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Short communication

The growth performance and nonspecific immunity of red swamp crayfish *Procambarus clarkia* affected by dietary *Rhodiola rosea* polysaccharide

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ABSTRACT

This study aims to investigate the effects of *Rhodiola rosea* polysaccharide (RRP) on the growth performance and nonspecific immunity of red swamp crayfish *Procambarus clarkia*. RRP was prepared by hot water extraction and partly characterised by high-performance liquid chromatography and sugar composition analyses. Three diets supplemented with three different levels of RRP (0.2, 0.6 and 1 g kg diet^{-1}) were formulated and tested for growth performance and nonspecific immunity of red swamp crayfish *Procambarus clarkii,* while a diet without any RRP supplementation served as control. After 8 weeks of feeding, body weight gain, feed efficiency, survival rate, phenoloxidase activity, superoxide dismutase activity, glutathione peroxidase level, total haemocyte count and number of hyaline cells, semigranular cells and granular cells and resistance to *Aeromonas hydrophila* were higher than those of the control. Moreover, based on the efficiency of RRP on the growth performance and nonspecific immunity of crayfish, the optimum dose of RRP was found to be 0.6 g kg diet^{-1}. Hence, intake of diets containing RRP could enhance the growth performance, immune responses and improve resistance of crayfish to infection by *A. hydrophila.*

1. Introduction

Red swamp crayfish *Procambarus clarkii* belongs to arthropods and crustaceans and is the most widely cultivated species of freshwater crayfish worldwide, particularly in the middle and lower reaches of the Yangtze River in China [1]. The meat of crayfish is very delicious, nutritious and favoured by consumers. However, the expansion of crayfish farming and production has been limited by frequent outbreaks and spread of crayfish diseases mainly of viral and bacterial aetiologies [2]. In particular, *Aeromonas* species are common pathogens of vibriosis in crayfish farming and are a serious threat to aquaculture industry [3]. In recent years, the outbreak of white spot syndrome and other diseases in crayfish culture has brought huge losses to the farmers. Considering the negative effects of long-term use of antibiotics on human consumer health and the environment, native compounds with safety and efficiency must be developed to resolve these challenges in aquatic animals. Recently, native compounds, e.g. galactooligosaccharide [4], fulvic acid [5], chitosan [6], and fructooligosaccharides [7], have been used to resolve the challenges in crayfish.

Rhodiola rosea is a perennial herb that grows mainly under hillside forests or where grassy slopes are cold and non-polluting. This herb has a high environmental adaptability and is known as “plateau ginseng”. *R. rosea* is a rare medicinal plant and its dried rhizome can be used as a medicine in China. Chinese medicine believes that *R. rosea* can tonify the qi, clears the lungs, cultivate the heart, collect blood stasis and dissipate swelling. *R. rosea* polysaccharide (RRP) has many biological activities such as antioxidant [8], hepatoprotective [8], hypoglycaemic [9], hypolipidaemic [9] and antitumor [10] activities, sperm protection [11] and immunomodulatory activity [12]. Dietary *R.* rosea was reported to modulate the growth, body composition and antioxidant capacity of white shrimp *Litopenaeus vannamei* under normal conditions and combined stress of low salinity and nitrite [13]. However, data regarding the effects of RRP on the growth performance and immunity of crayfish are limited.

This work aims to investigate the effect of dietary RRP supplementation on the growth performance and nonspecific immunity of crayfish.

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2. Materials and methods

2.1. Ethics statement

This study was approved by the ethics committee of Jiangsu Ocean University, China. All procedures were conducted in compliance with relevant laws and institutional guidelines.

2.2. Materials

Dried roots of *R. rosea* were purchased from Gansu Hall Nutritional Health Food Co., Ltd. (Gansu, China). Standard monosaccharides such as glucose, fructose, galactose, rhamnose, xylose, arabinose and mannose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The microelisa stripplate provided in the kit was precoated with an antibody specific to the target enzyme. All other chemicals were of reagent grade.

