

Effect of zeaxanthin addition to feeding on non-specific immune performance in the *Penaeus vannamei*

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Abstract. In recent years, the market demand for aquatic products has also been increasing. However, due to environmental pollution, many aquatic organisms become weaker in immunity and thus develop various diseases, which affect overall aquatic production. Currently, people enhance the immune performance of aquatic animals by adding peptidoglycan, astragalus polysaccharide, and vitamins to aquatic feeds; the addition of zeaxanthin (Zeaxanthin, 3,3'-dihydroxy- β -carotene) has not been reported to enhance immunity. This experiment investigated the effect of zeaxanthin on some immune indicators in South American white shrimp. The results of the kit showed that zeaxanthin had a significant effect on the activity of lysozyme, NOS and CAT ($P < 0.05$), with a decreasing trend in the activity of lysozyme and an increase in the activity of nitric oxide synthase and catalase as the concentration of zeaxanthin added increased. Next, there were no significant differences in the activities of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX). Among them, the addition of 0.5% zeaxanthin had the greatest effect on NOS and CAT activity. As shown above and combined with the actual economic benefits, 0.5% zeaxanthin was selected as the best feed additive.

Keywords. Zeaxanthin, South American white shrimp, non-specific immune performance.

1. Materials and Methods

1.1. Zeaxanthin

Zeaxanthin (3,3'-dihydroxy- β -carotene, $C_{40}H_{56}O_2$), an isomer of lutein, is mainly found in the macular portion of the eye [1]. Zeaxanthin is safe and non-toxic and is commonly used in the food, feed and pharmaceutical industries. The zeaxanthin used in the experiments was purchased from a local feed mill.

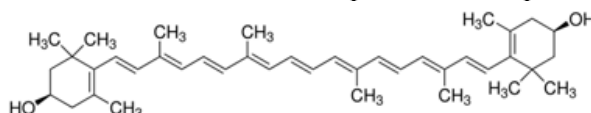


Figure 1. All-trans structure of zeaxanthin

1.1.1. Biological functions. Zeaxanthin is versatile and widely used in production practices for its excellent coloring and antioxidant properties.

In livestock production, zeaxanthin is used as a cost-effective colorant. Zeaxanthin can effectively improve the nutritional status and skin color of animals. In addition, zeaxanthin can be deposited in the yolk of egg-laying poultry, giving it a bright yellow color and improving the quality of the eggs. Zeaxanthin is also used in the food industry as a natural coloring agent to improve the color and appetite

of food, and has the antioxidant activity to prevent oxidation of nutrients in food, thus extending shelf life.

Zeaxanthin is also associated with preventing and treating eye diseases, heart disease and cancer. According to statistics in the United States, one of the main causes of vision loss in adults over the age of 40 is "age-related macular degeneration disease" (AMD), which affected approximately 14 million people in the United States in 2001. In a study of 365 people with AMD and 520 healthy people, the risk of AMD was significantly reduced in those who consumed yellow-green fruits and vegetables rich in zeaxanthin [2]. The risk of AMD was significantly reduced in those who consumed zeaxanthin-rich fruits and vegetables.

In addition, zeaxanthin can prevent cardiovascular diseases, slow down the process of atherosclerosis [3] prevent lipid peroxidation and thus inhibit the growth of tumors [4] and prevent DNA damage by UV radiation [5]. However, the dietary intake of zeaxanthin is very low. Increasing the amount of zeaxanthin in common foods through traditional plant breeding or genetic engineering techniques can help increase the amount of zeaxanthin [6].

In the aquatic industry, zeaxanthin and others have not only significant coloring effects, but also have other important roles, such as (1) protection from ultraviolet light; (2) improving immunity and promoting growth and maturation; (3) acting as a fertilization hormone and improving egg plasma; (4) improving the tolerance of fish to high ammonia and low oxygen environments; (5) being a precursor to vitamin A; (6) forming a marital color during the breeding and spawning season, forming a protective color for offspring a protective color [7].

1.1.2. Application of zeaxanthin in aquaculture. The production reflects the physiological health of fish by referring to their growth indicators. As a colorant, zeaxanthin can improve both feed utilization and the body color of fish. However, there are only a few studies on how zeaxanthin affects fish growth and immune mechanisms in domestic and international research and production. China accounts for 70% of global aquaculture production, and many species of yellow fish, such as *Pelteobagrus fulvidraco*, *Pelteobagrus fulvidraco*, and Golden *pompano* have a yellow body color in the wild. However, since fish and shrimp lack the ability to synthesize carotenoids from scratch, their food intake must contain the corresponding carotenoids or their precursors, but under aquaculture conditions, the carotenoid content in the artificial feed is low, which causes the problem that the body color of artificially farmed fish and shrimp is often less brilliant than that of wild individuals, especially in salmon and trout culture [8]. Therefore, exogenous zeaxanthin supplementation can be used to improve the body color of farmed aquatic animals.

