Effects of dietary astaxanthin on the immune response, resistance to white spot syndrome virus and transcription of antioxidant enzyme genes in Pacific white shrimp *Litopenaeus vannamei*

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Abstract

Three different experiments were conducted to study the effect of dietary astaxanthin on the immune response, resistance to white spot syndrome virus (WSSV) and transcription of antioxidant enzyme genes in Pacific white shrimp *Litopenaeus vannamei*. Each experiment included one triplicate treatment group (fed with 80 mg/kg astaxanthin supplemented diet) and one triplicate control group (fed with basal diet), and the feeding period lasted for 4 weeks. The experiment of immune responses showed that dietary astaxanthin significantly affects the hemolymph immunological index, including total haemocyte counts, phagocytic activity of haemocyte, serum anti-superoxide radical activity, serum phenoloxidase activity, serum anti-bacterial activity and serum bacteriolytic activity. In the experiment of resistance, the shrimp was challenged with WSSV after the feeding stage was finished. The result showed that the mortality of the experimental group 11 days post infection was 76.3%, while that of the control group was 100%. WSSV infection result via polymerase chain reaction (PCR) detection method showed that 95.6% of the dead shrimp in the experimental group were PCR positive, whilst 71.4% of the survived shrimps were WSSV positive. At the end of the experiment, 90% of the shrimps in the experimental group were WSSV positive, while all the the control group were WSSV positive. The result indicated that dietary astaxanthin could increase the resistance to WSSV. In the experiment of transcription of antioxidant enzyme (cyt-MnSOD, CAT and GP_X) genes, the effect of astaxanthin on the transcription of these enzyme genes in the hepatopancreas was studied using semi-quantitative RT-PCR analysis. The results suggested that astaxanthin could promote the antioxidant enzyme gene mRNA expression in the hepatopancreas of *L. vannamei*.

Keywords: Rainbow trout, *Oncorhynchus mykiss,* Stock density, Texture-colour quality, Chemical composition

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Introduction

Crustaceans have no immune memory and are easily infected with pathogenic bacteria and viruses, and they rely on innate immunity system to protect themselves against infections from the pathogen and other external factors. Enhancement of their immune activity is a primary consideration for the control of diseases. Many studies have tested the effect of dietary immune-stimulants in order to increase their immune responses and resistance against diseases. Many kinds of substances such as polysaccharides, vitamins, metals and antioxidants can serve as immune-stimulants. Carotenoids, as one kind of antioxidants, are known to enhance the immune function and disease resistance in higher animals (Supamattaya *et al*., 2005). However little is known about the effects of the immune stimulants on the crustacean and their possible mode of action in enhancing shrimp immunity. Astaxanthin is the predominant carotenoid in the crustacean. Since the crustacean can't synthesize carotenoids *de novo*, astaxanthin or appropriate precursors must be supplied in the diet (Matsuno, 2001). The effects of astaxanthin on the survival, growth, pigmentation and resistance of physical stresses in the crustacean have been widely studied (Yamada,1990; Menasveta *et al*., 1993; Merchie *et al*., 1998; Pan *et al*., 2001; Chien *et al*., 2003; Pan and Chien, 2003; Pan *et al.,* 2003; Chien and Shiau, 2005; Niu *et al*., 2009; Daly *et al*., 2013), while the studies of astaxanthin as an immune-stimulant in the crustacean were limited (Boonyaratpalin *et al*., 2001; Niu *et al*., 2009).

The effects of astaxanthin on the resistance of shrimp to infectious diseases have been widely reported, and mainly focused on the bacterial pathogens (Merchie *et al*., 1998; Boonyaratpalin *et al*., 2001; Pan and Chien, 2003; Angeles *et al*., 2009). Among viral pathogens of shrimp, WSSV has become the most threatening infectious agent in shrimp culture since it first appeared in 1992, among pathogens of shrimp (Guan *et al*., 2003). Some researchers reported that WSSV-infection in penaeid shrimp *Penaeus monodon* and *Fenneropenaeus indicus* caused oxidative stress by the release of reactive oxygen species (ROS) which were toxic to the cells, and led to a significant increase in the lipid peroxidation in all tissues and a substantial decrease in the activity of antioxidants enzymes (Mohankumar and Ramasamy, 2006; Rameshthangam and Ramasamy, 2006; Mathew *et al*., 2007; Liu *et al*., 2010; Ji *et al*., 2011). As an effective antioxidant, astaxanthin might alleviate the oxidative stress due to WSSV, and then alleviate the infection of WSSV. The dietary supplementation of synthetic and natural antioxidants including astaxanthin possessing health promoting and antimicrobial properties might be an alternative option for amelioration of WSSV infection (Mathew *et al*., 2007). As an effective antioxidant, astaxanthin might alleviate the oxidative stress due to WSSV, and then alleviate the infection of WSSV. Till now, report on astaxanthin on the resistance of shrimp to viral disease is scanty. Among the penaeid shrimp,

Litopenaeus vannamei has become the most important cultured species worldwide due to its tolerance of low salinity and successful development of techniques. In the present study, the effect of dietary astaxanthin on the resistance of Pacific white shrimp *L.vannamei* to WSSV, (the most threatening infectious agent in shrimp aquaculture), was investigated.

One of the most important characters of astaxanthin is its antioxidant properties, which has been reported to surpass those of β-carotene or even α-tocopherol (Miki, 1991; Edge *et al*., 1997; Higuera-Ciapara *et al*., 2006). As an effective antioxidant, astaxanthin is an important part of the antioxidant defence system in the body of the crustacean. In our previous study, we found that astaxanthin supplementation could increase the total antioxidant capability (T-AOC) and affect the activities of antioxidant enzymes including catalase (CAT) and superoxide dismutase (SOD) in *L. vannamei* (Pei *et al*., 2009). Angeles *et al*., (2009) also reported that injected astaxanthin significantly enhanced activities of antioxidant enzyme (SOD, glutathione peroxidase (GPx) and glutathione reductase) in giant freshwater prawn *Macrobrachium rosenbergii* De Man to some extent. However, whether it can affect the regulation of antioxidant enzyme genes of the shrimp is uncertain yet. In this paper, the effect of astaxanthin on the transcription of antioxidant enzyme genes (CAT, cyt Mn-SOD and GPx) in *L. vannamei* was studied.