2.3. RRP preparation

Dried roots of *R. rosea* (100 g) were pulverised and passed through an 80-mesh sieve to yield a fine powder. The powder was soaked in distilled water under agitation at room temperature (~20 °C) and 180 rpm for 30 min to obtain a suspension with a concentration of ~3% (w/v). The suspension was incubated in a water bath at 80 °C for 8 h, cooled to ~20 °C and then centrifuged at 5000 × *g* and ~20 °C for 15 min. The resulting supernatant was deproteinized using Sevag method [14], concentrated (~15%) using a vacuum rotary evaporator (RE-2010, Shanghai Qiang Qiang Industrial Development Co., Ltd., Shanghai, China) and precipitated with three volumes of absolute ethanol [9]. The precipitate was filtered through a Whatman GF/A filter paper and freeze dried. The RRP sample was redissolved in distilled water, loaded on a DEAE-52 cellulose anion-exchange chromatography column (30 cm × 2.6 cm, GE Healthcare, UK) and eluted successively with 0.05 mol L^{-1} phosphate-buffered saline and gradient solution of 0.05–1.0 mol L^{-1} NaCl at a flow rate of 1.0 mL min^{-1}. The eluted polysaccharide fractions were collected for subsequent experiments.

2.4. RRP characterisation

Proximate components, such as total sugar, protein and ash, of the products were assayed using phenol–sulphuric acid colorimetric method [15], Kjeldahl method [15] and the method described by the Association of Official Analytical Chemists [15]. The molecular weight (MW) of RRP was determined through high-performance liquid chromatography (HPLC) analysis (LC-10A, Shimadzu, Japan) by using an ultrahydrogel size exclusion column (LKB-Produkter AB, Bromma, Switzerland) and highly sensitive refractive index detector (Model ERC-7515 A, ERC Inc., Japan). The RRP was eluted with 0.1 N $NaNO_3$ at a flow rate of 0.9 mL min^{-1}. Standard pullulan samples (P20–P800, JM Science, Inc., NY, USA) were used as MW standard. Monosaccharide composition of RRP was analysed according to the methods described by Sheng et al. [16].

2.5. Diet preparation

RRP was directly added to a basal diet at four doses (0, 0.2, 0.6 and 1 g kg $diet^{-1}$). The composition of the basal diet is provided in Table 1. The experimental diets were fully mixed, coldly extruded, cut into pellets (2 mm), dried at 50 °C and stored at −20 °C until use.

2.6. Crayfish culture

The crayfishes with an average body weight of 10.31 g ± 1.9 were purchased from Freshwater Fisheries Research Institute of Jiangsu Province and then acclimated to the experimental conditions and fed with commercial diet for 2 weeks before feeding experiments.

Table 1
Composition of the red swamp crayfish diets (g kg $diet^{-1}$) supplemented with *Rhodiola rosea* polysaccharide (RRP).

Ingredient	RRP diets (g kg $diet^{-1}$)			
	Control	0.2	0.6	1
Fish meal	430	430	430	430
Soybean meal	65	65	65	65
Yeast meal	25	25	25	25
crayfish shell meal	70	70	70	70
Wheat flour	345	345	345	345
Cellulose	6	5.8	5.4	5
RRP	0	0.2	0.6	1
Gluten	25	25	25	25
Fish oil	8	8	8	8
Mineral mixture[a]	20	20	20	20
Vitamin mixture[b]	6	6	6	6
Proximate composition				
Protein	291	291	291	291
Lipid	69	69	69	69
Ash	72	72	72	72
Energy (MJ kg^{-1})	182	182	182	182

[a] Vitamin mixture (g kg $diet^{-1}$): β-Carotene, 3 M.I.U.; Cholecalciferol, 0.6 M.I.U.; Thiamin, 3.6; Riboflavin, 7.2; Pyridoxine, 6.6; Cyanocobalamine, 0.02; α-Tocopherol, 16.5; Menadione, 2.4; Niacin, 14.4; Pantothenic acid, 4; Biotin, 0.02; Folic acid, 1.2; Inositol, 30; Ascorbic acid, 100.