In addition to the color enhancement effect, when zeaxanthin was added to the feed of silver carp and swordtail, the survival rate of the young fish after UV irradiation was significantly higher than that of the control group. Under normal conditions, most of the carotenoids contained in fish and shrimp come from colorants ingested in food, and the ingested carotenoids are converted in some form to the body and flesh color of the fish and shrimp themselves. Feed additives can be used not only to color fish's skin, muscles and scales, but also to promote growth performance [9].

In addition to this, zeaxanthin is the most important of the carotenoids required by shrimp and crabs, and its intake determines the shade of their body color, while shrimp and crabs protect themselves from sun damage by accumulating zeaxanthin in their pigment cells. This masking mechanism blends in with its surroundings by adapting to changes in its living environment to make corresponding color changes [10]. Aquatic animals do not have a mechanism to synthesize zeaxanthin, so it must be taken from outside food. Feeding zeaxanthin to Vannamei shrimp resulted in higher survival rates, more significant coloration, and increased carotenoid content; feeding adult Vannamei shrimp 60 mg/kg of carotenoids for two weeks resulted in significant body coloration [11]. The Linan-Cabello test showed that carotenoids also had a beneficial effect on the immune reproductive capacity of crayfish [12]. The Linan-Cabello test showed that carotenoids also contributed to the immune reproductive capacity of crayfish. Feeding 80 mg/kg of zeaxanthin improved the liver's muscle color resistance and total antioxidant

capacity, reduced serum CAT, SOD and MDA levels in muscle, and prolonged muscle shelf life in spot prawns.[13].

1.2. South American white shrimp (*Penaeus vannamei*)

South American white shrimp (*Penaeus vannamei*) is the scientific name for the *Vannamei* shrimp, belongs to the Crustacea, Decapoda, shrimp family, the genus *Penaeus*, naturally distributed on the Pacific coast of the tropical species of widespread salinity, is one of the three most productive shrimp farmed in the world today. Since its introduction to China in 1988, with the successive breakthroughs in factory nursery technology and artificial culture technology, the rapid development of South American white shrimp aquaculture production, so far has spread from north to south of the coastal areas, and even many inland areas have also begun to practice trying, has become one of the most important aquaculture species in China [14]. The aquaculture industry has become one of the most important species in China. In recent years, intensive aquaculture has brought many problems, such as shrimp in the absorption of endogenous nutrients to feeding on exogenous nutrients in the process of transformation and development. The emergence of stress greatly increases the risk of infection with pathogens, making the occurrence of disease more and more frequent. The main reasons for this are the rapid spread of pathogens and the lack of disease resistance in shrimp. The main diseases of South American white shrimp are red leg disease and bacterial black blush disease of South American white shrimp [15]. The use of antibiotics and other chemicals can suppress some diseases to some extent, but can lead to the development of pathogen resistance [16]. Residual drugs can contaminate water quality, causing secondary pollution and endogenous infections [17]. The prevention and control of diseases have become an urgent problem in the current shrimp aquaculture industry [18].

1.2.1. *South American white shrimp fry*. The South American white shrimp used in the experiment were 30-day-old juvenile shrimp (TL=17.89 mm in total length and W=0.157 g in weight) purchased from a farm in Binhai District, Weifang, and tested by the kit. The shrimp were negative for WSSV [19]. Before the start of the experiment, 10 000 South American white shrimp were transported back to the laboratory for 7 d after temporary rearing to pick the individual uniformity and vigor of the shrimp assigned to the tank, a total of 4 groups, namely JC1, XP2, XP3, XP4 groups, each group of 6 replicates, each replicate of 500 tails. The seawater used for the experiment was fresh groundwater and brine, and the culture period was 30 days, with three daily feedings, full feeding, sucking out residual bait and feces before feeding, water temperature 25.9-28.1°C, salinity 18.6-20.4g/L, pH 7.6-8.0, dissolved oxygen 5.12-5.69 mg/L, continuous aeration [20].