In brief, the effects of dietary astaxanthin on the immune responses, the resistance to WSSV and mRNA levels of antioxidant enzyme genes of *L. vannamei* were investigated in the present study. The aim was to evaluate the role of astaxanthin as an immune-stimulant during supplementation in the diet of shrimp.

Materials and methods

Tested shrimp

The shrimp *L. vannamei* was obtained from Nanpaihe, Huanghua, Hebei province, where the shrimps had been acclimated to brackish water (4 ppt) in the husbandry farm before being transferred to the laboratory.

Diet preparation

The basal diet was prepared according to Wang *et al*., 2006. The experimental diet was prepared through the following stages. First, astaxanthin was extracted from dry algae powder (microalgae *Haematococcus pluvialis*) and then was determined quantitatively by HPLC (Pei *et al*, 2009). Astaxanthin was supplemented in the basal diets by 80 mg/kg according to the results of Flores (2007) and our previous report (Pei *et al*., 2009). The diets were prepared by thoroughly mixing the dry ingredients with oil, and then added cold water until a stiff dough resulted. It was passed through a mincing machine, and the resulting strings were air dried. After drying, the diets were broken into pellets, then sieved and stored at -20°C.

Experimental design

The study contained three independent experiments. Experiment I: the effect of astaxanthin on the immune responses of *L. vannamei*. Experiment II: the effect of

astaxanthin on the resistance of *L. vannamei* after challenged with WSSV. Experiment III: the effect of astaxanthin on the transcript of antioxidant enzyme genes in the hepatopancreas of the shrimp. The initial body weight of the shrimp was 4.53-4.97 g. Each experiment had one triplicate control group and one triplicate treatment group. The control group was fed with basal diet and the experimental group was fed with the diet supplemented with astaxanthin (80 mg/kg). In all experiments the feeding period was 4 weeks.

The shrimp were cultured in 200 L glass tanks (150×75×50 cm). Appropriate volumes of aerated tap water and seawater were mixed together to obtain water with 4 ppt salinity. Water temperature and pH were maintained at 27±2˚C and 8.0±0.2˚C, respectively. The tanks were continuously aerated and one third of the water volume was changed daily. The unconsumed diet and the faeces were removed before the exchange of water. The daily feeding rate was 2-4% of the shrimp biomass in each tank, and three times per day with the rate of 30%, 30% and 40% of the feed, respectively.

Experiment I: The effect of astaxanthin on the immune responses of L. vannamei.

Experiment procedure. Each triplicate tank contained 40 shrimps. Six shrimps of each tank were sampled at 0, 1, 2, 3 and 4 weeks after the onset of the experiment.

Two shrimps of each sampled lot were used to determine THC, two shrimps to measure the phagocytic activity, and the last two shrimps to measure all other immune parameters.

Total Haemocyte Count (THC). The hemolymph was drawn from the cardiocoelom of the shrimp using a 1 ml sterile syringe. One hundred µl hemolymph was mixed with same volume of pre-chilled anticoagulant (trisodium citrate 30 mM, sodium chloride 0.34 M, EDTA 10 mM, pH 7.55, osmolarity was adjusted to 780 mOsm/L using glucose) in an Eppendorf tube to determine THC using a haemocytometer and calculated by taking into account the volume of the anticoagulant used during bleeding (Smith and Johnston, 1992).

The phagocytic activity. One hundred µl hemolymph was mixed with 900 µl anticoagulant to determine the phagocytic activity of haemocytes according to the method of Fan and Shen (1980). Briefly, hemolymph was diluted by anticoagulant until a final density of 10^5 haemocyte cells /ml was obtained, and then 10µl of bacterial suspension of *B. subtilis* with the concentration of 1.0×10^7 CFU/ml was added into the diluted hemolymph and mixed thoroughly. The mixture was incubated at 27℃ for 1 hour, then centrifuged at 2683 rpm at 4℃ for 4 minutes. The sample was washed, re-suspended and spread on a glass slide. After drying the slide was fixed by formalin for 3-5 minutes. The fixed slide was stained with Gimsa stain for 1-1.5 hours, decolorized in ultrapure water, air dried and observed under an Olympus light microscope (10×ocular, 100×oil immersion objective). The number of phagocytic haemocytes among random 200 haemocytes was counted. The phagocytic percentage (PP) and

Phagocytic index (PI) were expressed as follows:
PP=(Phagocytic haemocytes)/ (total haemocytes)×100;
PI= (Total counts of phagocytic bacteria)/ (phagocytic haemocytes).

Other immune parameters. The hemolymph was drawn and transferred into an Eppendorf tube and kept at 4℃ overnight. Then it was centrifuged at 800×g for 3 minutes at 4℃. The obtained serum was transferred into another Eppendorf tube to determine the anti-superoxide anion activity, phenoloxidase activity, antibacterial activity and bacteriolytic activity. The activities were all expressed as U/ml serum.

