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Effects of different combinations of *Bacillus* on immunity and antioxidant activities in common carp

Longtao Wang[1] · Chenxia Ge[1] · Jianchao Wang[1] · Jing Dai[1] · Peijun Zhang[1] · Yuehong Li[1]

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Abstract *Bacillus* can not only improve the ecological environment of aquaculture water, but also improve the immunity of aquaculture animals, inhibit the reproduction of pathogenic microorganisms and reduce the incidence of disease. The present study represented the effect of different combinations of *Bacillus* on immunity and antioxidant activities in common carp. A total of 240 fish were randomly divided into four groups: control group (basal diet), experimental group I (add *B. subtilis*), experimental group II (add *B. subtilis* and *B. licheniformis*), and experimental group III (*B. subtilis, B. licheniformis*, and *B. cereus*). Compared with the control group, the carp in experimental group II displayed a significantly elevated phagocytic percentage (up to a 20.2% increase; $P < 0.05$), phagocytic index (up to a 41.5% increase; $P < 0.05$), serum immunoglobulin M (up to a 26.1% increase; $P < 0.05$), serum lysozyme activity (up to a 21.9% increase; $P < 0.05$), intestinal mucosal secretory immunoglobulin A content (up to a 53.1% increase; $P < 0.05$), and peripheral blood lymphocyte proliferation ratio (up to a 32.8% increase; $P < 0.05$). Further analysis of liver antioxidants showed that, compared with the control group, carp in experimental group II displayed

✉ Yuehong Li
1209050884@qq.com

Longtao Wang
wanglongtao2005@126.com

Chenxia Ge
gechenxia@126.com

Jianchao Wang
562432945@qq.com

Jing Dai
2291667665@qq.com

Peijun Zhang
1387547546@qq.com

[1] College of Animal Science and Technology, Jilin Agriculture University, Changchun, China

elevated superoxide dismutase (69.4%; $P < 0.05$), glutathione peroxidase (25.4%; $P < 0.05$), catalase (76.4%; $P < 0.05$), total antioxidant (41.1%; $P < 0.05$), maleic dialdehyde (28.9%; $P < 0.05$), and glutathione (35.6%; $P < 0.05$) activities. There was no significant difference between the experimental group I and the experimental group III ($P > 0.05$). Our results indicated that dietary *Bacillus* enhanced the performance of the common carp immune response and that a mixture of strains elevated the antioxidant ability.

Keywords Combinations of *Bacillus* · Immunity · Antioxidant activity · Common carp

Introduction

Common carp is one of the most economically valuable commercial farming fish species in China. However, the intensive aquaculture with high density always resulted in the decrease of immune level. In recent years, the outbreak of common carp diseases was more and more frequently. As we all know that the immune defense was one of the most efficient ways in preventing from the disease. The antibiotics have been used for years to control the outbreak of diseases. However, the antibiotics remain in the aquatic environment for a long time. While the antibiotics also lead to the development of antibiotic-resistant genes by exerting selective pressures to bacteria. Accordingly, there is an urgent demand for an alternative to antibiotics that can encourage fish growth and improve their immunity.

Probiotics are a safe and efficient alternative to antibiotics that act by modulating microbial communities and improving microbial balance (Zorriehzahra et al. 2016). *Bacillus* bacteria secreted many exoenzymes which could enhance the immune response against pathogenic microorganisms and antiviral effects (Simonen et al. 1993; Pohl et al. 2010). Thus, *Bacillus*, such as *B. subtilis*, *B.licheniformis*, and *B.cereus*, has been used widely as putative probiotics (Huang et al. 2015; He et al. 2013; Ringø 2011). *B. subtilis* added into water can increase serum immunoglobulin levels and most of non-specific immune parameter content and enhance the antioxidant ability of grass carp (Geng et al. 2011; Weifen et al. 2012). *B. licheniformis* was mainly promoted by the development and maturation of thymus and spleen to improve the immune function of fish Liu, C., (Lu et al. 2010). *B. cereus* could adjust ecological balance of the gastrointestinal tract. It plays an important role in promoting the healthy growth of animals (Berthold-Pluta et al. 2015). Now, the research of bacterial species is mainly focused on the single species comparison of the feeding of *B. subtilis* and *B. subtilis*. But the combination of *B. subtilis*, *B. licheniformis*, and *B. cereus* was few used in the production of aquatic animals.