1.3. Experimental feed and grouping

The base feed (containing 20% fishmeal and 2% soybean oil, with fishmeal as the animal protein source and soybean oil as the fat source) was fed as the control group, and the experimental group was composed of 3 groups, with the base feed as the basis, and four experimental feeds (requiring equal nitrogen and energy) were prepared by adding zeaxanthin 0%, 0.25%, 0.50% and 1%, respectively, marked with JC1, XP2, XP3 and XP4 as the group numbers. The detailed feed formulations are shown in Table 1-1. The raw materials were crushed and passed through 80 mesh sieve, weighed accurately and mixed thoroughly step by step, and then extruded into feed by granulator after adding the appropriate amount of water, placed in a cool place to dry, packed in bags and stored in 4°C refrigerator for spare.

Table 1. Base feed formulation and experimental groups

Basic feed formulation		Experimental group			
Name of raw material	Content(%)				
Fish Meal	20.0				
Shrimp Powder	3.0				
Flour	22.0				
Soybean meal	25.0				
Corn protein powder	5.0	Base	Base	Base	
Peanut meal	12.0	feed×99.75%	feed×99.50%	feed×99.00%+	
Corn husk	8.0	+Zeaxanthin×	+Zeaxanthin	Zeaxanthin×1.	
Soybean Oil	2.0	0.25%	×0.50%	00%	
VC phosphatidic acid	0.2				
Vitamin premixes	0.6				
Mineral premixes	0.5				
Calcium dihydrogen phosphate	1.5				
Choline	0.2				
Total	100.0				
Grouping	JC1	XP2	XP3	XP4	

1.4. Sample collection and processing

After the start of the experiment, 10 to 15 South American white shrimp were randomly selected for blood sampling in each experimental group every five days, then injected into sterile 1.5 ml centrifuge tubes and placed in the refrigerator at four °C overnight. The next day, the blood clot was cut with a sterile needle and the precipitated serum was used to measure immunological indicators.

1.5. Measurement of experimental indicators

At the end of the culture, each experimental group took the whole shrimp and stored them in liquid nitrogen, and then brought them back to the laboratory for rapid determination of each index of non-specific immune performance. The determination of lysozyme, NOS, MDA, SOD, CAT and GSH-PX viability, all viability indicators of the determination of kits are produced in Nanjing Jiancheng Institute of Biological Engineering, the determination method is detailed in the instructions.

1.5.1. Determination of lysozyme activity. Lysozyme is mainly derived from hemolymph or hemocytes, and the test was performed by turbidimetric assay to calculate the lysozyme activity based on the transmittance. A substrate suspension was first prepared using 0.1% mol/L (pH 6.4) potassium phosphate buffer (PBS) [21]. The absorbance value at 570 nm was measured as A0, and then the solution was quickly transferred to a 37°C water bath for 30 min, and then immediately placed in an ice bath for 10 min to stop the reaction, and the A value was measured. The lysis viability was (A0-A)/A [22].

1.5.2. Determination of nitric oxide synthase (NOS) activity. Nitric oxide synthase (ratio) activity is defined as: 1mg of histone reacts with the substrate at 37°C, and 1nmol of nitric oxide is generated for 1min as 1 activity unit. The principle is that NOS can catalyze the reaction between L-arginine and oxygen to produce NO, and NOS and nucleophilic substances to produce colored compounds. The absorbance value was measured at 530 nm and the viability of NOS was calculated.

1.5.3. Malondialdehyde (MDA) content determination. Through the condensation of MDA with TBA, a red substance can be formed and has a maximum absorption peak at 532 nm, and the content of MDA can be calculated according to its absorbance value.

1.5.4. Determination of superoxide dismutase (SOD) activity. Follow the assay kit in detail [23]. This assay is defined as 50% inhibition of NBT photoreduction expressed as 1 viability unit (U) [24]. About 0.1g of shrimp hepatopancreas was taken, put into 2mL of 0.9% NaCl pre-chilled at 4°C, mixed at 2500r/min in an ice-water bath, then centrifuged at 4000r/min at 4°C for 10min to remove the supernatant, and the OD value was measured at a wavelength of 550nm in accordance with the method of the SOD kit instructions.

1.5.5. Catalase (CAT) activity determination. CAT viability was calculated as follows: the decomposition of H₂O₂ by CAT was stopped rapidly by the addition of ammonium molybdate, after which a yellowish complex was generated and the amount produced was measured at 405 nm to find the result. The specific activity of hydrogen peroxide is defined as follows: at 37°C, the activity of one CAT unit is equal to the amount of 1 μmol H₂O₂ decomposed by 1 mg of histone per second.