[b] Mineral premix (g kg $diet^{-1}$): P, 120; Ca, 120; Mg, 15; Fe, 1.5; Zn, 4.2; Cu, 2.1; K, 75; Co, 0.11; Mn, 1.6; Se, 0.01; Mo, 0.005; Al, 0.025; I, 0.4.

At the beginning of the feeding trial, a total of 480 crayfishes were randomly assigned to 12 tanks, resulting in 3 tanks per group and 40 crayfishes per tank. The crayfishes were individually fed with control diet (without RRP) and RRP-containing diets at 0.2, 0.6 and 1 g kg $diet^{-1}$ dosages. The crayfishes were placed in a 1 ton circular fiber glass-reinforced plastic tank with 0.8 tons of freshwater. Twenty crayfishes were subjected to susceptibility test, and the remaining 20 crayfishes were subjected to immune response assay.

Feeding ration was initially set to 0.5 g crayfish $^{-1}$ twice daily at 08:00 and 18:00. On the following day, the unused feed was sucked out with a siphon. The culture conditions were maintained as follows: water temperature, 25 ± 1 °C; pH, 7.4–7.9; total hardness of water, 100–250 mg L^{-1} (calculated as $CaCO_3$); and dissolved oxygen concentration, 7.1–8.7 mg L^{-1}. The culture water was daily renewed with a volume of 30% fresh water. The experiments were performed in triplicate.

2.7. Growth and survival rate

The crayfishes from each tank were weighed at the end of the 8-week feeding trial. Growth was measured by the body weight gain of surviving crayfishes in each tank and calculated as (final body weight − initial body weight). Feed efficiency (FE) was calculated as [(final body weight − initial body weight)/feed intake].

2.8. Nonspecific immune response assays

Ten crayfish was blotted up after the final weighing. Haemolymph was withdrawn from the ventral sinus of each crayfish with a 1 mL syringe fitted with a 25-gauge needle, which was rinsed with 50 μL of precooled (4 °C) 10% sodium citrate as anticoagulant. The following immunologic parameters were used to evaluate the stimulating effect of RRP: phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, total haemocyte count (THC), number of hyaline cells (HC), semigranular cells (SC) and granular cells (GC).

PO activity was measured using the methods described by Huang et al. [17]. In brief, 200 μL of the anticoagulant (4 °C) and 200 μL of

crayfish haemolymph were added to a 2 mL syringe with 2.5 G needle and mixed. The solution was centrifuged at 800 × *g* and 4 °C for 10 min. The resulting plasma (20 mL) was strewed in a spectrophotometer cuvette and used as unknown sample. The anticoagulant (20 mL) was strewed in another cuvette and used as control. After 1 min, 880 μL L-DOPA solutions were added to the two cuvettes. Absorption was recorded at 490 nm every 10 s, totally for 120 s. One unit of enzyme activity was defined as a linear increase in absorption of 0.001 per min per mL haemolymph.

SOD activity in the supernatant of haemolymph samples was determined according to the method described by Hao et al. [18] by using an assay kit (Nanjing Jiancheng Bioengineering Institute, China). The serum (100 μL) from each replicate was used. The optical density of SOD at 550 nm was recorded on a spectrophotometer (752, Shanghai Jinghua Instrument, Shanghai, China). One unit of SOD activity was defined as the amount of enzyme required to inhibit superoxide-induced oxidation by 50%.

GPx activity in the supernatant of haemolymph samples was assayed following the method of Subramanian et al. [19]. GPx activity of the haemolymph samples was determined spectrophotometrically at 412 nm (Libra S22 UV/Visible, Biochrom, England). Anti-oxidative enzyme detection kits were purchased from Randox Laboratories Limited (Country Antrim, UK). One unit of GPx activity was defined as the amount of enzyme that reduced glutathione concentration in the reaction system at 1 mmol L^{-1} min^{-1}.

