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Several Indicators of Immunity and Antioxidant Activities Improved in Grass Carp Given a Diet Containing Bacillus Additive

W.F. Li, B. Deng, Z.W. Cui, L.Q. Fu, N.N. Chen, X.X. Zhou, W.Y. Shen and D.Y. Yu
Key Laboratory of Molecular Feed Science, Institute of Animal Nutrition and Feed Science,
College of Animal Science, Zhejiang University, 310058 Hangzhou, China

Abstract: The effect of Bacillus additive on immunity and antioxidant activities of grass carp (*Ctenopharyngodon idellus*) was studied in this experiment. Animals of the control group were fed the basal diet and those of treatment group were fed the basal diet augmented with Bacillus additive at a dose of 10^5 Colony Forming Units per gram (CFU g^{-1}) feed. Data showed that serum immunity parameters including IgM, complement C3 and Alkaline Phosphatase (AKP) significantly increased ($p<0.05$) by 30.77, 34.62 and 107.79% in the treatment group. However, there was no significant difference in serum total protein, globulin, albumin, A/G ratio, Lysozyme (LZM), Glutamic Oxaloacetic Transaminase (GOT) and Glutamic Pyruvic Transaminase (GPT) activities. Serum antioxidant parameters such as serum Glutathione (GSH), Total Antioxidant Capacity (T-AOC), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GSH-Px) activities significantly ($p<0.05$) enhanced by 32.76, 41.75, 20.06 and 11.30%, respectively and hepatopancreatic antioxidant parameters including SOD activity and GSH increased significantly ($p<0.05$) by 33.83 and 25.86%. In addition, GSH-Px and Malondialdehyde (MDA) content in hepatopancreatic decreased by 20.69% ($p<0.05$) and 25.39% ($p<0.05$) individually and the anti-superoxide anion (anti-O_2^-) activity in hepatopancreatic decreased by 32.21% ($p<0.05$) in the treatment group. These results indicate that the Bacillus addtive can promote the immunity and antioxidant function of grass carp.

Key words: Bacillus additive, grass carp, immunity, antioxidation, control group, China

INTRODUCTION

Grass carp is the most widely cultivated freshwater fish in China, intensive culture practices have resulted in the deterioration of water environment (Paez-Osuna *et al*., 1998) and frequent occurrence of diseases (Bondad-Reantaso *et al*., 2005) which would lead to significant economic losses. Probiotics are used for a quite long time but only in last few years probiotics became a very important part of the culture practices for improving growth and resistance to disease (Amin *et al*., 2009; Asli *et al*., 2007; Vali, 2009; Nikfar *et al*., 2010; Vijayabaskar and Somasundaram, 2008; Kabir *et al*., 2005; Bansal *et al*., 2011). Probiotics have many advantages over antibiotics and other drugs which may result in the development of resistant pathogens. Some studies have proved that probiotics could inhibit pathogens and modulating immunity of animals (Nayak, 2010). Many probiotics consist of bacterial spores, principally of genus *Bacillus* (Casula and Cutting, 2002; Hong *et al*., 2005). Several spiecies are isolated as probiotics including *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. clausii*, *B. megaterium*, *B. firmus* and species of the *B. cereus* group (Barbosa *et al*., 2005).

Like the trails on other animals, probiotics as feed additives could promote the immnity of fish. Several studies have shown mechanism of probiotics which is mainly relevant to improvement of water quality (Lalloo *et al*., 2007), regulation of gut microflora (Venkat *et al*., 2004; Burr *et al*., 2005) and enhancement of immunity and antioxidant activities (Shen *et al*., 2010). It has been demonstrated that fed with *Lactobacillus plantarum*, *Lactobacillus acidophilus* or *Saccharomyces cerevisiae* supplemented diet to fish have enhanced the non-specific immunity of fish (Harikrishnan *et al*., 2011). However, studies of probiotics on the immune and antioxidant function of grass carp are few. In this study, the researchers investigated the effect of dietary supplementation with Bacillus on immunity and antioxidant function in grass carp.