Thus, this experiment was conducted to investigate the effects of different combinations of *B. subtilis*, *B. licheniformis*, and *B. cereus* on immunity and antioxidant activities in common carp and provide the reference for the application of Bacillus in aquaculture.

Material and methods

Fish and bacteria

Healthy common carp (mean weight, 57.42 ± 0.58 g) were obtained from Xinlicheng reservoir in Changchun. The animals were maintained at 25 ± 3 °C in recirculating tanks

and fed regularly with artificial feed for 2 weeks before the experiment was started. All fish experiments described in this paper were conducted according to the Chinese regulation for experimental animals. The institutional animal care and use committee of Jilin Agricultural University had approved the animal usage in the present study. *B. subtilis*, *B. licheniformis*, and *B. cereus* were provided by the laboratory of College of Animal Science and Technology in Jilin Agricultural University. *Staphylococcus aureus* was cultured in Luria broth at 37 °C and inactivated with 0.4% (*V*/*V*) formalin (F-SA) for the phagocytic activity assay.

Experimental design

A total of 240 healthy common carp were randomly divided into four groups: control group, group I, II, and III. Each group had three replicates with 20 common carps in each. The control group was fed the basal diet without *Bacillus*. Treatment group I was fed the basal diet and supplemented with 1×10^5 cfu/g of *B. subtilis*. Treatment group II was fed the basal diet and supplemented with 1×10^5 cfu/g of *B. subtilis* and *B. licheniformis*. Treatment group III was fed the basal diet and supplemented with 1×10^5 cfu/g of *B. subtilis*, *B. licheniformis*, and *B. cereus*. Fish were hand-fed 3% of their body weight every 8 h. The common carp were maintained at 25 ± 3 °C in tanks. The experiment duration was 45 days.

Sampling for analysis

Serum, intestines, and liver samples

At the end of feeding trial, after 24-h fasting period, all the fish were placed under anesthesia for the collection of blood samples from the caudal vein. Blood samples were refrigerated at 4 °C for 24 h vertically and then centrifuged at 2000 rpm for 15 min at 4 °C. The supernatant was collected and stored at −20 °C. Intestine samples of common carp were collected and length to 5 cm. And pre-cooling PBS (0.01 mol/L, pH = 7.4) was added. Supernatant was separated at 8000 rpm for 30 min at 4 °C and stored at −20 °C. Liver samples of common carp were collected and weighed to 0.5 g. And then, 4.5 mL pre-cooling sterilized normal saline was added. An automatic homogenizer was used to homogenize the solution while kept on ice. Supernatant was separated at 2500 rpm for 10 min at 4 °C and quickly stored at −80 °C for future study.

Phagocytic activity

0.2 mL of blood sample and 0.05 mL of F-SA were thoroughly mixed. The mixture was incubated at 25 °C for 60 min with gentle shaking every 10 min. The mixture was centrifugated at 1000 rpm for 5 min, and then the cell pellet was resuspended with 0.2 mL DPBS, It was smeared on a glass slide, stained with Wright–Giemsa. And then 100 phagocytic cells were randomly observed under oil immersion. Results were calculated as phagocytic percentage (PP) using the formula PP = N100/100 × 100% and phagocytic index (PI) with the formula PI = N1/N2. In the formula, N100 was the number of cells exhibiting phagocytosis of the 100 phagocytic cells observed. N1 and N2 represented the total number of F-SA and the total number of phagocytic cells, respectively.

Determination of serum immune response

The contents of IgM were determined by the kit provided by Shanghai Langdon Biotechnology Co. Ltd. Rate nephelometric technique was used for the determination of immune transmission turbidity of IgM content. Serum lysozyme activity was determined by the kit provided by Nanjing Jiancheng Biological Engineering Research Institute.

Determination of SIgA in intestinal mucosa

The contents of SIgA were determined in intestinal mucosa by the kit provided by Shanghai Langdon Biotechnology Co. Ltd.

Determination of peripheral blood lymphocyte proliferation ratio

MTT was used to detect the peripheral blood lymphocyte proliferation. Lymphocytes were isolated by the kit provided by Tianjin Haoyang Biological Technology Co. Ltd. Stimulation Index (SI) represented peripheral blood lymphocyte proliferation ratio. SI = OD of test hole/ OD of control hole.