Anti-superoxide anion activity was assayed with commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Phenoloxidase activity of serum was measured spectro-photometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) according to the method of Huang *et al* (2006a). In brief, the supernatant of haemolymph (100 μl), or in the case of control, with 100 μl distilled water, was added in 3 ml phosphate buffer solution (0.1 mol/L, pH 6.4) before 100 μl of L-DOPA (0.01 mol/L) was added. Then the mixture was incubated at 30 ℃ for 5 minutes. The reaction was allowed to proceed and the absorbance at 490 nm was measured using a spectrophotometer every 2 minutes. An increment in the absorbance at 490 nm of 0.001 every minute under this condition was defined as one unit of PO activity. Antibacterial activity in the serum was measured using *E.coli* according to the references (Hultmark *et al*., 1980; Wang *et al*., 1994), and bacteriolytic activity was measured using *Micrococcus lysodeikticus* according to Yue *et al*. (2010).

Experiment II: the effect of astaxanthin on the resistance of L. vannamei after challenged with WSSV.

Experiment procedure. Each tank contained 10 shrimps. The shrimps were challenged with WSSV after 4 weeks feeding. WSSV-infected shrimp was obtained from Shilihai shrimp farm, Tanghai, Hebei Province, China, and was demonstrated WSSV positive infected by PCR method (Huang *et al*., 2006b). Virus stock solution was prepared according to our previous description (Guan *et al*., 2003). The shrimp was injected with 200 μl virus solution. During the 11 days' challenge stage, the survival number was recorded daily and the dead shrimp were collected and preserved at -80℃. At the end of the experiment, each shrimp (the survival and the dead) was detected by PCR method to determine WSSV infection.

WSSV infection by the PCR technique. WSSV-infection was detected by the PCR technique described by Huang *et al* (2006b). In brief, template DNA for PCR tests was prepared by the extraction of the gills. Primer F1: 5'GCG GAT CCA TGG ATCT TTC TTT CAC TCT 3', Primer R1: 5' GCG AGC TCT TAC TCG GTC TCA GTG CC 3'. The primers designed were used to amplify a 631-bp fragment of WSSV-DNA. PCR products were

analyzed by electrophoresis in 0.8% agarose gel stained with ethidium bromide, and visualized by 2020D gel image analysis systems (Beijing New Technology Application Institute, Bejing, China).

Experiment III: the effect of astaxanthin on the transcription of antioxidant enzyme genes in the hepatopancreas of shrimp.

Experiment procedure. Each tank contained 10 shrimps. One shrimp was randomly selected from each tank for the extraction of the total RNA from hepatopancreas in order to determine the transcription of the antioxidant enzyme genes.

Transcript of the antioxidant enzyme genes. The mRNA level of antioxidant enzymes in each treatment of experiment III was measured by semi-quantitative RT-PCR. Total RNA was extracted from the hepatopancreas of the shrimp by Trizol kit (Invitrogen, USA) according to the procedure. The first-strand cDNA synthesis was carried out using the total RNA as the template and the M-MLV RT (Promega) as the reverse transcriptase. Reactions were incubated at 42˚C for 1 hour, terminated by heating at 95˚C for 5 minutes. cDNA sequences of β-actin (AF300705), cytosolic-Mn superoxide dismutase (cyt-MnSOD) (DQ005531; Tavares-Sánchez *et al*., 2004), catalase (CAT) (AY518322; Gómez-Anduro *et al*., 2006) and glutathione peroxidase (GPx) (AY973252) were obtained from the GenBank. Primers for these genes were designed using Primer Premier 5.0 (http://www.premierbiosoft.com/primerdesign/index.html) and sequences for these primers were listed in Table1. The β-actin served as internal control for amount and quality of cDNA of the interest genes.

Table 1: PCR primers and reaction conditions of PCR.

Gene	Primer sequence	Product length(bp)	Annealing temperature(℃)	Cycle times
β-actin	Sense 5' GCATCACCAGGGCTACAT 3' Antisense 5' GTCGCCACGAGAAGTCAA 3'	692	55	25
Cyt-Mn SOD	Sense 5' GCATCACCAGGGCTACAT 3' Antisense 5' GTCGCCACGAGAAGTCAA 3'	537	55	30
CAT	Sense 5' AACAGACGGCTCCCGATAA 3' Antisense 5' GAACGCAATCTGCTCCACC 3'	944	56	35
GP_X	Sense 5' GAGCCCAAGATGGTTATG 3' Antisense 5' TGTTGAGGTCCCAAGGTC 3'	471	58	35

All PCR reactions were performed according to the following protocol: 1 µl of cDNA solution was mixed with 0.4 µl dNTPs (10 mmol/L), 0.3µL Taq polymerase (5 U/L), 1 µl of each gene specific primer (10 µmol/L), 2.5 µl 10× PCR Mg^{2+} -free PCR buffer, 1.5 µl $MgCl_2$ (25 mmol/L) and 17.3 µl RNAase-free water. The PCR reactions were performed in a MyCycler Thermal cycler (Bio-Rad, USA) for 5 minutes at 94˚C, followed by 25 cycles (for β-actin), 30 cycles (for Cyt-MnSOD), and 35 cycles (for CAT and GPx). The annealing temperature of β-actin, Cyt-MnSOD, CAT and GPx in each cycle were 55˚C, 55˚C, 56˚C and 58˚C for 40 seconds. The denaturation temperature in each cycle was 94˚C for 40 seconds and

the extension temperature in each cycle was 72˚C for 60 seconds. A final extension step is at 72˚C for 10 minutes.

The PCR products were separated in 2% agarose gel and stained with ethidium bromide. Electrophoretic image and the densities of target bands were analyzed using the Quantity One analysis software in the 2020D gel image analysis system. The ratio of amplicon of antioxidant enzyme gene and β-actin indicate the mRNA level of each antioxidant enzyme.

