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Short communication

Dietary chitosan oligosaccharides modulate the growth, intestine digestive enzymes, body composition and nonspecific immunity of loach *Paramisgurnus dabryanus*

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ABSTRACT

Three test diets containing three different levels (1, 3, and 5 g kg^{-1}) of dietary chitosan oligosaccharides (COs) were formulated and used to test the growth performance, body composition, intestine digestive enzymes, antioxidant responses and resistance to *Aeromonas hydrophila* of loach *Paramisgurnus dabryanus*. A basal diet without any COs served as the control. After 60 days of feeding, the growth performance, intestine digestive-enzyme activities, body protein content and total polyunsaturated fatty acids, antioxidant responses, and resistance to *A. hydrophila* of loach *P. dabryanus* were higher than those of the control when the loach *P. dabryanus* was fed with CO-containing diets. The optimum dose of dietary COs required for the maximum growth of loach was 3 g kg^{-1} of the diet. Results indicated that dietary COs can improve the growth performance, body composition, intestine digestive enzymes, antioxidant responses, and resistance to *A. hydrophila* of loach *P. dabryanus* and can thus be used as a diet supplement for them.

1. Introduction

Loach, a prevalent freshwater fish in East Asia, is mostly cultured and produced in China, Korea, and Japan [1]. Loach (*Paramisgurnus dabryanus*), a small freshwater benthic fish, belongs to genus *Paramisgurnus*, family *Cyprinus* [2]. *P. dabryanus* is a widely cultured loach species because of its good nutritional composition, good growth performance, high tolerance to hostile environments and high market demand. Among various loach species, *P. dabryanus* is dubbed ‘ginseng in water’ and is among the larger and faster-growing species.

However, high stocking density and deteriorating aquaculture environments have resulted in increased incidence of disease every year, expanded epidemic area, increased mortality rate and worse harm [3]. In particular, *Aeromonas hydrophila* is common pathogen in loch farming and is a serious threat to aquaculture industry [4]. Therefore, farmers are compelled to use antibiotics to mitigate these challenges in aquatic animals. However, the use of antibiotics has been met with increasing opposition because of their negative long-term effects on the environment and potential harm to human consumers [5]. Therefore, developing an immune enhancer for *P. dabryanus* is an ideal method to prevent diseases by enhancing the immunity of the fish itself.

Chitosan oligosaccharides (COs), water soluble chitosan, which are oligomers of β-1,4-linked 2-amino-2-deoxy-D-glucopyranose (GlcN) and have traces of 2-acetamido-2-deoxy-D-glucopyranose, have many special physical, chemical, and biological properties, such as antibacterial, antioxidant, immunomodulatory, antidiabetic and hypolipidemic activities, which differ from those of ordinary chitosan [6–10]. The addition of COs through diet significantly improved the growth performance of weaned pigs [11], tiger puffer [12], sea cucumber [13], Japanese quail [14], turbot [15] and koi [16]. However, data on the effects of COs on the growth performance, intestine digestive enzymes, body composition and nonspecific immunity of *P. dabryanus* are limited.

Accordingly, the present study was conducted to elucidate the dietary effects of COs on the growth performance, intestine digestive enzymes, body composition and nonspecific immunity of *P. dabryanus*.

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Table 1
Composition of the loach basal diets (g kg^{-1}).

Ingredient	Content
Fish meal	210
Soybean meal	360
Wheat flour	100
wheat bran	70
Rapeseed meal	40
Peanut oil	20
Corn flour	170
$Ca(H_2PO_4)_2$	15
Sodium methylcellulose	10
NaCl	3
Vitamin mixture[a]	1.2
Mineral[b]	0.8

[a] Vitamin mixture provided per kg of diet: VA 12,500,000 IU; VD 2,000,000 IU; VE 7000 IU; VK 2000 mg; VB_1 800 mg; VB_2 2500 mg; VB_6 800 mg; VB_{12} 10 mg; niacin 3000 mg; pantothenic acid 10,000 mg; folic acid 300 mg; biotin 20 g; VC 20,000 mg.

[b] Mineral provided per kg of diet: Mn 19 mg; Mg 230 mg; Co 0.1 mg; I 0.25 mg; Fe 140 mg; Cu 2.5 mg; Zn 65 mg; Se 0.2 mg.

2. Materials and methods

2.1. Ethics statement

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

2.2. Materials

COs were prepared according to the methods described by Wu [17]. The yield and purity were 91.23% and 96.17%, respectively. The micro-ELISA strip plate provided in this kit (48T, Shanghai Changjin Biotechnology Co., Ltd., Shanghai, China) had been pre-coated with an antibody specific for the target enzyme. All other chemicals were of reagent grade.