THC, HC, SGC and GC were assayed using a haemocytometer (Neubauer chamber) and a phase-contrast microscope (40× magnification) in accordance with the methods described by Gullian et al. [20].

2.9. Susceptibility of crayfish to Aeromonas hydrophila

A. hydrophila was purchased from Shanghai Ocean University, China. Stocks were cultured on tryptic soy agar medium (supplemented with 0.5% NaCl; Difco, Detroit, MI, USA) for 24 h at 28 °C and transferred to 10 mL of tryptic soy broth (supplemented with 0.5% NaCl; Difco, Detroit, MI, USA) at 28 °C for 24 h. The cultured broth was centrifuged at 10,000 × *g* and 4 °C for 10 min. The pellets were re-suspended in saline solution (0.5% NaCl) at 10^7 CFU for susceptibility test. At the end of the 8-week feeding trial, crayfishes from the four groups were counted, weighed and used for susceptibility assay to *A. hydrophila*. Each treatment consisted of 20 crayfishes. Challenge test was carried out by injection of each 20 μL of bacterial suspension containing 2×10^5 CFU bacteria to each crayfish into the ventral sinus of the cephalothorax for the four challenge groups. Experimental crayfishes were kept in 80 L glass aquaria containing 60 L of water at 28 °C. The water was renewed daily, and the experiment lasted for 8 days. Challenge test was conducted in triplicate.

2.10. Statistical analysis

Data were reported as means ± standard deviation and checked for the normality and homogeneity of variance. The variance and significant differences among the means were tested through one-way ANOVA by using SPSS software (version 17.0 for Windows, SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was used to compare means at the $P < 0.05$ level of significance. Percentage data were arcsine-transformed before analysis.

3. Results and discussion

3.1. Product characterisation

The total carbohydrate and protein contents in the RRP were 92.37% and 4.68%, respectively. Analysis of the elution curve indicated that the RRP consisted of one polysaccharide (Fig. 1). HPLC analysis showed the MW of RRP was 11.82 kDa (Fig. 2). AMP consisted of rhamnose, arabinose, xylose, mannose, glucose, galactose and galacturonic acid with a ratio of 1:2.71:0.16:0.21:0.11:0.58:0.14, which are similar to previously reported values [11].

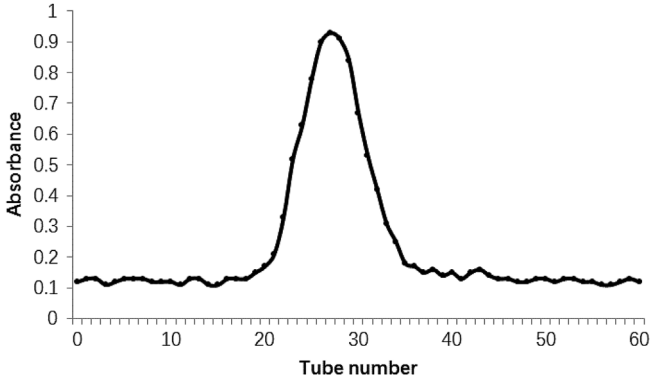

Fig. 1. Elution curve of *Rhodiola rosea* polysaccharide on a DEAE Sepharose Fast Flow ion exchange chromatography column.