Statistical analysis

Statistical analysis was performed using SPSS program version 17.0 (SPSS, Chicago, IL, USA). The possible differences of cumulative mortality, immune responses and transcript of antioxidant enzyme gene of the hepatopancreas between the experimental group and the control group were tested by independent t-test. The possible differences of immune responses among the control group or the experiment group at different sampling time were tested by one-way ANOVA. First, the Levene's test was performed to determine whether the homogeneity of variance existed or not. If homogeneity existed, then Duncan's multiple range tests were carried out. Differences were considered significant at a level of 0.05.

Results

The effect of astaxanthin on the immune responses of L. vannamei

The immune responses of the shrimps of the two treatments were listed in Table 2. After feeding with astaxanthin supplemented diet, THC was significantly higher than that of the control group at the same week (p<0.05). THC of the experimental group at week 1, 2 and 3 were 124%, 135% and 139% of THC of the control group, respectively. THC of the experimental group at week 3 was $8.600 \pm 1.040 \times 10^5$ cell/mL, which reached the peak. At week 4, THC was 94.9% of that of week 3. The differences among THC of experimental group at each week were not significant (p>0.05). The result indicated that THC increased to some extent after feeding with astaxanthin supplemented diet and showed the tendency of increasing first and then decreasing.

Table 2: The effect of astaxanthin on the immune responses of *Litopenaeus vannamei*.

Items	Treatment	Week 0	Week 1	Week 2	Week 3	Week 4
THC	Control	6.667±0.288	6.167±0.577	6.330±0.288	6.160±1.040	6.000±1.320
($\times10^5$ cell/ml)	Experiment	6.833±1.040	7.667±0.763*	8.500±1.322*	8.600±1.040*	8.160±1.890
Phagocytic	Control	22±4	21±4	21±7	20±7	21±5
percentage (%)	Experiment	23±7^{a}	28±5^{ab}	31±5^{ab}	43±7^{*b}	32±4^{*ab}
Phagocytic	Control	1.53±0.29	1.32±0.16	1.46±0.25	1.36±0.38	1.35±0.24
index(ind./cell)	Experiment	1.43±0.27	1.6±0.03	1.73±0.13	1.77±0.12	1.71±0.27
Anti-superoxide	Control	0.24±0.01	0.25±0.01	0.25±0.01	0.23±0.02	0.22±0.01
radical (U/ml)	Experiment	0.22±0.01^{a}	0.31±0.01^{*b}	0.35±0.05^{*b}	0.38±0.02^{*c}	0.36±0.01^{*c}
Phonoloxidase	Control	15.33±0.67	18.83±1.2	15.83±1.17	17.33±1.92	14.67±1.48
activity(U/ml)	Experiment	18.67±0.33^{a}	21.06±0.69^{*a}	24.39±1.64^{*b}	29.61±1.36^{*c}	27.78±1.92^{*c}
Antibacterial	Control	0.25±0.05	0.22±0.02	0.25±0.02	0.30±0.03	0.29±0.02
activity(U/ml)	Experiment	0.28±0.02^{a}	0.38±0.01^{*b}	0.47±0.04^{*c}	0.51±0.02^{*cd}	0.54±0.03^{*d}
Bacteriolytic	Control	0.14±0.01	0.14±0.02	0.15±0.01	0.16±0.03	0.17±0.05
activity(U/ml)	Experiment	0.17±0.01^{a}	0.26±0.03^{*b}	0.24±0.02^{*b}	0.34±0.03^{*c}	0.31±0.02^{*c}

Note: The value was expressed as mean±S.D. Superscript letters (a, b and c) indicate significant difference among the control group or the experimental group at different weeks (one way ANOVA, $p<0.05$); Superscript asterisk (*) indicate significant difference between dietary treatments (*t*-test, $p<0.05$) at the same week. n=6 for each mean value. THC stands for "Total Haemocyte Counts".

Phagocytic percentages of haemocyte in the experimental group were higher than that of control group. The average of phagocytic percentage of experimental group and control group were 31.4% and 21%, respectively. At week 3, phagocytic percentage was 43%, which showed to be the highest, and was significantly higher than that of experimental group at week 0 ($p<0.05$). Changes in the phagocytic index of the experimental group was more obvious compared with the control group, but the differences between the experimental group and the control group at each week were not significant ($p>0.05$). Serum anti-superoxide radical ability of the experimental group (from week 1 to week 4) was significantly higher than the control ($p<0.05$). The activity at week 3 was the highest and was 165% of the control group. The activities of the experimental group at each week (from week 1 to week 4) were significantly different ($p<0.05$).

Serum phenoloxidase activity of the experimental group (from week 1 to week 4) was significantly higher than the control group at each week ($p<0.05$). PO activity at week 3 was the highest and was 124% of the control group at week 3.

Antibacterial activity of the experimental group was significantly higher than that of the control ($p<0.05$). It tended to increase after feeding with astaxanthin and reached its peak (0.54 U/ml) at week 4. Serum bacteriolytic activities fluctuated after feeding with astaxanthin and were significantly higher than the corresponding control ($p<0.05$). The difference between bacteriolytic activities of the experimental group at week 1 and week 2 were not significant ($p>0.05$), but were significantly lower than bacteriolytic activities at weeks 3 and 4 ($p<0.05$). Bacteriolytic activity at week 3 was 0.34 U/ml and reached the maximum.

The effect of astaxanthin on the resistance of L. vannamei after challenged with WSSV

Cumulative mortalities of both groups were shown in Fig.1. After challenged for

3 days, the shrimp started to die substantially. Mortality increased considerably in both treatments at 4th and 5th days, and slowed down at 6th day. At 8th day, it tended to be stable, and the cumulative mortality of the control group and the experimental group were 100% and 76.3%, respectively. The difference between the cumulative mortality of the two groups at the final stage was significant ($p<0.05$). WSSV infection result showed that 95.6% of the dead shrimp (22 shrimps among 23 shrimps) in the experimental group were PCR positive, and only one shrimp (sample 7 in Fig. 2e) was PCR negative. Seven shrimps survived in the experimental group and 5 of them were WSSV positive (sample 9 and 10 in Fig. 2d, sample 8 in Fig. 2e, and sample 9 and 10 in Fig. 2f) and WSSV positive rate of the survived shrimp were 71.4% (5 shrimps among 7 shrimps) . Altogether the shrimps in the experimental group was 90% WSSV positive. All the shrimps in the control group were WSSV positive and died during the experiment (Fig. 2.a-f). From the results of the cumulative mortality and WSSV detection, it was indicated that astaxanthin supplementation could enhance the resistance of *L. vannamei* to WSSV.

