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Effects of dietary supplementation with *Clostridium butyricum* on the growth performance and humoral immune response in *Miichthys miiuy**

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Abstract: The effects of dietary supplementation with *Clostridium butyricum* on growth performance and humoral immune response in *Miichthys miiuy* were evaluated. One hundred and fifty *Miichthys miiuy* weighing approximately 200~260 g were divided into five groups and reared in 15 tanks with closed circuiting culture system. The animals were fed 5 diets: basal diet only (control) or supplemented of the basal diet with *C. butyricum* at doses of 10^3 (CB1), 10^5 (CB2), 10^7 (CB3) or 10^9 (CB4) CFU/g. Compared with the control, the serum phenoloxidase activity was significantly increased by the supplementation ($P<0.05$), acid phosphatases activity was increased significantly ($P<0.05$) at the doses of 10^9 CFU/g. Serum lysozyme activity peaked at dose of 10^7 CFU/g and in the skin mucus at dose of 10^9 CFU/g. Immunoglobulin M level in the serum and skin mucus was increased except at dose of 10^3 CFU/g ($P<0.05$). The growth at the dose of 10^9 CFU/g was higher than that of the control ($P<0.05$). It is concluded that supplementation of *C. butyricum* can mediate the humoral immune responses and improve the growth performance in *Miichthys miiuy*.

Key words: *Clostridium butyricum*, Growth performance, Humoral immune response, *Miichthys miiuy*
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INTRODUCTION

It is well known that antibiotics are used as effective agents to control diseases and as growth promoters in domestic animals. However, the continuous use of antimicrobials increases the bacterial resistance. Therefore, it is essential to find effective and non-resistance replacements. Probiotics are viable cell preparations that have beneficial effects on health of the host by improving its intestinal balance (Fuller, 1989) via producing nutrients, enhancing immune responses and improving the water quality in aquaculture (Gatesoupe, 1999; Verschuere *et al*., 2000). In recent years, probiotics have been increasingly used in the biological control to prevent diseases in aquaculture (Verschuere *et al*., 2000), which will make aquaculture products more acceptable to consumers.

Clostridium butyricum has been used as probiotics for many years in Eastern Asia countries, such as Japan, Korea and China (Ito *et al*., 1997; Kamiya *et al*., 1997) and is a butyricum-acid producing, spore-forming, gram-positive and oblige anaerobe rod bacterium, found in soil and intestines of healthy animals and humans, and is used clinically to prevent disturbances of microflora in the human intestine and to

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treat diarrhea. *C. butyricum* also has cellular immunostimulatory effects, such as stimulation of production of macrophage and NK (natural killer) cells (Young *et al*., 1987). Sakai *et al*.(1995) demonstrated that enhancement of resistance to *Vibrosis* in rainbow trout by oral administration of *C. butyricum* bacteria was mediated by leucocyte activation, including phagocytosis and increased superoxide anion production. We hypothesize that *C. butyricum* enhances humoral immune response in marine fish. This hypothesis was examined by measuring growth performance and humoral immune response in *Miichthys miiuy* that originally grow in the North Pacific west coast in response to dietary supplementation with *C. butyricum*.

MATERIALS AND METHODS

Bacterial strain and preparation of *C. butyricum*

The bacterial strain *C. butyricum* was isolated from healthy chicken intestine, and identified by biochemistry and molecular experiments in the School of Science, Zhejiang University, China.

C. butyricum was fermented in a 70-liter-fermentation tank (GUJS7A-70, Dongfang Bioengineering Co., China). After 48 h fermentation, *C. butyricum* was collected, centrifuged and freezer-dried. The freezer-dried powder was then enumerated by plate counting on MRS agar (Sigma Chemical Co., USA). The viability of the freezer-dried bacteria was 1.86×10^{11} CFU/g. They were stored at −20 °C until further use. In the supplementation, dried bacteria were added into the basal diet (see below), mixed thoroughly and pelleted into 5 mm diameter (Mingo Tech-Bank Co., Ltd.). Subsequently, the pellets were dried immediately in oven at 50~60 °C, and then stored at −20 °C. Plate counting method was used to detect the number of *C. butyricum* in dried pellets. The viability of *C. butyricum* was approximately 90% in the four dried experimental diets.