Determination of antioxidant index

The kit provided by Nanjing Jiancheng Biological Engineering Research Institute was used for the determination of antioxidant indices, including total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-PX), catalase (CAT), total antioxidant (T-AOC), maleic dialdehyde (MDA), and glutathione (GSH). One unit of SOD activity was defined as the amount that resulted in 50% inhibition in 1 mL serum. One unit of GSH-PX was defined as the amount of GSH-PX that transformed 1 mol NADPH to $NADP^+$ per minute. One unit of CAT was defined as the amount of CAT that degrades 1 mol H_2O_2 per second. One unit of T-AOC was defined as the concentration of T-AOC that made the absorbance of 1 mL of serum increase 0.001 per minute at 37 °C. MDA content was determined by the thiobarbituric acid (TBA) technique. GSH content was examined by the dithio-bis-nitrobenzoic acid (DTNB) method.

Statistical analysis

All statistical analysis were performed using Statistical Package for the Social Sciences 22.0 (SPSS 22.0) package and expressed as mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA), and significance was set at $P < 0.05$. Multiple comparisons among means were made with Duncan's new multiple range test.

Results

Growth performance

The dietary effects of carp fed for 45 days (Table 1). It can be seen that the weight gain and specific growth rate of carp in treatment group I, treatment group II, and treatment group III

Table 1 Carp growth performance

Dietary treatment	Control	Group I	Group II	Group III
Initial body weight (g)	57.40 ± 0.43	57.67 ± 0.21	57.52 ± 0.35	57.23 ± 0.48
Final body weight (g)	66.37 ± 0.32	68.56 ± 0.49^{b}	71.03 ± 0.85^{a}	70.30 ± 0.79^{a}
WG (%)	0.15 ± 0.22	0.18 ± 0.28	0.23 ± 0.32	0.22 ± 0.17
SGR (%)	0.32 ± 0.34^{c}	0.35 ± 0.25^{bc}	0.50 ± 0.44^{a}	0.42 ± 0.36^{b}

Note: different letters mean significant difference ($P < 0.05$), and the same letters mean no significant difference

were higher than those in control group, and the results showed that adding compound bacteria could significantly promote the growth of carp, and the effect of compound bacteria was better than that of control group.

Immune response

In common carp, we investigated the effect of different combinations of *Bacillus* on immune factors including phagocytic activity, serum LSZ activity, the content of IgM and SIgA, and peripheral blood SI. Our results showed that compared with the control group, PP of the experimental group II was significantly increased up to 20.2% ($P < 0.05$), the PI was significantly increased up to 41.5% ($P < 0.05$), serum IgM content was significantly increased up to 26.1% ($P < 0.05$), serum LSZ activity was significantly increased up to 21.9% ($P < 0.05$), and peripheral blood SI was significantly increased up to 32.8% ($P < 0.05$), but other groups were not significantly different. It was noteworthy that, compared with the control, the experimental group I and II common carp SIgA content were higher than the control group by 92.3% ($P < 0.05$) and 53.1% ($P < 0.05$), respectively (Table 2). It was indicated that adding the mixing of *B. subtilis* and *B. licheniformis* could increase serum immunoglobulin levels and some immune parameters of the common carp.

Liver antioxidant index

In common carp, we investigated the effect of different combinations of *Bacillus* on liver antioxidant index including T-SOD, GSH-PX, CAT, T-AOC, MDA, and GSH. The first, T-SOD from treatment group I, II, and III were higher than control group by 35.1% ($P < 0.05$),

Table 2 Effects of different combinations of *Bacillus* on blood immune factors and some immune parameters

	Control	Group I	Group II	Group III
PI	1.13 ± 0.06^{a}	1.31 ± 0.09^{a}	1.60 ± 0.09^{b}	1.29 ± 1.13^{a}
PP (%)	35.22 ± 1.10^{a}	37.41 ± 0.50^{a}	42.34 ± 0.99^{b}	36.25 ± 0.56^{a}
IgM (g/L)	0.88 ± 0.04^{a}	0.90 ± 0.06^{a}	1.11 ± 0.10^{b}	0.89 ± 0.08^{a}
LSZ (μg/mL)	1.05 ± 0.03^{a}	1.12 ± 0.04^{a}	1.28 ± 0.03^{b}	1.13 ± 0.03^{a}
SIgA (μg/g)	2.07 ± 0.18^{a}	3.98 ± 0.55^{c}	3.17 ± 0.86^{b}	1.92 ± 0.44^{a}
SI	0.61 ± 0.012^{a}	0.65 ± 0.022^{a}	0.81 ± 0.024^{b}	0.66 ± 0.012^{a}