The effect of astaxanthin on the transcript of antioxidant enzyme genes in the hepatopancreas of the shrimp

Cyt-Mn SOD, CAT, GPx gene and internal control gene β-actin were detected in the hepatopancreas of the shrimp of the experimental group and the control group. Compared with the control, the mRNA level of cyt-Mn SOD, CAT and GPx of the experimental group increased by 56.4%, 48.4% and 133%, respectively and were all significantly higher than those of the control group ($p<0.05$). It indicated that the antioxidant enzyme genes of the shrimps, including cyt-Mn SOD, CAT and GPX could be enhanced at the mRNA level after feeding with astaxanthin supplemented diet for 4 weeks (Table 3, Fig.3.a-c).

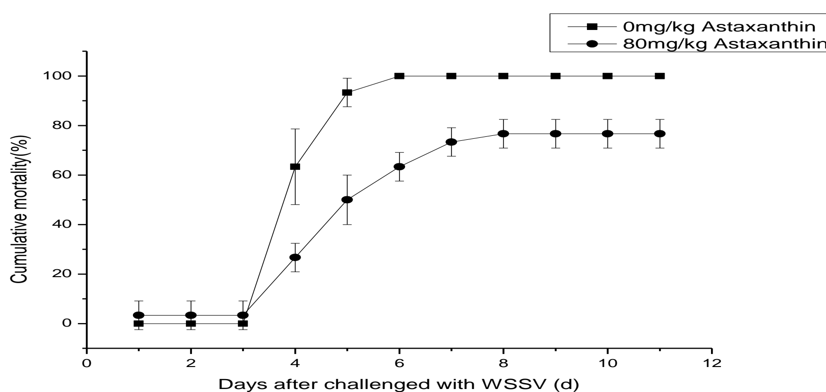

Figure 1: The effect of astaxanthin on the mortality of *Litopenaeus vannamei* after challenged with WSSV.

Square stands for the control group (0 mg/kg astaxanthin), while diamond stands for the experimental group (80 mg/kg astaxanthin).