2.3. Diet preparation

The composition of the basal diet is provided in Table 1. COs were added to the basal diet at three levels (1, 3 and 5 g kg^{-1} dry diets). The trial diets were extruded, cut into pellets (2 mm), dried at 60 °C, and stored at −20 °C until use.

2.4. Loach culture

Loaches with an average body weight of 3.14 ± 0.17 g were obtained from a local farm in Lianyungang, China. The loaches were safely transferred and acclimated in a semi-intensive culture pond for 2 weeks. During the acclimation period, the loaches were fed with the basal diet twice daily (08:00 and 18:00).

For the feeding trial, 240 loaches were randomly assigned into four groups: one control group (received basal diet without COs addition) and three CO-containing groups (received diet containing 1, 3 and 5 g kg^{-1} of COs). The loaches of each group were assigned randomly to three 24 L PVC tanks that contained aerated/dechlorinated freshwater.

Feeding ration was initially set at 5%–7% of the body weight twice daily at 08:00 and 18:00 and then adjusted as per the feeding response of the loaches in each tank. The culture conditions were set as follows: water temperature 25 ± 1 °C, pH 7.1 ± 0.2, dissolved oxygen 6.0–6.8 mg L^{-1}, total ammonia nitrogen < 0.06 mg L^{-1}, and nitrite < 0.02 mg L^{-1}. The culture water was renewed daily with a volume of 30% freshwater. The experiments were conducted in triplicates.

2.5. Growth performance

At the end of the 60 day feeding trial, the loaches in each tank were weighed to calculate the feed conversion ratio (FCR), specific growth rate (SGR), condition factor (CF) and survival rate 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 rate (%) = 100 × (final loach number/initial loach number).

2.6. Sample

After the 60 day feeding trial, the loaches in each tank were starved for 24 h and bulk-weighed. Ten loaches were killed with MS222 (Sigma-Aldrich, St. Louis, MO, USA). Loach skin was disinfected with 70% ethanol. The peritoneal cavity was opened aseptically using a sterile scalpel, and part of the intestine between approximately 1 cm anterior to the anus and pyloric caeca was excised. The faeces with mucus were stripped off using sterile forceps, and the intestinal wall was washed with sterile saline. The loach intestines were mixed with 10 vol (v/w) of ice-cold phosphate-buffered saline, homogenised, and centrifuged at 3000 × *g* and 4 °C for 20 min. The resulting supernatant was collected and stored in a refrigerator at −20 °C and used to analyse the various enzyme activities.

At the end of the 60 day feeding trial, blood was collected from the tail vein of 10 loaches selected randomly from each tank. The blood samples were centrifuged at 3000 × *g* for 10 min. The resulting serum was stored in a refrigerator at −20 °C and used to assay the various antioxidant parameters.

2.7. Assay methods

Body moisture, protein and lipid contents were determined according to the standard methods of AOAC [18]. Fatty acid composition was assayed in accordance with the previously described method [19]. The biochemical parameters, i.e. amylase activity, protease activity, lipase activity, superoxide dismutase (SOD) activity, phenoloxidase (PO) activity and glutathione peroxidase (GPx) activity, were assayed using ELISA kits in accordance with the methods of Gao et al. [3]. In a typical procedure, standards or samples were added to the appropriate micro-ELISA strip plate wells and combined with the specific antibody, followed by a horseradish-peroxidase-conjugated antibody specific for the target enzyme. The free components were washed away. Tetramethylbenzidine 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 conducted in triplicates.

2.8. Resistance of the loaches to *A. hydrophila*

A. hydrophila was purchased from Shanghai Ocean University, China and cultured at 25 °C and pH 7.1. At the end of the 60 day feeding trial, 30 loaches were selected randomly from each group, subjected to acclimation for five days in tanks and then subjected to intraperitoneal

Table 2
Growth performance and survival rate of loaches feeding different diets after 60 days.

Parameters	Control	COs (g kg^{-1})		
		1	3	5
Initial weight (g)	3.11 ± 0.03^a	3.09 ± 0.04^a	3.15 ± 0.04^a	3.08 ± 0.03^a
Body weight gain (%)	103.07 ± 2.24^a	114.15 ± 3.21^b	126.53 ± 4.61^c	113.18 ± 2.39^b
FCR (%)	1.85 ± 0.21^a	1.59 ± 0.12^b	1.48 ± 0.13^c	1.58 ± 0.11^b
SGR (%)	0.79 ± 0.03^a	0.91 ± 0.03^b	0.93 ± 0.04^b	.92 ± 0.04^b
CF (%)	3.09 ± 0.22^a	4.06 ± 0.37^b	5.21 ± 0.62^c	4.03 ± 0.49^b
Survival rate (%)	87.24 ± 1.13^a	93.52 ± 1.46^b	95.26 ± 1.54^b	94.08 ± 1.28^b

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

injection of *A. hydrophila* (0.2 mL, 3.0×10^9 cfu/mL). The loaches of the control group were subjected to intraperitoneal injection of an equal volume of saline. The challenge test period was performed for 3 weeks. The feeding management was the same as the abovementioned feeding experiment conditions. During the challenge test, the behaviour of the loaches was recorded.

