Fish and Shellfish Immunology 87 (2019) 615–619

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

The growth performance and nonspecific immunity of juvenile grass carp (*Ctenopharyngodon idella*) affected by dietary *Porphyra yezoensis* polysaccharide supplementation

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ARTICLE INFO

Keywords:
Porphyra yezoensis polysaccharides
Grass carp
Growth performance
Immunity

ABSTRACT

This study aims to investigate the effects of dietary *Porphyra yezoensis* polysaccharides (PPs) on growth performance and nonspecific immunity of juvenile grass carp (*Ctenopharyngodon idella*). Four hundreds of juvenile grass carps were randomly divided into four groups, i.e. one control group received basal diet and three treatment groups received diets supplemented with three different levels of PPs (1, 3, and 5 g kg^{-1}) and tested for growth performance, survival percent, intestine digestive-enzyme activities, superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione peroxidase (GPx) activity, serum total protein, lysozyme, alkaline phosphatase, complement C3 and complement C4 and resistance to *Aeromonas hydrophila* of juvenile grass carp. After 60 days of feeding, the diet supplemented with an appropriate dose of PPs significantly increased growth performance, survival percent, intestine digestive-enzyme activities, SOD activity, CAT activity, GPx activity, serum lysozyme, alkaline phosphatase, complement C3, and complement C4 and resistance to *Aeromonas hydrophila* of juvenile grass carp compared with the control group and the optimum dose of PPs was found to be 3 g kg^{-1}. Results showed that dietary PPs can improve growth performance and nonspecific immunity of juvenile grass carps and can thus be used as a diet supplement for them.

1. Introduction

Porphyra yezoensis, one of the economically important seaweeds, is rich in nutrition; it contains 25–40% of carbohydrates and 25–50% of protein based on dry matter basis [1]. *P. yezoensis* prevents atherosclerosis, hypertension and coronary heart disease. *P. yezoensis* polysaccharides (PPs) is a homogeneous polysaccharide composed of precursor [(1 → 3)-β-D-galactose-(1 → 4)-6-OSO_3-α-L-galactose] and repetitive units of agarobiose [(1 → 3)-β-D-galactose-(1 → 4)-3,6-inner ether-2-OCH_3-α-L-galactose] and [(1 → 3)-β-D-galactose- (1 → 4)-α-L-galactose] in appropriate ratio of 9: 9: 1 [2]. PPs reportedly have antioxidant activity [3], hypolipidemic activity [4], immunomodulatory activity [5], therapeutic effects on rats with cerebral ischemia/reperfusion injury [6], anti-cancer activity [7,8] and protective effect against carbon tetrachloride induced hepatotoxicity in mice [9].

Grass carp (*Ctenopharyngodon idellus*) is one of the most important freshwater species cultured in China due to its rapid growth, high yield and low price [10]. Recently, famers have suffered high economic losses due to the rapid development of intensified farming model and increased bacterial diseases. Farmers have been compelled to use antibiotics to control traditional disease for decades in aquaculture. Nevertheless, long-term use of the antibiotics resulted in adverse effects on the health of human consumers and environment pollution [11]. Therefore, safety native compounds with efficiency must be developed to resolve these challenges in aquaculture.

In recent years, many native compounds, e.g. reduced glutathione [10], microencapsulated organic acids [11], geniposidic acid [12], yeast culture [13], bile acids [14], phytic acid [15], Eucommia ulmoides [16], geniposide [17], sodium butyrate [18], silymarin [19], gamma aminobutyric acid [20], choline [21], vitamin A [22], phenylalanine [23] and arginine [24], were developed to improve growth performance of grass carp. In addition, red alga *Porphyra yezoensis* was reported to stimulate growth of Strongylocentrotus intermedius (Echinoidea: Echinodermata) and Haliotis discus hannai (Gastropoda:

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https://doi.org/10.1016/j.fsi.2019.02.013
Received 20 November 2018; Received in revised form 6 February 2019; Accepted 8 February 2019
Available online 10 February 2019

Mollusca) juveniles [25]. However, the effects of PPs on the growth performance and nonspecific immunity of grass carp remain to be clarified.