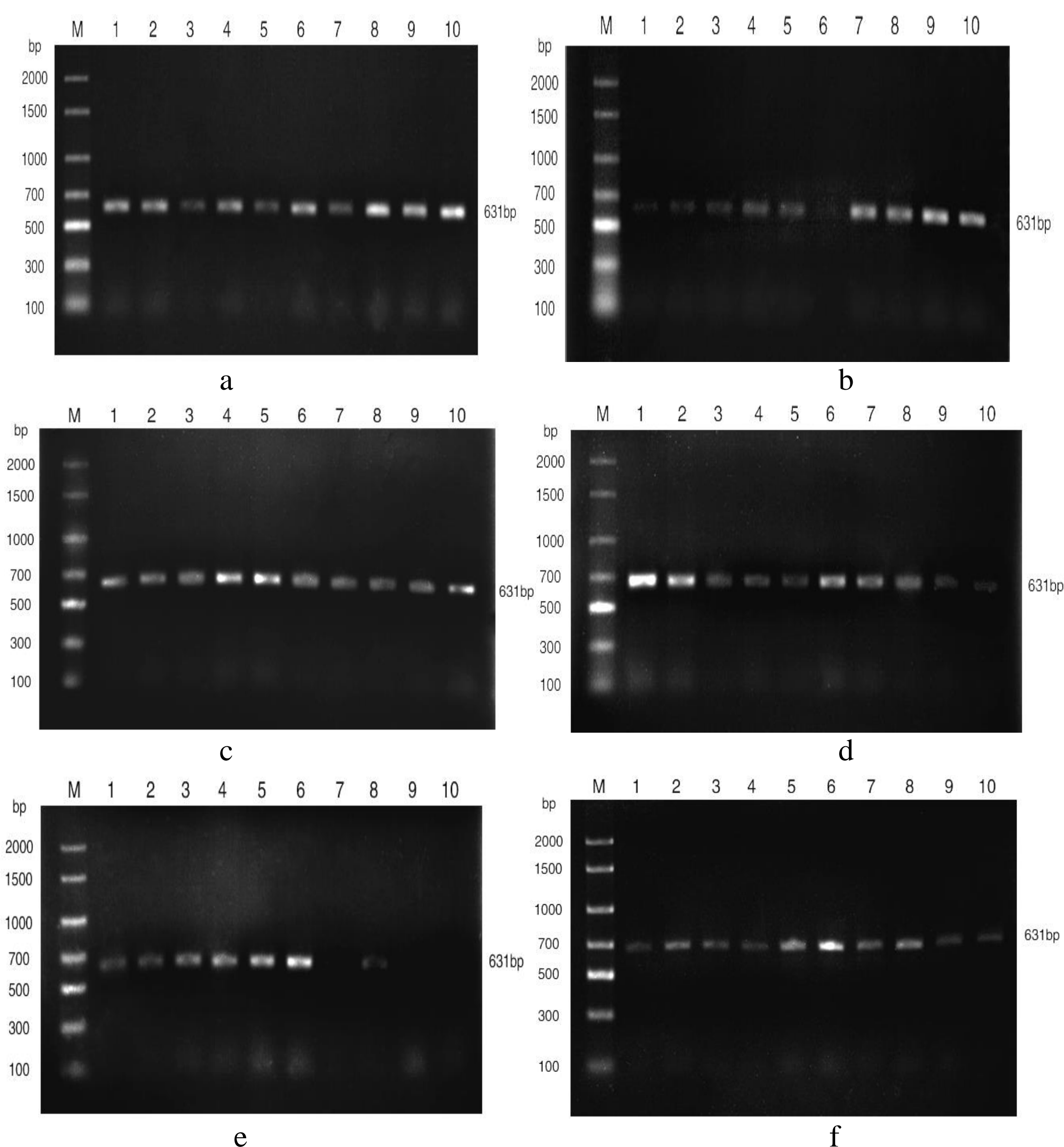

Figure 2: WSSV detection by PCR of *Litopenaeus vannamei* challenged with WSSV.
a-c, Tank 1-3 of the control group (0 mg/kg astaxanthin), Lane 1-10 dead shrimp; d, Tank 1 of the experimental group (80mg/kg astaxanthin), Lane 1-8 dead shrimp, Lane 9-10 survival shrimp; e, Tank 2 of the experimental group, Lane 1-7 dead shrimp, Lane 8-10 survival shrimp; f, Tank 3 of the experimental group, Lane 1-8 dead shrimp, Lane 9-10 survival shrimp. M: Marker.

Table 3: Antioxidant enzyme gene transcripts of hepatopancreas of *Litopenaeus vannamei* revealed by RT-PCR.

Treatment	Cyt-Mn SOD /β-actin	CAT/β-actin	GPx/β-actin
Control	0.3977±0.0450	1.1588±0.1689	0.2015±0.0242
Experimental	0.6223±0.0640*	1.7198±0.2434*	0.4697±0.0683*

Note: The ratio of antioxidant enzyme gene and β-actin were expressed in mean±S.D. (n=3). Superscript asterisk (*) indicate significant difference between the two dietary treatments (*t*-test, $p<0.05$).

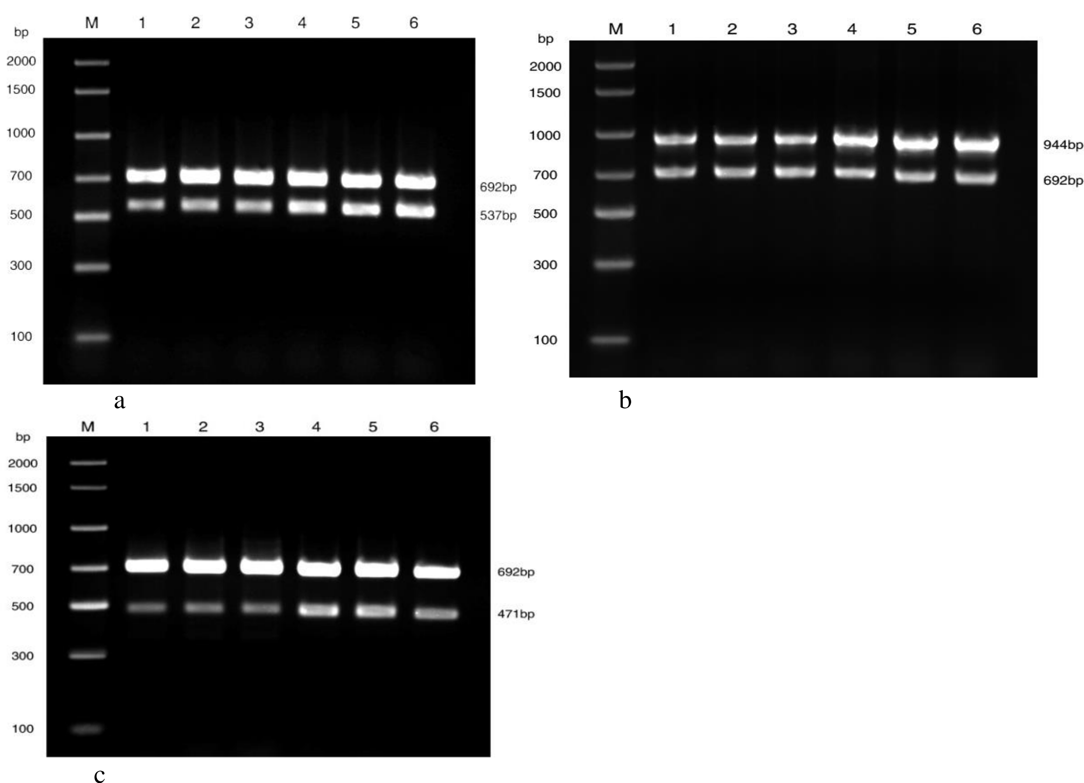

Figure 3: Antioxidant enzyme gene transcripts of hepatopancreas of *Litopenaeus vannamei* revealed by RT-PCR.

A, RT-PCR analysis of cyt-Mn SOD mRNA expression; b, CAT; c, GPx. β-actin served as an internal control, M, Marker.Lane 1-3, the shrimp in the control group (0 mg/kg). Lane 4-6, the shrimp in the experimental group (80 mg/kg). 537 bp, 944 bp, 471 bp and 692 bp fragment stand for fragment of cyt-Mn SOD, CAT, GPx and β-actin gene, respectively.