2.9. Statistical analysis

All tests were conducted in triplicates, and data were reported as the mean ± standard deviation. The variance and significant differences among the means were tested through one-way ANOVA using SPSS software (version 17.0 for Windows; SPSS Inc., Chicago, IL, USA).

3. Results

The effects of oral CO administration on the growth performance of loaches are shown in Table 2. The groups orally administered with COs significantly showed increased body weight gain, SGR, CF and survival rate, as well as decreased FCR, compared with the model group ($p < 0.05$). However, a high level of CO administration (5 g kg^{-1}) significantly decreased the body weight gain and CF, as well as increased the FCR, of the CO-fed loaches compared with those of the moderate group (3 g kg^{-1}, $p < 0.05$). Moreover, no significant differences in the SGR and survival rates of all levels of CO administration groups were observed ($p < 0.05$).

The changes in the activities of intestine digestive enzymes (i.e. protease, lipase and amylase) after the 60 day feeding trial are presented in Table 3. The oral administration of COs significantly improved the activities of protease, lipase and amylase compared with those of the model group ($p < 0.05$). Nevertheless, a high level of CO administration (5 g kg^{-1}) significantly decreased the activities of protease, lipase and amylase compared with those of the moderate group (3 g kg^{-1}, $p < 0.05$). The oral administration of COs significantly improved the protein and lipid contents and the ratio of total saturated to total polyunsaturated fatty acids in meats and significantly decreased body water content and the saturated fatty acid ratio of fatty acids in meat lipids compared with the control group (Tables 4 and 5; $p < 0.05$).

Table 3
Effect of dietary chitosan oligosaccharides (COs) level on intestine digestive enzymes of loaches.

Parameters	Control	COs (g kg^{-1})		
		1	3	5
Protease (U/ml)	1.06 ± 0.02^a	1.48 ± 0.06^b	1.91 ± 0.07^c	1.46 ± 0.05^b
Lipase (U/L)	37.71 ± 5.06^a	45.29 ± 6.31^b	49.24 ± 8.37^c	46.03 ± 6.23^b
Amylase (U/ml)	0.29 ± 0.03^a	0.42 ± 0.05^b	0.64 ± 0.07^c	0.43 ± 0.06^b

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

Table 4
Effect of dietary chitosan oligosaccharides (COs) level on body composition of loaches.

COs (g kg^{-1})	Water (%)	Protein (%)	Fat (%)
Control (0)	72.31 ± 2.74^a	18.41 ± 0.71^a	3.15 ± 0.16^a
1	68.91 ± 2.15^b	22.39 ± 1.14^b	2.42 ± 0.12^b
3	68.13 ± 2.03^b	23.16 ± 1.21^b	2.39 ± 0.11^b
5	68.97 ± 2.13^b	22.28 ± 1.12^b	2.41 ± 0.14^b

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

Table 5
Effect of dietary chitosan oligosaccharides (COs) level on fatty acid composition of loaches.

COs (g kg^{-1})	Total saturated fatty acid	Total polyunsaturated fatty acid
Control (0)	41.57 ± 2.15^a	41.46 ± 2.17^a
1	37.42 ± 1.85^b	45.34 ± 2.29^b
3	36.54 ± 1.72^b	46.16 ± 2.34^b
5	36.27 ± 1.62^b	46.53 ± 2.39^b

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

Table 6 shows the PO, SOD and GPx activities in the loaches fed with CO-containing diets and the control diet for 60 days. The activities of PO, SOD and GPx were higher in the loaches fed with the CO-containing diets than that in the control group ($p < 0.05$).

At the end of the 60 day feeding trial, 30 loaches were selected randomly from each group and subjected to challenge test. The mortality of loaches occurred in the control group for the first week of feeding after injection, and the loaches this group exhibited the highest among all groups (Table 7, $p < 0.05$). However, a high dose of COs (5 g kg^{-1}) did not further increase the survival rate compared with that by a moderate dose of COs (3 g kg^{-1}, $p > 0.05$).