1.5.6. Determination of glutathione peroxidase (GSH-PX) activity. The GSH-PX (specific) activity is usually defined as follows: 1mg of histone protein at 37°C is defined as one unit of enzyme activity per 1 min of non-enzymatic reaction by deducting 1 μmol/L of GSH from the reaction system. GSH-PX can promote the reaction between hydrogen peroxide and GSH to produce water and GSSG, and the speed of the enzymatic reaction is used to express the size of GSH-PX activity.

2. Results and Analysis

2.1. Effect of different concentrations of zeaxanthin on lysozyme activity

The lysozyme activity tended to decrease as the concentration of zeaxanthin addition increased. The lysozyme activity of XP2 group was not significantly different from that of JC1 group, while that of XP3 and XP4 groups was significantly lower than that of JC1 group, and there was no significant difference in lysozyme activity among XP2, XP3 and XP4 groups.

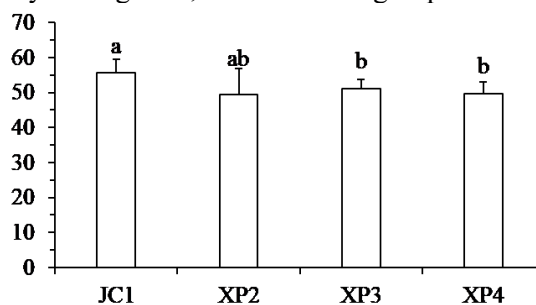


Figure 2. lysozyme activity (U/mg protein)

2.2. Effect of different concentrations of zeaxanthin on nitric oxide synthase (NOS) activity

NOS activity tended to increase with increasing zeaxanthin addition concentration, and was significantly higher in the XP2 group than in the JC1 group and substantially lower than in the XP3 and XP4 groups, while there was no significant difference in activity between the XP3 and XP4 groups.

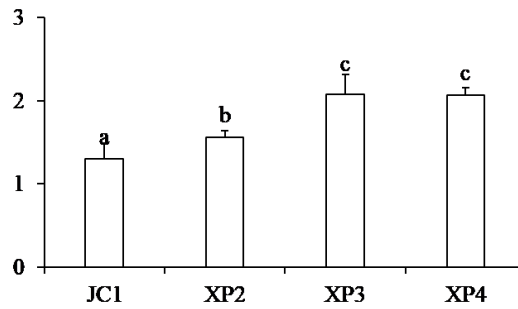


Figure 3. NOS activity (U/mg protein)

2.3. Effect of different concentrations of zeaxanthin on malondialdehyde (MDA) content

There was no significant change in malondialdehyde activity after the addition of zeaxanthin to the feed, with a slight increase in the XP2 group. As the concentration gradient of zeaxanthin increased step by step, malondialdehyde activity showed a low-promoted and high-suppressed performance.

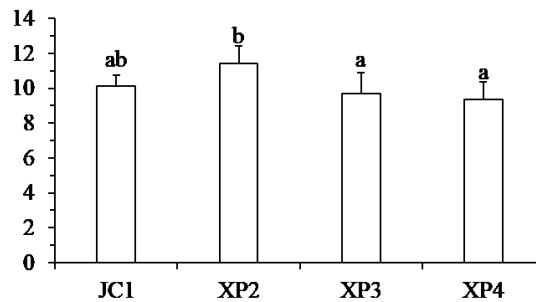


Figure 4. MDA activity (nmol/mg protein)

2.4. Effect of different concentrations of zeaxanthin on superoxide dismutase (SOD) activity

The SOD activity of all three experimental groups was not significantly different from the control group. Therefore, the addition of a certain amount of zeaxanthin to the feed did not have a significant effect on the SOD activity of South American white shrimp.

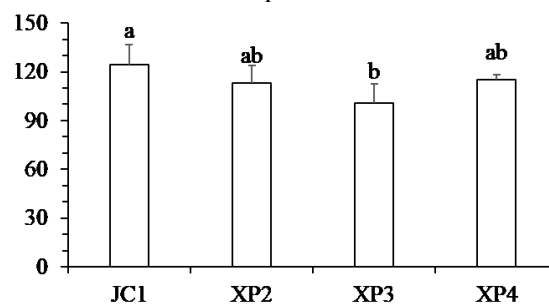


Figure 5. SOD activity (U/mg protein)

2.5. Effect of different concentrations of zeaxanthin on catalase (CAT) activity

CAT activity was increased after the addition of zeaxanthin to the feed, and the activity values in the JC1 group were significantly lower than those in the XP2, XP3 and XP4 groups, while there was no significant difference between the latter three groups. Therefore, the addition of zeaxanthin to the feed significantly enhanced the CAT activity.