MATERIALS AND METHODS

Probiotics preparation: *Bacillus additive* (*B. subtilis* and *B. amyloliquefaciens*, 1:1, bacterial counts of 10^8 CFU g^{-1}) was prepared in the laboratory. Ingredients and nutritional composition of the basal diet are shown in Table 1. In the

Corresponding Author: D.Y. Yu, Key Laboratory of Molecular Feed Science, Institute of Animal Nutrition and Feed Science, College of Animal Science, Zhejiang University, 310058 Hangzhou, China

Table 1: Ingredients and nutritional composition of the basal diet

Ingredients	Composition (%)	Nutritional index	Proximate composition (% wet weight)
Fish meal	1.0	Moisture	9.31
Soybean meal	5.0	Crude protein	28.40
Cottonseed meal	23.8	Crude fat	2.97
Rapeseed meal	27.2	Crude ash	12.08
Wheat flour	18.0	Calcium	0.80
Rice bran	8.0	Total phosphonium	1.29
Lee power	6.0	-	
Malt root	6.0	-	-
Premix[a]	5.0	-	-

[a]Premix provides following per kilogram of diet: VA: 150 000 IU, VD3: 30000 IU, VE: 750 mg, VK3: 150 mg, Fe: 2.5 g, Cu: 0.075 g, Zn: 0.75 g, Mn 0.5 g, Mg 5 g, I 22.5 mg, Se 3.5 mg and Co 7.5 mg

test group, 1% Bacillus additive was added into the diet in which bacteria counts reached 10^5 CFU g^{-1}.

Fish and experimental design: Grass carps used in this experiment were provided by a freshwater fish farm in Shaoxing, China and were fed in the indoor recirculating aquaculture system (water volume was 1.33 m^3). Water temperature was maintained 18-25°C and compressor aeration system was used in the trail for dissolved oxygen supply continuously. Water used in the experiment was pumped from a nearby reservoir and replaced 1/3 of its volume every week. A total of 300 healthy grass carps with initial weight of 51.0±2.3 g were selected and randomly divided into two groups, control group and treatment group. Each group had 3 duplicates with 50 grass carps in each duplicate. Basal diet was fed to the animals of control group and diet containing Bacillus additive was fed to those of the treatment group for 52 days (10^5 CFU g^{-1} feed) ingredients were shown in Table 1. Fishes were fed three times daily at 6:30 am, 12:00 am and 21:00 pm. All animals were fed the same diet for 1 week prior to the feeding trail and feed intake was about 3% total body weight per day and regulated according to the actual intake.

Probiotics preparation sampling blood and hepatopancreas: At the end of feeding trial, grass carps were fasted for 24 h to sacrifice. About 10 fishes randomly selected from each replicate were anaesthetised with MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Tricaine; Sigma, 1:5000). Blood was sampled from the fish caudal vein using 1 mL syringes and then placed in the refrigerator at 4°C for 24 h standing. The blood samples were centrifuged at 4000 g for 15 min at 4°C and the supernatants were transferred to Eppendorf tubes (1.5 mL) and immediately stored at -80°C. Hepatopancreas collected from each fish were packed in sealed bags, quickly frozen with liquid nitrogen and stored at -80°C.

Determination of serum immune parameters: Total protein of serum was analyzed using Coomassie Brilliant Blue G-250 Dye Method and albumin by Bromocresol Green Method. The globulin content is the protein content of the total protein content minus albumin content. Lysozyme (LZM), Myeloperoxidase (MPO), Alkaline Phosphatase (AKP), Glutamic Oxaloacetic Transaminase (GOT) and Glutamic Pyruvic Transaminase (GPT) were determined by the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The serum LZM activity was measured by turbidity assay using 0.25 mg mL^{-1} micrococcus lysoleikticus as the substrate and one enzyme unit is defined as the amount of LZM which could make the absorbency value decrease 0.001 min^{-1}. AKP activity was detected using disodium phenyl phosphate method and one enzyme unit is defined as the amount of AKP which could make the substrate produce 1 mg of phenol after the reaction of 1 mL serum and substrate at 37°C for 15 min. The activity of GOT and GPT were measured by the method of Reitman. The contents of active complement C3 and IgM were assayed by the commercial kit (Shanghai Huasheng Bioengineering Co., Shanghai, China). Rate nephelometric method was used for the determination of C3 content and immune transmission turbidity for that of IgM content.

Determination of antioxidant indices: About 0.5 g hepatopancreatic sample of grass carp was accurately weighed and placed in the 10 mL sterile centrifuge tube, adding 4.5 mL 0.85% pre-cooled and sterile normal saline and then homogenized on ice using automatic homogenizer to obtain the 10% tissue homogenate. The homogenate was centrifuged at 4000 g for 20 min at 4°C. Remove the protein precipitation and the supernatant was collected in 1.5 mL Eppendorf tube and immediately stored at -80°C.