In this study, the dietary effects of PPs on the growth performance, intestine digestive-enzyme activities and nonspecific immunity of grass carp were investigated. Accordingly, we evaluated the growth performance, survival percent, intestine digestive-enzyme activities, superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione peroxidase (GPx) activity, serum malondialdehyde (MDA) level, serum total protein, lysozyme, alkaline phosphatase, complement C3 and complement C4 and resistance to *Aeromonas hydrophila* of juvenile grass carp.

2. Materials and methods

2.1. Ethics statement

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

2.2. Materials

Dried *P. yezoensis* were purchased from a local farmers market (Xinpu, China), and the PPs were prepared according to the method described by Zhou et al. [3]. The ash, moisture, protein and total sugar contents in PP product were 1.26%, 2.27%, 3.15% and 92.64%, respectively. All other chemicals used were of reagent grade.

2.3. Diet preparation

PPs were added to a basal diet at four concentrations (0, 1, 3 and 5 g kg^{-1} dry diets). The composition of the basal diet is presented in Table 1. The experimental diets were extruded, cut into pellets (1 mm), dried at 60 °C, and then stored at −18 °C until use.

2.4. Grass carp culture

The grass carps with an average body weight of 7.03 ± 0.24 g were purchased from a commercial farm in Lianyungang, China, transferred and acclimated for two weeks in a semi-intensive culture pond. During the acclimation period, the grass carps were fed with a basal diet twice daily (08:00 and 18:30).

Table 1
Composition of basal diets of grass carp.

Ingredient	Content (g/kg)
Fish meal	50
Soybean meal	250
Rapeseed meal	225
Cottonseed meal	100
Rice bran	50
Wheat flour	268
Soybean oil	20
Choline chloride (50%)	2
Zeolite powder	5
$Ca(H_2PO_4)_2$	18
NaCl	2
Vitamin mixture	5
Mineral	5
Proximate analysis (% dry matters)	
Crude protein	31.73
Crude lipid	4.91
Carbohydrate	54.31
Ash	7.58
Gross energy (kJ g^{-1})	16.79

The ingredients were purchased from Jiangsu Tian Fu Lai Group Co., Ltd., Jiangsu, China.

At the beginning of the feeding trial, 480 grass carps were randomly distributed into 12 cylindrical fiberglass tanks (500-L), and each tank was stocked with 40 grass carps. Each diet was randomly assigned to triplicate tanks.

Each tank was supplied with a continuous water flow (1.5 L/min) and aeration via air-stones to maintain enough dissolved oxygen (DO). The grass carps were hand-fed with the test diets to apparent satiation twice (8:00 and 16:30) daily for 60 days. The culture conditions were maintained as follows: water temperature, 26 ± 1 °C; pH, 7.5 ± 0.2; dissolved oxygen, 6.0 mg L^{-1}; total ammonia nitrogen < 0.05 mg L^{-1}; and nitrite < 0.01 mg L^{-1}.

2.5. Growth performance

At the end of the 60-day feeding experiment, the grass carps in each tank were weighed to calculate the feed conversion ratio (FCR), the specific growth rate (SGR), the condition factor (CF) and the survival percent by using the following equations:

FCR (%) = 100 × total dry feed consumption/net weight gain;
SGR (% day^{-1}) = 100 × [Ln (final mean body weight) - Ln (initial mean body weight)]/time (days);
CF (%) = final body weight/body length;
Survival percent (%) = 100 × (final number of loach/initial number of loach).

2.6. Sample

At the end of the 60-day feeding trial, the grass carps in each tank were bulk-weighed after being starved for 24 h. Six grass carps from each tank were sacrificed using MS222 (Sigma-Aldrich) and the blood samples were collected by caudal venipuncture with 1-mL medical syringe, allowed clotting at 4 °C for 1–2 h, and centrifuged at 3000 × *g* and 4 °C for 10 min, and the serum was collected and frozen at −80 °C until use. The peritoneal cavity was aseptically opened using a sterile scalpel, and the part of the intestine between the pyloric caeca and approximately 1 cm anterior to the anus was excised. The intestinal wall was washed with sterile saline, and the faeces with mucus were stripped off using sterile forceps. The intestines of the grass carps were homogenised in 10 vol (v/w) of ice-cold phosphate buffered saline and centrifuged at 3000 × *g* at 4 °C for 20 min. The resulting supernatant was stored in a refrigerator at −20 °C and used to analyse intestine digestive enzymes activities.