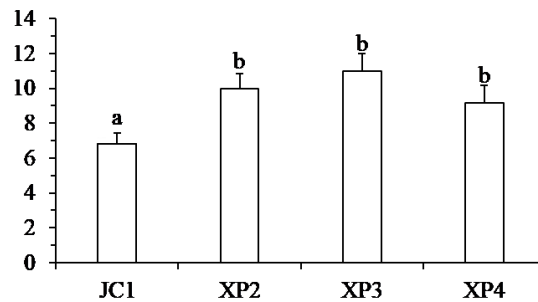


Figure 6. CAT activity (U/mg protein)

2.6. *Effect of different concentrations of zeaxanthin on glutathione peroxidase (GSH-PX) activity*
 There was no significant difference ($P < 0.05$) between the experimental groups compared to the control group overall after the addition of zeaxanthin to the feed.

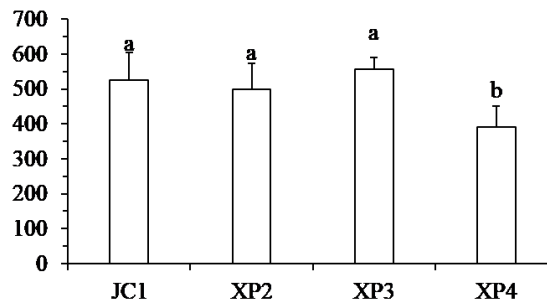


Figure 7. GSH-PX activity (U/mg protein)

3. Discussion and Conclusion

The South American white shrimp belongs to the phylum Arthropoda, and this classification determines that its circulatory system is open, and this open circulatory system lags behind that of higher animals. This lower circulatory system, in turn, determines that its immune system is also lower. South American white shrimp does not have the function of specific immunity, so it can not produce specific antibodies to specific antigens. In this case the shrimp's own disease resistance mechanism relies mainly on non-specific immune mechanisms to eliminate invading pathogenic microorganisms. The immune system of shrimp has two main components, one is the physical barrier protection: the outer shell of its body to ward off external pathogenic microorganisms, while once the pathogen invades the body, it relies on its internal defense system to eliminate the pathogen. The internal defense system is mainly realized through the phagocytosis of blood cells and some humoral immune factors such as superoxide dismutase and lysozyme. Lysozyme can hydrolyze the acetylamino glycan of the bacterial cell wall and change the osmotic pressure so that the bacteria rupture and die, which can be used as an indicator of immunity in South American white shrimp; NOS can be divided into calcium-dependent and calcium-non-dependent, and its immune principle is to fight pathogens by using oxidative stress (free radicals) of nitric oxide; superoxide dismutase can play a role in restoring damaged cells and recovering from cellular damage caused by free radicals. In addition, zeaxanthin has multiple conjugated double bonds in its molecular structure, which can scavenge free radicals in animals and enhance SOD activity, so as to quickly and effectively scavenge free radicals in the body, protect proteins, lipids and nucleic acids and other important substances that make up the cells, and improve the immunity of the body through this pathway. And the GSH-PX enzyme system mainly includes four different kinds of GSH-PX, of which selenium is the main component of the GSH-Px enzyme system, which can reduce toxic peroxides to non-toxic hydroxyl compounds, so as to protect the structure and function of the cell membrane. Catalase is both an oxidizing and reducing agent, and it belongs to hemoglobinase, which can catalyze the generation of water and molecular oxygen from hydrogen peroxide, thus acting as a transferred electron.

When the nutrients in the feed can be utilized in different ways and forms, thus causing the organism to exhibit different degrees of health status, the results of this experiment showed that zeaxanthin had a significant effect ($P < 0.05$) on the activity of lysozyme, CAT and NOS, and that the lysozyme activity gradually decreased, NOS activity gradually increased and CAT activity increased during the addition of the concentration of zeaxanthin from 0 to 1%. Secondly, it was found that there was no significant difference in the activities of MDA, SOD and glutathione peroxidase, and some substantial patterns were not concluded. In conclusion, the addition of different concentrations of zeaxanthin to the feed can improve the non-specific immune performance of South American white shrimp to varying degrees, thus improving its growth performance. Zeaxanthin at 0.5% had the highest effect on nitric oxide synthase and CAT activity, and feeding zeaxanthin at this concentration could be beneficial to the non-specific immune performance of the shrimp.

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