Commercial kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) were used for the determination of antioxidant parameters including Superoxide Dismutase (SOD), Catalase (CAT), Total Antioxidant Capacity (T-AOC), Glutathione Peroxidase (GSH-Px), Glutathione (GSH), Malondialdehyde (MDA) and anti-O_2^-. One unit of SOD activity is defined as the amount which could make SOD inhibition rate reach 50% in 1 mL serum; one unit of CAT is defined as the content of CAT which could degrade 1 mol H_2O_2 sec^{-1}; one unit of T-AOC is defined as the concentration of T-AOC that could make the absorbance of 1 mL serum increase 0.001 min^{-1}; one unit of GSH-Px is defined as the amount of GSH-Px that could transform 1 mol NADPH-$NADP^+$ min^{-1}. GSH content was measured using Dithio-bis-nitrobenzoic acid (DTNB) Method, anti-O_2- was detected by Pyrogallol Autoxidation Method and MDA content was assayed by the Thiobarbituric Acid (TBA) Method.

Statistcal analysis: Data are reported as means±standard deviation. All data means were compared using Student's t-test (Statistical Package Social Science, SPSS, Version 13.0). A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The effects of Bacillus additive supplemented in the diet on the concentration of total serum protein, albumin, globulin, IgM, complement C3, LZM, A/G ratio and the activity of AKP, MPO, GOT, GPT in the serum of grass carp are shown in Table 2. Compared with the control group, the content of IgM and complement C3 and AKP activity in the serum of the treatment group increased by 30.77% ($p<0.05$), 34.62% ($p<0.05$) and 107.79% ($p<0.05$), respectively and MPO activity decreased by 19.70% ($p<0.05$). However, Bacillus additive had no significant effect on the concentration of total serum protein, albumin, globulin, A/G ratio, GOT and GPT activity and LZM content. It indicated that dietary Bacillus additive could improve specific and nonspecific immunity of grass carp.

Serum antioxidant parameters including T-AOC, CAT, SOD, anti-O_2^- and the content of GSH, GSH-Px, MDA in the serum and hepatopancreas are showed in Table 3. According to the results, Dietary probiotics remarkably ($p<0.05$) increased the activities of T-AOC and SOD, GSH content and GSH-Px content in the serum of grass carps by 41.75, 20.06, 32.76 and 11.30%, respectively ($p<0.05$). Hepatopancreatic antioxidant parameters including SOD activity and GSH increased significantly ($p<0.05$) by 33.83 and 25.86%. In addition, GSH-Px and Malondialdehyde (MDA) content in hepatopancreatic decreased by 20.69% ($p<0.05$) and 25.39% ($p<0.05$) individually and the anti-superoxide anion (anti-O_2^-) activity in hepatopancreatic decreased by 32.21% ($p<0.05$) in the treatment group. These results indicated that Bacillus additive could improve the antioxidant activities of grass carps and reduce the damage from free radicals.

Studies on the application of probiotics in aquaculture mainly focus on the improvement of water quality (Lalloo *et al*., 2007; Zhou *et al*., 2009) and promotion of the growth of aquatic animals (Kumar *et al*., 2006; Ziaei-Nejad *et al*., 2006; Merrifield *et al*., 2010; Wang, 2011) while reports concerning effects of probiotics on the immunity and antioxidant activities of aquatic animals are very few (Zhou *et al*., 2010). The results have revealed that Bacillus additive supplemented in diet could not only significantly promote growth of grass carp but also improve the immunity and antioxidant activities of them.

In this research, fish given probiotics as diet additive had higher concentration of IgM and complement C3 and AKP activity in the serum of the grass carp than the control. Similar result was obtained by Salinas *et al*. (2008) who observed higher serum IgM in the juvenile seabream fed diets supplemented with inactivated *Lactobacillus debreckii* ssp. *lactis* and *Bacillus subtilis* (individually or combined). Previous reports have also revealed a remarkable positive effect of different probiotics on the level of complement (Douillet, 2000; Pangrahi *et al*., 2005).

However, there are no significant differences between the treatment and the control fish in total serum protein, albumin, globulin, A/G ratio. Wang *et al*. (2008) also reported that probiotics *Enterococcus faecium* ZJ4 had no significant effect on the serum parameters including total serum protein, albumin, globulin and A/G ratio of tilapia (*O. niloticus*). But Zhou *et al*. (2010) reported an inconsistent result: *B. coagulans* B16