4. Discussion

Although oral CO administration increased the growth performance of weaned pigs [11], tiger puffer [12], sea cucumber [13], Japanese quail [14], turbot [15] and koi [15], the effects of CO on the growth performance, intestine digestive enzymes, body composition and antioxidant responses of *P. dabryanus* remain unclear. In this study, the body weight gain, SGR, FC and survival rates were higher in the loaches fed with COs than those in the control group, whereas FCR exhibited the opposite trend. This finding can be ascribed to the fact that the oral administration of COs increased the activities of intestine digestive enzymes. However, a high level of CO administration decreased the body weight gain, SGR and FC values, as well as increased the FCR, compared with a moderate dose of COs. The high levels of COs

Table 6
Phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity of loaches after 60 days of feeding different diets.

Parameters	Control	COs (g kg^{-1})		
		1	3	5
PO activity (O.D. 490 nm)	0.29 ± 0.04^a	0.47 ± 0.05^b	0.68 ± 0.07^c	0.71 ± 0.08^c
SOD activity (U g protein^{-1})	22.67 ± 3.07^a	26.14 ± 5.23^b	31.92 ± 6.64^c	32.01 ± 6.15^c
GPx (U g protein^{-1})	103.81 ± 16.62^a	117.54 ± 7.18^b	131.47 ± 8.25^c	132.26 ± 9.23^c

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

Table 7
Survival rate (%) of loaches fed with chitosan oligosaccharides (COs) diets, after being challenged by *Aeromonas hydrophila*.

Parameters	Control	COs (g kg^{-1})		
		1	3	5
Survival rate (%, 1 week)	92.3 ± 5.1^a	100^b	100^b	100^b
Survival rate (%, 2 week)	69.2 ± 4.1^a	78.6 ± 5.2^b	87.2 ± 6.3^c	88.1 ± 6.4^c
Survival rate (%, 3 week)	43.5 ± 3.1^a	64.7 ± 4.5^b	84.6 ± 5.8^c	85.6 ± 6.2^c

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

presumably induced mainly hypolipidemic activity [20].

The digestive enzymes, i.e. protease, lipase and amylase, are responsible for the hydrolysis of protein, lipid and carbohydrate to yield hydrolysates that can be absorbed in the intestine. Therefore, evaluating these digestive enzymes in the aquaculture industry is important. In this study, all doses of COs increased the intestine digestive enzymes, indicating that COs can induce the expression of these enzymes; this phenomenon is presumably due to the antibacterial activity of COs [6], which inhibited bacterial growth and suppressed the expression of digestive enzymes of bacteria, thereby inducing the expression of the intestine digestive enzymes of loaches.

Meat quality is affected by its nutritional composition, such as water, protein and lipid contents, and fatty acid composition. Therefore, evaluating the nutritional composition of meat is important in the aquaculture industry. Loaches fed with all doses of COs exhibited higher protein content and total polyunsaturated fatty acid ratio in fatty acids and lower body water content and lipid content in meat and total saturated fatty acid ratio in fatty acids than those in the control group; this phenomenon can be due to the antioxidant and hypolipidemic activities [7,9,10].

The significant differences in the immunological indicators (PO, SOD and GPx) of the loaches fed with COs and the control diet indicated that the immune responses of the loaches were changed by the oral administration of dietary COs. In this study, the PO, SOD and GPx activities were higher in the loaches fed with COs than those in the control group, indicating that the immunity defences of the loaches fed with COs were positively improved; this phenomenon can also be due to the antioxidant activity [7]. Similarly, dietary COs significantly increased the immune response of weaned pigs [11], sea cucumber [12], turbot [15] and koi [16], indicating that COs can be a promising immunostimulant for the enhancement of animals.

The loaches received CO diets showed significantly less susceptibility to *A. hydrophila* than those in the control group; this could be due to the antibacterial, antioxidant activities and immunomodulatory activities [6–8].

In brief, COS promoted promoting growth performance of loaches through the ways as follows: (1) the antibacterial and immunomodulatory activities of COs inhibited pathogenic bacterial and viral infections and decreased susceptibility to *A. hydrophila* of loaches; (2) the antibacterial activity of COs induced the expression of the intestine digestive enzymes of loaches; (3) the antioxidant activity of COs increased PO, SOD and GPx activities and improved nonspecific immunity of loaches; and (4) the antioxidant and hypolipidemic activities of COs improved body composition of loaches.

In conclusion, dietary COs improved the growth performance, intestine digestive enzymes, body composition and nonspecific immunity of loach. However, a high level of COs (5 g kg^{-1}) did not further increase its efficiency. Therefore, 3 g kg^{-1} was considered the appropriate dose of COs. This study brought new insights into the role of COs in improving growth performance, intestine digestive enzymes, body composition and nonspecific immunity of loaches. Moreover, it is confidently predict that COS could be widely used as an antibiotic alternative for preventing bacterial and viral infections and improving immunity in other animals and humans.

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