2.7. Biochemical assays

The biochemical parameters, i.e. protease activity, amylase activity, lipase activity and serum superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione peroxidase (GPx) activity, total protein, lysozyme, alkaline phosphatase, complement C3, and complement C4, were analysed using ELISA kits by following the methods by Gao et al. [26]. Briefly, in the appropriate microelisa stripplate wells, standards or samples were added and combined to the specific antibody, followed by a horseradish peroxidase (HRP)-conjugated antibody specific for the target enzyme. The free components were washed away. Tetramethylbenzidine (TMB) substrate solution was added to each well. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The OD was proportional to the activity of the target enzyme, which was calculated by comparing the OD of the samples to the standard curve. A unit of enzyme activity was defined as the amount of enzyme that decreased the absorbance by 0.001 min^{-1}. All assays were performed in triplicates.

2.8. Resistance of the grass carps to Aeromonas hydrophila

At the end of the 60-day feeding trial, 12 grass carps were randomly selected from each of the four groups and raised in tanks. After an acclimation period of five days, the grass carps were subjected to intraperitoneal injection of 1.4×10^5 cfu/g fish body weight of *A. hydrophila* based on our previous results. The grass carps of control group received intraperitoneal injection of an equal volume of saline. The challenge test period was performed for 15 days. The feeding management was identical to the aforementioned feeding experiment conditions. During the challenge test, the behaviour of the grass carp such as observing and feeding, and the mantissa was recorded.

2.9. Statistical analysis

All tests were performed in triplicates, and the data were reported as means ± standard deviation. When the differences were significant at $p < 0.05$ level, Duncan's multiple range test was used to compare the mean values among groups. Statistical analysis was performed using SPSS Statistics 17.0 for Windows.

3. Results

Effects of oral PP administration on the growth performance of grass carps are shown in Table 2. Moderate ($3\ g\ kg^{-1}$) and high ($5\ g\ kg^{-1}$) dose of oral PP administration significantly increased body gain weight body gain, SGR, and survival percent and decreased FCR compared with the model group ($P < 0.05$). However, a high level of PP administration ($5\ g\ kg^{-1}$) significantly did not further increase the body gain weight body gain, SGR, and survival percent and further decrease the FCR compared to those of the moderate group ($3\ g\ kg^{-1}$, $P < 0.05$). In addition, no significant differences were observed in the CF of all groups ($P > 0.05$).

The changes in the intestine digestive-enzyme (protease, lipase and amylase) activities after the 60-day feeding trial are presented in Table 3. Oral PP administration significantly increased the protease, lipase and amylase activities compared with those in the control group ($P < 0.05$). However, a high level of PP administration ($5\ g\ kg^{-1}$) did not further increase the protease, lipase and amylase activities compared with those in the moderate group ($3\ g\ kg^{-1}$, $P < 0.05$).

The changes in PO, SOD and GPx activities in the grass carps fed with PP-containing diets and the control diet for 60 days are presented in Table 4. The PO, SOD and GPx activities were higher in the grass carps fed with the PP-containing diets than those in the control group ($P < 0.05$).

Table 5 presents total protein, lysozyme, alkaline phosphatase, complement C3, and complement C4 in the grass carps fed with PP-containing diets and the control diet for 60 days. No significant differences were observed in the total protein of all groups ($P > 0.05$). Oral PP administration significantly increased lysozyme, alkaline phosphatase, complement C3, and complement C4 compared with those in the control group ($P < 0.05$). However, a high level of PP administration ($5\ g\ kg^{-1}$) did not further increase lysozyme, alkaline phosphatase, complement C3, and complement C4 compared with those in the moderate group ($3\ g\ kg^{-1}$, $P > 0.05$).

Survival percent (%) of grass carp after being challenged by *A. hydrophila* is presented in Table 6. The grass carp mortality was observed in the control group for the first five days of feeding after injection, and this group showed the lowest disease resistance among all groups (Table 6, $P < 0.05$). However, a high level of PPs ($5\ g\ kg^{-1}$) did not further increase the survival percent compared with that by a moderate level of PPs ($3\ g\ kg^{-1}$, $P > 0.05$).

