.00
Fish oil	1.00
Soy lecithin	2.00
Wheat flour	27.53
Choline chloride (50%)	0.30
Stay C[2,3]	0.05
$Ca(H_2PO_4)_2$	0.37
Vitamin premix[3]	0.50
Mineral premix[3]	1.00
Antimycin[3,4]	0.10
Molt hormone[3,5]	0.10
Ethoxyquin[3]	0.05

[1] Crude protein 67.5% (dry weight basis), crude lipid 7.8% (dry weight basis).
[2] Stay C: L-ascorbyl-2-monophosphate (35% ascorbic acid activity, Haffman La Roche, Swiss).
[3] Kindly provided by Qingdao Master Biotechnology Co. Ltd, Qingdao, China.
[4] Contained 50% calcium propionic acid and 50% fumaric acid.
[5] Contained 8% chitin and 10% gentian extract.

Table 2
Groups and feeding strategy.

Treatment abbreviations	Feeding strategy
Control	Shrimps were fed with basal diet continuously
Treatment 1	Shrimps were fed with dietary β-glucan (2000 mg/kg) continuously
Treatment 2	Shrimps were fed with dietary glycyrrhizin (600 mg/kg) continuously
Treatment 3	Shrimps were fed with dietary β-glucan (2000 mg/kg) for seven days and then with basal diet for seven days alternately
Treatment 4	Shrimps were fed with dietary β-glucan (2000 mg/kg) for two days and then with basal diet for five days alternately
Treatment 5	Shrimps were fed with dietary β-glucan (2000 mg/kg) seven days and then with dietary glycyrrhizin (600 mg/kg) for seven days alternately

Three thousand shrimps (initial mean weight 4.70 ± 0.20 g) were randomly distributed to six treatments and each treatment had ten replicates. Each 300-L cylindrical fiberglass tank with 50 shrimps was used as a replicate. The shrimps were fed to apparent satiation four times a day at 06:00, 12:00, 18:00 and 24:00. For each time, the tank was cleaned first to remove the waste matter. Then the air pump was turned off and a certain amount of feeds were put into the tank. One hour later, the air pump was turned on and the uneaten feeds were removed.

During the 6-week feeding trial, water temperature was maintained at 27–29 °C, pH 7.8–8.2, salinity 35‰.

2.3. Experimental procedure

From the 1st day of the experiment, five shrimps in the intermolt stage were randomly chosen from one tank to assay immune parameters every two days. Three tanks in one treatment were randomly chosen at each sampling time point. 100 μl haemolymph was withdrawn from the ventral sinus of each shrimp into a 1-ml sterile syringe containing 200 μl anticoagulant solution (30 mM trisodium citrate, 10 mM EDTA, 0.34 mM sodium chloride 0.12 mM glucose, adjust pH to 7.55 and osmotic pressure to 780 mOsm/kg). The haemolymph from five shrimps in one tank was pooled. The molt stage was determined by the examination of uropoda in which partial retraction of the epidermis could be distinguished (Robsertson et al., 1987). At the end of the 6-week growth experiment, the body weight of remaining shrimps was weighed and the specific growth rate (SGR) was evaluated as follows:

$$\mathrm{SGR}(\%) = [\ln(\text{final weight}) - \ln(\text{initial weight})] / \text{time(day)} \times 100$$

2.4. Immune parameters assay

A drop of the anticoagulant-haemolymph was placed on a Buker hemocytometer to measure total haemocyte count (THC) under optical microscope (XPS-BM-2GA, shanghai BM optical institution manufacture CO. LTD.). The haemocytes were counted manually in all 25 squares (= 0.1 mm^3). A 1-ml anticoagulant-haemolymph sample was centrifuged at 700 × *g* at 4 °C for 10 min, and supernatant was used to measure phenoloxidase (PO) activity and superoxide dismutase (SOD) activity. About 500 μl anticoagulant-haemolymph was used to measure the superoxide anion in haemocytes.

PO was estimated spectrophotometrically by recording the formation of dopachrome using L-3,4 dihydroxyphenylalanine (L-DOPA; Sigma, USA) as substrate according to Hernández-López et al. (1996). Briefly, 50 μl haemolymph supernatant was incubated with 50 μl trypsin (0.1% in CAC buffer: 0.45 M sodium chloride, 0.10 M trisodium citrate, 0.01 M sodium cacodylate, pH 7.0) in 96 wells micro plate at 25 °C for 10 min, and then 50 μl L-DOPA (0.3% in CAC buffer)