Table 2: Effect of *Bacillus additive* on serum immune factors of grass carp

Immune factors	Control group	Treatment group
Total serum protein (g L^{-1})	31.15±2.600	35.05±4.3900
Albumin (g L^{-1})	15.34±1.290	15.72±2.2100
Globulin (g L^{-1})	18.58±1.370	20.13±1.4100
A/G ratio	0.88±0.120	0.93±0.0980
IgM (g L^{-1})	0.13±0.019^b	0.17±0.0180^a
AKP (King unit/100 mL)	4.62±0.580^b	9.60±0.8900^a
MPO (U L^{-1})	84.89±8.090^a	68.17±6.8500^b
C3 (g L^{-1})	0.52±0.051^b	0.70±0.0087^a
LZM (μg mL^{-1})	0.97±0.140	0.96±0.1400
GOT (U L^{-1})	2.36±0.410	2.18±0.4700
GPT (U L^{-1})	7.44±0.700	7.23±0.6500

^{a,b}denotes a statistical significant difference between values for a 95% confidence interval

Table 3: Effect of Bacillus additive on hepatopancreas and serum antioxidant indices of grass carp

	Hepatopancreas		Serum	
Antioxidant indices	Control group	Treatment group	Control group	Treatment group
T-AOC (U $mgprot^{-1}$)	17.34±1.730	16.31±1.630	6.61±0.640^b	9.37±0.980^a
CAT (U $mgprot^{-1}$)	10.98±1.110	12.73±1.240	3.64±0.590	3.46±0.920
SOD (U $mgprot^{-1}$)	90.77±11.73^b	121.48±6.800^a	20.79±0.790^b	24.96±1.730^a
GSH-Px (μmol $gprot^{-1}$)	418.45±39.97^a	331.88±30.22^b	311.74±13.24^b	346.96±16.19^a
GSH (mg $gprot^{-1}$)	228.27±23.77^b	287.29±19.96^a	136.55±13.12^b	181.28±18.28^a
Anti-O_2^- (U $gprot^{-1}$)	425.26±25.23^a	288.27±13.33^b	94.43±9.200	92.02±9.160
MDA (nmol $mgprot^{-1}$)	7.05±0.550^a	5.26±0.510^b	7.24±0.150	6.79±0.270

increased the serum protein and globulin content and the other two did not significantly affect them. Different results may be related to different probiotics used in the trails.

In addition, *B. subtilis* and *L. acidophilus* increased lysozyme activity of Nile tilapia (Aly *et al*., 2008) but the present result revealed that the probiotics did not affect the lysozyme activity of grass carp. Therefore, further studies are necessary to explore the relationship between probiotic strain or fish species and lysozyme activity. The MPO is an important enzyme with capacity to kill pathogens and can use hydrogen peroxide and chloridion to produce hypochlorous acid and radical. The MPO activity was lower in the experimental group than in the control which is consistent with another report about a potent immunosuppressor, cyclophosphamide, remarkably decreasing the MPO activity of Asian catifish, *Clarias batrachus* (L.) (Kumari and Sahoo, 2005). However, Kumari and Sahoo (2006) observed the yeast β-glucan could increase the MPO actiity. In this study, there is no difference of GPT and GOT content between treatment and comparison group indicating that Bacillus additive did not damage hepatopancreatic function of grass carp.

The mechanism of adding Bacillus additive into the diet to enhance the immunity may be related to the improved intestinal microflora (Li *et al*., 2007) and immunoactivator (Aly *et al*., 2008; Wang *et al*., 2010) which are beneficial to animals and prevent them from pathogens and related diseases (Kumar *et al*., 2006). Moreover, *Bacillus* species can bind the antigen binding sites of intestinal lymph nodes and they can play an important role in stimulating immune cells (Thurnher *et al*., 1997). Through regulating the microbiota of animals, probiotics can enhance the resistance against pathogens and improve weight gain. The present results also suggests that adding Bacillus additive into the diet could significantly enhance inner antioxidant function of grass carp through increasing the related antioxidant enzyme activities in serum and hepatopancreas. This is proved by the significant increase of serum and hepatopancreatic T-AOC, SOD and reduction of the oxidative metabolism product (MDA and anti-O_2^-).

The result was similar to Shen *et al*. (2010) who also reported that dietary supplementation with *Bacillus subtilis* could improve the antioxidant capacity of shrimp. Besides, most of the probiotics can produce SOD enzyme and glutathione to prevent the oxidation of the organism. Moreover, Bacillus can also secrete antioxidant enzymes itself or be an activator to promote the secretion of antioxidant enzymes by the organism, effectively removing free radicals and preventing lipid peroxidation.

CONCLUSION

Adding Bacillus additive in diet could improve the immune and antioxidant function of grass carp which is also a main reason of using Bacillus additive as feed additive to promote the growth of grass carp.

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