4. Discussion

In this study, the body weight gain, SGR, and survival percent were higher in the juvenile grass carps fed with PPs than those in the control group, whereas FCR was displayed the opposite trend. In addition, a high level of PPs ($2\ g\ kg^{-1}$) did not further increase the body weight gain, SGR values and survival percent and further decrease the FCR relative to those by a moderate level of PPs. PPs have immunomodulatory activity and reduced the bacterial and viral infections, thus promoting growth performance of juvenile grass carps. The high levels of PPs presumably exhibited mainly hypolipidemic activity due to its oil absorption effect in the intestine [4].

Intestine digestive enzymes such as protease, lipase and amylase, play important roles in the growth of aquatic animals [27]. In this study, all doses of PPs increased the activities of protease, lipase and amylase, indicating that PPs can induce high expression of these enzymes. This phenomenon is probably due to the growth promoting effect of PPs and the dilution of protease, lipase and amylase by PPs, thereby inducing high expression of the intestine digestive enzymes. The increases in the activities of protease, lipase and amylase can improve digestibility and availability of nutrients from feed stuffs.

The significant differences in the immunological indicators of the grass carps fed with PPs and the control diet indicated that the immune responses of the grass carp were changed by oral PP administration. In our study, the activities of SOD, CAT, and GPx and total protein, lysozyme, alkaline phosphatase, complement C3, and complement C4 were higher in the grass carps fed with PPs than those in the control group, suggesting that the immunity defences of the grass carps fed PPs effectively increased; this could be due to the immunomodulatory activity of PPs [5]. The increased SOD, CAT, and GPx and total protein, lysozyme, alkaline phosphatase, complement C3, and complement C4 decreased bacterial and viral infections and increased the growth performance of the grass carps. Similarly, the immunity defences of the grass carps increased when they were fed with diets supplemented with reduced glutathione [10], microencapsulated organic acids [11], silymarin [19], choline [21], phenylalanine [23] and arginine [24].

During the challenge trial against *A. hydrophila*, the grass carps fed with PP-containing diets displayed significantly less susceptibility to *A. hydrophila* than that in the control group. PPs have immunomodulatory activity and the elevated immune response of the grass carps might be an important factor in reducing the percentage mortality and protecting

Table 2
Growth performance and survival percent of grass carps feeding control diet and *P. yezoensis* polysaccharide diets after 60 days.

Parameters	*P. yezoensis* polysaccharides ($g\ kg^{-1}$)			
	0	1	3	5
Initial weight (g)	7.03 ± 0.21^{a}	7.05 ± 0.23^{a}	7.02 ± 0.25^{a}	7.04 ± 0.26^{a}
Body weight gain (%)	205.23 ± 12.46^{a}	216.14 ± 13.57^{ab}	247.34 ± 14.27^{b}	246.07 ± 14.06^{b}
FCR (%)	2.11 ± 0.06^{a}	1.91 ± 0.05^{ab}	1.82 ± 0.04^{b}	1.86 ± 0.04^{b}
SGR (%)	1.79 ± 0.07^{a}	1.94 ± 0.08^{ab}	2.06 ± 0.09^{b}	2.03 ± 0.06^{b}
CF (%)	2.09 ± 0.43^{a}	2.07 ± 0.39^{a}	2.04 ± 0.37^{a}	2.02 ± 0.35^{a}
Survival percent (%)	76.24 ± 7.13^{a}	78.35 ± 7.29^{ab}	93.74 ± 7.41^{b}	92.06 ± 7.03^{b}

Different superscript letters indicate significant differences between the same row ($p < 0.05$) for grass carp. Values are the mean ± SD (n = 3).

Table 3
Effect of dietary *P. yezoensis* polysaccharides level on intestine digestive enzymes of grass carps feeding control diet and *P. yezoensis* polysaccharide diets.