Discussion

The effect of astaxanthin on the immune responses of L. vannamei

Crustacean haemocyte play an important role in the hosts' immune response including recognition, phagocytosis, melanization, cytotoxicity and cell-cell communication. The haemocyte count varies greatly in response to infection, environmental stress and endocrine activity during moulting cycle (Johansson *et al*., 2000). THC is one of the major parameters used to evaluate immune response and can reflect the resistance to the foreign pathogen to some extent. Phagocytosis of the haemocyte is also an important immune response of the crustacean, which represents an important way to eliminate microorganisms or foreign particles, and has been described and documented in the invertebrate (Van de Braak *et al*., 2002). Some immune-stimulants could enhance the above immune responses of the crustacean. For example, after injected with astaxanthin, THC of *M. rosenbergii* increased (Angeles *et al*., 2009). Astaxanthin can also affect the immune response of other aquatic

animals. For example, Amar *et al*. (2001) reported that astaxanthin and some other carotenoids could enhance the cellular immunity of rainbow trout such as cytotoxicity and phagocytosis. In our present experiment, THC and phagocytosis (indicated by phagocytic index and phagocytic percentage) of *L.vannamei* were enhanced after feeding with astaxanthin supplemented diet. The result showed the tendency of initial increasing followed by subsequent decreasing trend, and all of these parameters reached their peaks at week 2 or week 3. Many studies reported that some other immune-stimulants can also improve the immune responses of the shrimp. After feeding with diet supplemented with 1 g/kg or 4 g/kg β-glucan, laminarin or sodium alginate, THC of *L. vannamei* significantly increased after 1-2 days' treatment and the concentration of superoxide anion reached its highest after 2-4 days' treatment (Huang *et al*, 2006a). Phagocytic percentage of haemocyte and phagocytic index in Chinese shrimp *Fenneropenaeus chinensis* Osbeck increased by 11.4% and 39.7%, respectively, after it was injected with Fungus polysaccharide, extracted from *Cordyceps militaris* for 48 hours (Jiang *et al*, 1999). Cheng *et al*. (2004) reported that after received sodium alginate at 10μg/g or more, *L. vannamei* increased its immune ability and resistance from *Vibrio alginolyticus* infection. In detail, *L. vannamei* injected with sodium alginate at 50 μg /g maintained a higher phagocytic activity and clearance efficiency to *V. alginolyticus* after 4 days.

Respiratory burst is a post-phagocytic event, which released ROS including superoxide anion. Starting from superoxide anion, a number of reactions lead to the production of hydrogen peroxide, singlet oxygen, hydroxyl radical and numerous other reactive products. Anti-superoxide anion ability can indicate the activity of scavenging the superoxide anion in the body, and it can also reflect the releasing of superoxide anion by respiratory burst. The result showed that the anti-superoxide anion abilities of the experimental shrimp were significantly higher than the respective control from week 1 to week 4, and it indicated that the ability of scavenging superoxide anion was improved after feeding with astaxanthin supplemented diet. Phenoloxidase is the terminal enzyme of the so-called prophenoloxidase (proPO) activating system, a non-self-recognition system present in arthropods and other invertebrates. Several components or associated factors of the proPO system have been found to play several important roles in the defence reactions of crustacean. Sutthangkul *et al*. (2015) showed that proPO system is an important component of the *Penaeus monodon* shrimp immune defense towards WSSV. Wang (2002) reported that lipopolysaccharide, 1, 3-β-glucan, inactivated Vibrio harveyi and inactivated Vibrio anguillarum could stimulate the production of phenoloxidase. Cheng *et al*., (2004) reported that after received sodium alginate at 10 μg/g or more, *L. vannamei* increased its immune ability and resistance from *Vibrio alginolyticus* infection. In

detail, *L. vannamei* injected with sodium alginate at 50 µg/g maintained a higher phagocytic activity and clearance efficiency to *V. alginolyticus* after 4 days.

In our experiment, phenoloxidase activities of the experimental group were significantly higher than the control group from week 1 to week 4. Cornet *et al* (2007) demonstrated that maintenance and use of the prophenoloxidase system is strongly correlated with carotenoid concentration in haemolymph within and among natural populations of the crustacean *Gammarus pulex*. It was speculated that carotenoids played a role as an immunostimulant and/or antioxidant by scavenging free radicals produced during the PO cascade, thus allowing the enhancement of the proPO system, although it should be further verified.

Concerning about humoral parameters, the antibacterial activity of plasma can be considered as one of the criteria of health status. Wang *et al* (1995) reported that antibacterial activity and bacteriolytic activity can be taken as immune indicators and can be utilized to evaluate the immune responses of the shrimp. Liu and Li (1998) reported that some immnostimulants such as polysaccharides could enhance the antibacterial activity and bacteriolytic activity of shrimp. In our experiment, the experimental group showed higher antibacterial activity and bacteriolytic activity than the control group. Amar *et al*. (2001) reported that astaxanthin and some other carotenoids could not only enhance the cellular immunity of rainbow trout, but also enhance the humoral immunity such as serum defense in and bacteriolytic activity. Another report of Amar *et al* (2004) showed that dietary carotenoids from both marine green algae *Dunaliella salina* (β-carotene) and red yeast *Phaffia rhodozyma* (astaxanthin) can modulate some of the innate defence mechanisms including serum alternative complement activity, phagocytic rate and index in rainbow trout. Our result was in accordance with those.