Results are presented as means ± SD of triplicate observations. Means in the same column with different letters are significantly different ($P < 0.05$)

PI blood phagocytic index, *PP* blood phagocytic percentage, IgM serum, IgM content, LSZ serum lysozyme activity, SIgA intestinal mucosal secretory, SI lymphocyte conversion rate

69.4% ($P < 0.05$), and 60.4% ($P < 0.05$), respectively. Compared with the group I, the group II and III common carp T-SOD were significantly different ($P < 0.05$). The second, compared with the control group, the experimental group II common carp GSH-PX was significantly increased up to 25.4% ($P < 0.05$), but other groups were not significantly different. The third, CAT from treatment group I, II, and III were higher than control group by 20.8% ($P < 0.05$), 76.4% ($P < 0.05$), and 11.6% ($P < 0.05$), respectively. Compared with the group I and III, the group II common carp CAT was also significantly different ($P < 0.05$). The fourth, T-AOC from treatment group I, II, and III were higher than control group by 10.8% ($P < 0.05$), 41.1% ($P < 0.05$), and 29.2% ($P < 0.05$), respectively. Interestingly, there were significant differences between any two groups. The fifth, compared with the control group, MDA level from treatment group II and III were decreased significantly by 28.9% ($P < 0.05$) and 31.4% ($P < 0.05$), respectively. The sixth, GSH level from treatment group I, II, and III were higher than control group by 12.6% ($P < 0.05$), 35.6% ($P < 0.05$), and 22.5% ($P < 0.05$), respectively. Compared with the group I and III, the group II common carp GSH level was also significantly different ($P < 0.05$) (Table 3). Our results were indicated that feed with the mixing of *B. subtilis* and *B. licheniformis* can enhance the liver antioxidant ability of common carp.

Discussion

As the first line of defense against microbial pathogens, the innate immune system plays a crucial role in teleost fish, which are lower vertebrates (Magnadottir et al. 2006). Previous research suggested that addition of *Bacillus* preparations into water could increase most nonspecific immune parameters, but the immune responses of different combinations of *Bacillus* were rarely reported (Weifen et al. 2012). The results of our study were shown that, compared with the control group, addition of the mix of *B. subtilis* and *B. licheniformis* into the feed can increase leukocytes phagocytic activity and LSZ activity ($P < 0.05$). These suggested that different combinations of *Bacillus* could increase the level of non-specific immunity in common carp. Through further experiments, we were surprised to find that the mixing of *B. subtilis* and *B. licheniformis* into the feed not only promoted the innate immunity, but also enhance the acquired immune in common carp. Some specific immune parameters such as IgM, SIgA, and SI were detected.

Compared with the control group, addition of the mix of *B. subtilis* and *B. licheniformis* into the feed can increase the content of IgM, SIgA, and peripheral blood lymphocyte proliferation ratio of the common carp ($P < 0.05$). Above all, dietary *Bacillus* played a certain role in enhancing the performance of the carp immune response, where in the mixing of *B. subtilis* and *B. licheniformis* worked better in enhancing the specific and non-specific aspects of immune function. Intestinal tract is an important place for fish digestion, absorption, and intestinal mucosal immunity, especially for non-stomach carp. The integrity of the intestinal structure reflects the health state of the animal to a certain extent and also reflects the basis for the normal functioning of the intestinal tract. The intestinal mucosa folds into the lumen projecting wrinkles of intestinal epithelial cells is the distribution of functional cells to absorb nutrients in the above, so the intestinal fold height increasing the surface area represents the increase in urinary enteral area so that intestinal absorption, intestinal area and food contact area, and intestinal fluid increases, so the height and width of the intestinal tract the fold reflects the size of intestinal digestion (CasPary W F et al.1992). The results of this experiment showed that different combinations of *Bacillus* could promote the height growth of intestinal

Table 3 Effects of different combinations of *Bacillus* on liver antioxidation factors