Parameters	*P. yezoensis* polysaccharides (g kg^{-1})			
	0	1	3	5
Protease (U/mg protein)	1991.14 ± 471.32^{a}	2637.74 ± 504.44^{b}	3785.91 ± 638.76^{c}	3572.68 ± 584.65^{c}
Lipase (U/mg protein)	746.34 ± 103.35^{a}	957.81 ± 149.36^{b}	1258.72 ± 171.39^{c}	1217.52 ± 167.27^{c}
Amylase (U/g protein)	186.13 ± 31.17^{a}	237.35 ± 37.09^{b}	271.08 ± 51.79^{c}	256.08 ± 42.2^{bc}

Different superscript letters indicate significant differences between the same row ($p < 0.05$) for grass carp. Values are the mean ± SD (n = 3).

Table 4
Superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione peroxidase (GPx) activity after 60 days of grass carps feeding control diets and *P. yezoensis* polysaccharide diets.

Parameters	*P. yezoensis* polysaccharides (g kg^{-1})			
	0	1	3	5
SOD activity (U/mg protein)	106.18 ± 7.08^{a}	128.29 ± 8.15^{b}	139.82 ± 9.51^{c}	141.53 ± 9.72^{c}
Catalase (U/g protein)	118.27 ± 18.14^{a}	142.61 ± 23.49^{b}	183.61 ± 28.37^{c}	193.73 ± 31.02^{c}
GPx (U/mg protein)	96.52 ± 17.19^{a}	124.63 ± 21.23^{b}	126.41 ± 24.85^{b}	128.79 ± 26.61^{b}

Different superscript letters indicate significant differences between the same row ($p < 0.05$) for grass carp. Values are the mean ± SD (n = 3).

Table 5
Effects of dietary *P. yezoensis* polysaccharides on serum non-specific immune parameters of grass carps feeding control diet and *P. yezoensis* polysaccharide diets.

Parameters	*P. yezoensis* polysaccharides (g kg^{-1})			
	0	1	3	5
Total protein (g/l)	37.15 ± 5.02^{a}	38.02 ± 5.17^{a}	39.42 ± 5.41^{a}	38.57 ± 5.27^{a}
Lysozyme (μg/ml)	7.28 ± 1.17^{a}	8.19 ± 1.24^{ab}	9.41 ± 1.36^{b}	9.32 ± 1.31^{b}
Alkaline phosphatase (U/l)	221.41 ± 21.08^{a}	232.51 ± 24.74^{ab}	251.63 ± 27.61^{b}	248.68 ± 25.56^{b}
Complement C3 (g/l)	0.22 ± 0.06^{a}	0.28 ± 0.08^{ab}	0.36 ± 1.02^{b}	0.34 ± 1.01^{b}
Complement C4 (g/l)	0.05 ± 0.02^{a}	0.07 ± 0.03^{ab}	0.09 ± 0.05^{b}	0.08 ± 0.04^{b}

Different superscript letters indicate significant differences between the same row ($p < 0.05$) for grass carp. Values are the mean ± SD (n = 3).

Table 6
Survival percent (%) of grass carps feeding control diets and *P. yezoensis* polysaccharide diets, after being challenged by *Aeromonas hydrophila*.

Parameters	*P. yezoensis* polysaccharides (g kg^{-1})			
	0	1	3	5
Survival percent (%, 5 day)	90.2 ± 4.7^{a}	100^{b}	100^{b}	100^{b}
Survival percent (%, 10 day)	69.3 ± 4.1^{a}	81.3 ± 4.6^{b}	86.2 ± 5.3^{c}	86.8 ± 5.8^{c}
Survival percent (%, 15 day)	44.3 ± 2.2^{a}	66.7 ± 3.5^{b}	79.6 ± 4.4^{c}	80.3 ± 4.7^{c}

Different superscript letters indicate significant differences between the same row ($p < 0.05$) for grass carp. Values are the mean ± SD (n = 3).

the grass carps against *A. hydrophila* [5].

In conclusion, dietary administration of PPs improved the growth performance and nonspecific immunity of grass carps. Nevertheless, an excessive dose of PPs (> 3 g kg^{-1}) could not further improve the efficiency of PPs, thus 3 g kg^{-1} was considered the optimum dose of PPs. These results indicated that PPs can be used as an immunostimulant for enhancing the growth and immunity of grass carps.

Acknowledgements

This research was supported by A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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