The effect of astanthin on the disease resistance of L.vannamei after challenged with WSSV

Many researchers have carried out the experiments on using the immunostimulants to prevent the viral disease in cultured shrimp and have proved its effectiveness. These people mainly utilized polysaccharides as the immune-stimulant. The effects of astaxanthin on the resistance to infectious diseases of shrimp have scarcely reported. Pan and Chien (2003) reported that antioxidant capacity and hepatopancreatic enzymes of juvenile *P. monodon* changed significantly after being fed with astaxanthin supplemented diet. After exposed to *V. damsela* challenge, survival rate of *P. monodon* were higher than the control group, which were fed with basal diet. Angeles *et al*. (2009) reported that *M. rosenbergii*, which was previously injected with astaxanthin, exhibited higher survival rate than the control group, after exposed to *Lactococcus garvieae*. Supamattaya *et al* (2005) reported that *P. monodon* fed with 300 mg of the commercially available *Dunaliella* extract (a marine alga containing various carotenoids, especially

high amounts of β-carotene)/kg diet exhibited higher resistance to WSSV infection than other groups. These results indicated that astaxanthin or some other carotenoids could enhance the resistance of shrimp to bacterial or viral pathogens. In contrast, some researchers failed to find that astaxanthin can improve the immune responses of shrimp and resistance to pathogen. Merchie *et al*. (1998) reported that post-larval *P. monodon* were fed with graded concentrations of astaxanthin and ascorbic acid for 4 weeks, and then were challenged with *V. harveyi*. No conclusive result could be made since the challenge dose might be too low and no mortality was observed. Boonyaratpalin *et al.* (2001) thought that the supplementation with β-carotene or astaxanthin had no significant effect on the immune response of the shrimp *P. monodon* in terms of production of haemocyte phenoloxidase in the cytoplasmic granules of the haemocytes, microbial clearance ability and resistance to pathogenic bacteria *V. harveyi* infectious diseases and white spot syndrome (indicated by mortality). Since WSSV has become the most threatening infectious agent in shrimp aquaculture after it first appeared in 1992 among pathogens of shrimp, whether the resistance to WSSV in the shrimp after being fed with astaxanthin was enhanced or not should be more investigated.

Mathew *et al*. (2007) reported that WSSV infection induced a significant increase in lipid peroxidation in haemolymph, muscle and hepatopancreas of experimental *P. monodon* compared to normal controls. The antioxidant defence system in WSSV infected *P. monodon* is operating at a lower rate, which ultimately resulted in the failure of counteraction of ROS, leading to oxidative stress. Other results on *P.monodon* and *F. indicus* were similar with Mathew’s report (Mohankumar and Ramasamy, 2006; Rameshthangam and Ramasamy, 2006; Liu *et al*., 2010). These reports indicated that enhancement of the antioxidant defence system and alleviation of the oxidative stress due to viral infection might improve the resistance of WSSV in crustaceans. It could help to advance the therapeutic armamentarium for control of WSSV in shrimp. In our previous study, the shrimp fed with astaxanthin supplemented diet significantly increased its total antioxidant capability (Pei *et al*., 2009). In the present study, the shrimp was fed with astaxanthin supplemented diet for 4 weeks and was challenged by WSSV subsequently in order to determine whether it can be used to prevent the outbreak of white spot syndrome. The results showed that the group fed with 80 mg/kg astaxanthin showed significantly higher survival rate than the control group, and the rate of WSSV positive in the experimental group was lower than that of the control group. The result indicated that astaxanthin could enhance the resistance of WSSV in *L. vannamei*. Enhancement of the resistance of WSSV by astaxanthin might be due to astaxanthin could scavenge the ROS effectively as a strong antioxidant and consequently alleviate the oxidative stress induced by WSSV infection.

Effect of astaxanthin supplementation on the transcription of antioxidant enzyme genes in L. vannamei

Aerobic organisms could produce excessive ROS such as superoxide anion ($O_2^-\cdot$), hydroxyl radical, singlet oxygen and hydrogen peroxide after exposed to environmental stresses. If the excessive ROS could not be scavenged effectively, the accumulation of them could induce oxidative stress and may ultimately damage many biological macromolecules such as DNA, proteins and lipids. In the body of aerobic organisms, harmful effects of ROS are effectively neutralized by antioxidant defence system of organisms. The integrated antioxidant system, comprising endogenous antioxidant enzymes (e.g. superoxide dismutase SOD, and catalase CAT) and dietary antioxidants (e.g. carotenoids), helps to cope with immune-mediated oxidative stress (Babin *et al*., 2015). The antioxidase system, and ROS and lysozyme might play a crucial role in shrimp defense against bacterial and viral infection (Ji *et al.*, 2011). Among the antioxidant defensive system of the crustacean, there are few reports on how the antioxidant enzyme and antioxidant molecule interact. Dandapat *et al*. (2000) reported that dietary vitamin-E was capable of reducing lipid peroxidation level and could modulate antioxidant defence system in gills and hepatopancreas of *M. rosenbergii*.

Astaxanthin might protect the body through direct or indirect way. As an effective antioxidant, it can scavenge ROS directly and play an important role in the antioxidant defence system. For example, astaxanthin blocks the increase of thiobarbituric acid reactive substances (TBARS) and stimulates the cellular antioxidant system in the CCl_4-treated rat liver. On the other hand, it might exert through affecting the activity of antioxidant enzyme and/or the expression of antioxidant enzyme gene (Kang *et al*., 2001). Similarly, the oral administration of astaxanthin inhibits the lipid peroxide level in gastric mucosa in rats. In addition, pre-treatment of astaxanthin results in a significant increase in the activities of radical scavenging enzymes such as SOD, CAT, and GPx (Kim *et al*., 2005). In our previous report, we found that astaxanthin supplementation could affect the activities of antioxidant enzymes including CAT and SOD significantly of *L. vannamei* (Pei *et al*., 2009). Angeles *et al*. (2009) reported that injected astaxanthin significantly enhanced activities of antioxidant enzyme (SOD, GPx and glutathione reductase) in *M. rosenbergii* to some extent. The amphipod crustacean *Gammarus pulex* exhibited down-regulated SOD activity but high CAT activity after feeding with carotenoids supplemented dietary, compared to the control (Babin *et al*., 2015). In the present study we examined the effect of astaxanthin supplemented in the diet on the transcription of antioxidant enzyme genes. The semi-quantitative RT-PCR results in our study indicated that astaxanthin might enhance the expression of antioxidant enzyme genes including cyt-MnSOD, CAT and GPx gene at the mRNA level and then enhance the antioxidative capability indirectly.

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