	Control	Group I	Group I vs control (%)	Group II	Group II vs control (%)	Group III	Group III vs control (%)
T-SOD (U/mL)	20.61 ± 2.17^{a}	27.84 ± 0.71^{b}	35.1	34.91 ± 0.88^{c}	69.4	33.06 ± 0.44^{c}	60.4
GSH-PX (mol/L)	1135.07 ± 23.18^{a}	1284.90 ± 12.06^{a}	13.2	1423.38 ± 75.59^{b}	25.4	1372.30 ± 94.29^{a}	20.9
CAT (U/mgprot)	8.32 ± 0.86^{a}	10.05 ± 0.80^{b}	20.8	14.68 ± 0.73^{c}	76.4	9.29 ± 1.01^{b}	11.6
T-AOC (U/mg)	16.56 ± 0.49^{a}	18.35 ± 0.47^{b}	10.8	23.37 ± 0.80^{c}	41.1	21.40 ± 0.51^{d}	29.2
MDA (mol/mgprot)	8.66 ± 0.46^{b}	7.24 ± 1.67^{ab}	16.4	6.16 ± 1.62^{a}	28.9	5.94 ± 0.83^{a}	31.4
GSH (mg/gprot)	18.9 ± 0.48^{a}	21.28 ± 0.22^{b}	12.6	25.63 ± 0.32^{c}	35.6	23.15 ± 0.21^{b}	22.5

Results are presented as means ± SD of triplicate observations. Means in the same column with different letters are significantly different ($P < 0.05$)

T-SOD total superoxide dismutase, *GSH-PX* glutathione peroxidase, *CAT* catalase, *T-AOC* total antioxidant activity, *MAD* maleic dialdehyde, *GSH* serum glutathione

mucosal folds. Mongkol (2002) study of *Bacillus* in broilers feed in the production performance of broilers increased, the chicken intestinal villi increased significantly, mucosal crypt also deepened, and the increased surface area and lining the intestine results.

Intracellular redox balance plays an important role in the detection, control, and maintenance of the normal function of the cell. Once this balance is broken, it will lead to a variety of diseases, such as heart failure, kidney disease, diabetic, cancer (Gorin et al. 2016; Guzman et al., 2016; Taniguchi et al. 2016; Wray et al. 2016). Fish are also susceptible to the attack of reactive oxygen species. The antioxidants of fish include antioxidant enzymes like GSH-PX, T-SOD, and CAT. In our study, compared with the control group, addition of the mix of *B.subtilis* and *B.licheniformis* into the feed can increase liver GSH-PX activity ($P < 0.05$), and other two groups were not improved significantly. Due to other components involved in the antioxidant defense system leads to a reduced concentration of reactive oxygen species. SOD is an important enzyme in the elimination of the reactive anion superoxide O_2, which is transformed into H_2O_2. CAT helped H_2O_2 is further decomposed into H_2O and O_2. Superoxide anion radical scavenging could protect cells against oxidative damage. Our results showed that T-SOD and CAT from treatment group were higher than control group ($P < 0.05$). It indicated that *Bacillus* may maintain the free radical in carp liver a dynamic balance between "production" and "clear." Thus preventing damage to the cell membrane, enhances the defense capabilities of antioxidant enzyme defense system and improves the activity of antioxidant enzymes. T-AOC is used to detect oxidative stress phenomena in bodily fluids and tissues. In our study, T-AOC from all treatment groups was higher than that control group ($P < 0.05$). It suggested that *Bacillus* can maintain the body's own oxidation-antioxidant balance of the system, thereby enhancing the antioxidant capacity of the common carp. The concentration of GSH reflects the antioxidant capacity. In our study, the GSH content of treatment group II was the most improved ($P < 0.05$). Addition of the mix of *B.subtilis* and *B.licheniformis* into the feed can increase antioxidant capacity of the common carp. The concentration of MDA reflects the toxic processes caused by free radicals, and the MDA level is considered to be a suitable indicator of the extent of lipid peroxidation. In our study, MDA from all treatment groups was reduced, and treatment III decreased the most ($P < 0.05$). It indicated that by avoiding the accumulation of lipid peroxidation products, the bacillus could prevent the oxidative stress. In general, our results proved that the different combinations of *Bacillus* could improve the antioxidant capacity of the common carp, and then help to improve the immunity, ensure food safety, and increase economic benefits.

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