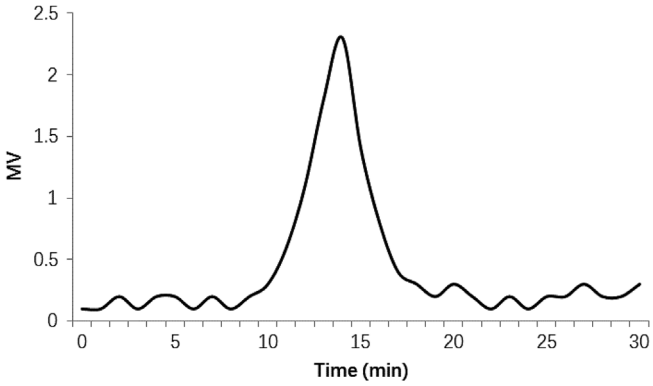

Fig. 2. HPLC spectrum of *Rhodiola rosea* polysaccharide.

Table 2
Body weight gain, feed efficiency (FE), and survival rate of red swamp crayfish, after 8 weeks of feeding the experimental diets.

RRP (g $kg\ diet^{-1}$)	Body weight gain (g)	FE (%)	Survival rate (%)
Control (0)	2.14 ± 0.49[a]	0.30 ± 0.03[a]	90.5 ± 3.2[a]
0.2	3.22 ± 0.69[b]	0.41 ± 0.04[b]	95.7 ± 3.5[b]
0.6	4.09 ± 0.80[c]	0.60 ± 0.05[b]	100 ± 0[b]
1	4.13 ± 0.71[c]	0.59 ± 0.05[b]	100 ± 0[b]

The different superscript letters indicate significant differences for each column ($p < 0.05$). Values are the mean ± SD (n = 3).

Table 3
Phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, and glutathione peroxidase (GPx) after 8 weeks of feeding the experimental diets.

RRP levels (g $kg\ diet^{-1}$)	PO activity (O.D. 490 nm)	SOD activity (U mg $protein^{-1}$)	GPx (U g $protein^{-1}$)
Control (0)	0.36 ± 0.03[a]	35.78 ± 2.03[a]	91.00 ± 13.51[a]
0.2	0.52 ± 0.04[b]	48.12 ± 3.36[b]	122.91 ± 17.89[b]
0.6	0.77 ± 0.06[c]	53.47 ± 3.49[c]	160.11 ± 21.23[c]
1	0.75 ± 0.07[c]	53.22 ± 3.61[c]	158.98 ± 22.06[c]

The different superscript letters indicate significant differences for each column ($p < 0.05$). Values are the mean ± SD (n = 3).

3.2. Growth and survival rate

Although native compounds, e.g. galactooligosaccharide [4], fulvic

Table 4
Total haemocyte count (THC), hyaline cells (HC), semigranular cells (SGC) and granular cells (GC) of crayfish, fed with *Rhodiola rosea* polysaccharide (RRP) diets, after 8 weeks.

RRP levels (g kg diet^{-1})	THC ($\times 10^4$ mL^{-1})	HC ($\times 10^4$ mL^{-1})	SGC ($\times 10^4$ mL^{-1})	GC ($\times 10^4$ mL^{-1})
Control (0)	3.51 ± 0.21^a	1.62 ± 0.11^a	1.22 ± 0.11^a	0.46 ± 0.04^a
0.2	4.42 ± 0.24^b	2.40 ± 0.25^b	1.45 ± 0.16^b	0.70 ± 0.06^b
0.6	6.96 ± 0.39^c	4.11 ± 0.29^c	2.58 ± 0.33^c	1.01 ± 0.09^c
1	7.04 ± 0.41^c	4.14 ± 0.31^c	2.59 ± 0.37^c	1.02 ± 0.11^c

The different superscript letters indicate significant differences for each column ($p < 0.05$). Values are the mean ± SD (n = 3).

Table 5
Survival rate (%) of red swamp crayfishes fed with *Rhodiola rosea* polysaccharide (RRP) diets, after being challenged by pathogen, *Aeromonas hydrophila*.

RRP levels (g kg diet^{-1})	Time after challenge (d)		
	2	4	8
Control (0)	84.4 ± 4.2^a	55.8 ± 3.3^a	42.8 ± 1.9^a
0.2	100 ± 0^a	92.7 ± 4.4^b	84.7 ± 3.5^b
0.6	100 ± 0^a	94.1 ± 4.9^b	85.6 ± 3.9^b
1	100 ± 0^a	93.7 ± 4.6^b	84.4 ± 4.3^b

Different superscript letters indicate significant differences for each column ($p < 0.05$). Values are the mean ± SD (n = 3).

acid [5], chitosan [6], and fructooligosaccharides [7], have been used to improve the growth performance of crayfish, the effects of *R. rosea* polysaccharide (RRP) on the growth performance and immunity of crayfish are not frequently reported. At the end of the 8-week feeding trials, the body weight gain, FE and survival rate of crayfishes were higher in crayfishes fed with RRP-containing diets than in the control group (Table 2, $p < 0.05$). However, a high level of RRP dose (1 g kg diet^{-1}) did not further increase body weight gain, FE and survival rate of crayfishes compared with those of the moderate group (0.6 g kg diet^{-1}). RRP has immunomodulatory activity and reduced the bacterial and viral infections, thus simulating the growth performance of crayfishes [12]. However, the high levels of RRP presumably exhibited mainly hypolipidemic activity due to its oil absorption effect in the intestine [9].

3.3. Immune parameters of crayfishes fed with RRP-containing diets

Evaluation of disease resistance is necessary in the aquaculture industry. Previous studies verified that RRP exhibits immunoregulatory activity [12]. Table 3 shows the changes in PO, SOD and GPx activities of crayfishes fed with RRP-containing and control diets for 8 weeks. The crayfishes fed with RRP-containing diets exhibited higher PO, SOD and GPx activities compared with the control group ($p < 0.05$); this could be due to the antioxidant activity of RRP [8]. The increases in PO, SOD and GPx activities indicated that the nonspecific immunity of crayfish was simulated by dietary RRP. Similarly, the PO, SOD and GPx activities of white shrimp *Litopenaeus vannamei* increased when fed with diets supplemented with RRP [13].

The haemocytes of crayfishes are responsible for clotting, exoskeleton hardening and elimination of foreign materials. After eight weeks, THC, HC, SC and GC in crayfishes fed with RRP diets were higher than those in the control group ($p < 0.05$, Table 4); this could be due to the antioxidant and immunomodulatory activities of RRP [8,12]. The increases in THC, HC, SC and GC indicated that the disease resistance of crayfishes increased by dietary RRP. However, there were no significant differences in THC, HC, SGC and GC between crayfishes fed with 0.6 and 1 g kg diet^{-1} RRP-containing diets. Similarly, the THC, HC, SGC and GC of red swamp crayfish *Procambarus clarkia* increased when fed with diets supplemented with fulvic acid [5].

3.4. Effect of RRP-containing diets on survival rate of crayfishes upon challenge with A. hydrophila

For the control group, crayfish mortality was detected 2 days after injection of *A. hydrophila,* while for crayfishes fed with RRP-containing diets, mortality was detected 4 days after injection of *A. hydrophila*. After the challenge test against *A. hydrophila*, the survival rate of crayfishes fed with RRP-containing diets at all doses was significantly higher than those of the control group ($p < 0.05$, Table 5). As mentioned above, dietary RRP increased THC, HC, SC and GC, which subsequently reduced *A. hydrophila* infection, thus increased the survival rate of crayfishes. This result could also be due to the antioxidant and immunomodulatory activities of RRP [8,12].

4. Conclusions

RRP supplements increased the body weight gain, FE, survival rate, PO activity, SOD activity, GPx activity, THC, HC, SC, GC and disease resistance against *A. hydrophila* of red swamp crayfish *Procambarus clarkia*. Based on the efficiency of RRP on the growth performance and nonspecific immunity of crayfish, the optimum dose of RRP was found to be 0.6 g kg diet^{-1}. Therefore, RRP may be used as an immunostimulant for enhancement of red swamp crayfish *Procambarus clarkia* growth and immunity.

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