Diet preparation and experiment design

The components of a basal diet presented in Table 1 should provide adequate nutrient supply to *Miichthys miiuy*. White fishmeal, soybean meal and protein powder of blood corpuscles were used for sources of protein; fish oil and alpha-starch for non-protein energy. Five treatments consisted of the basal diet only (control) and four doses of supplementation with *C. butyricum* respectively at 10^3 (CB1), 10^5 (CB2), 10^7 (CB3) and 10^9 (CB4) CFU/g in freezer-dried form.

Table 1 Formulation and proximate composition of the basal diet (on dry matter basis)

	Composition (%)
Ingredients	
White fish meal	66.5
Soybean meal	4.75
Alpha-cornstarch	19
Protein powder of blood powder	1.9
Vitamin pre-mixture*	0.675
Mineral pre-mixture*	2
Cellulose	0.175
Fish oil	5
Proximate	
Moisture	10.88
Crude protein	48.45
Ash	9.86
Crude fat	5.14

*Vitamin and mineral pre-mixtures were provided by the Mingo Tech-Bank Co., Ltd. China; Total energy is 15.59 kJ/kg

Experimental animals and rearing facilities

Miichthys miiuy weighing approximately 200~260 g were purchased from net-cage culture in Xihu Port, Zhejiang Province, China and transferred to the experimental laboratory (Institute of Mingo Tech-Bank Co., Ltd.). Upon arrival, after being bathed in penicillin solution, they were assigned to 600-liter-tanks and acclimatized to a circulating rearing system for three weeks, during which the fish were fed the basal diet. Subsequently, 150 healthy, energetic and no-disease fish were put into 15 tanks and divided into 5 groups. Each group was repeated in triplicates. The supplementation lasted for 8 weeks. The fish were fed twice daily at 07:30 a.m. and 17:30 p.m., with the feed offered being about 1.5% of the fish biomass.

The closed circulating rearing system consisted of microorganism-filtration, heating, cooling, protein-separating and ultraviolet sterilization devices. Each tank was a part of the whole system with a common reservoir of water at 29‰~32‰ salinity. The data on pH, DO (dissolved oxygen), temperature, conductance and ORP (oxidation-reduction potential) was recorded and saved in a computer. Water was

circulated 2 times per hour through a separate biofilter to remove impurities and reduce ammonia concentration. Photoperiod was 12 h light (08:00 a.m.~20:00 p.m.) and 12 h dark. Water temperature 25~30 °C was maintained. Total ammonia and nitrite concentrations were maintained at 0.01 mg/L and 0.01 mg/L, respectively.

Sample collection and assays

1. Sampling

At the end of the research, blood was drawn from the caudal vein of the individual fish after anaesthetization (Fengyuan Co., Ltd., China). The blood samples were collected in heparinized tube and plasma was harvested by centrifuging at 1500×g for 5 min at 4 °C. The whole blood was collected in a syringe, allowed to clot for an hour in microtubes at room temperature, followed by five hours at 4 °C and then serum was harvested by centrifuging at 1500×g for 5 min at 4 °C. Both plasma and serum samples were preserved at −20 °C prior to analysis. The plasma samples were used for immunoglobulin M (IgM) analysis and the serum samples for determining the activities of lysozyme, phenoloxidase and acid phosphatase.

The skin mucus sample was collected by scalpels. The mucus was mixed with four volumes of 8.5% salty solution and centrifuged at 1500×g for 5 min at 4 °C. The supernatant was collected for lysozyme and IgM assays.

2. Growth performance

At beginning and end of the feeding trial, the animals were fasted for one day and their weights were determined. Growth performance was expressed as the total weight gain (TWG), daily weight gain (DWG), feed utilization efficiency (FUE), feed conversion ratio (FCR), relative weight gain (RWG) and specific growth rate (SGR). The calculation formulas were as follows:

$$TWG=W_t-W_i,$$
$$DWG=(W_t-W_i)/t,$$
$$RWG=(W_t-W_i)\times 100\%/W_i,$$
$$SGR=(\ln W_t-\ln W_i)/t\times 100\%,$$
$$FUE=TWG\times 100\%/\text{feed consumption},$$
$$FCR=\text{total feed consumption}/TWG,$$

where W_t and W_i are the final and initial mean weight (g), respectively, and t is the feeding trial period (d).

3. Assay

Lysozyme activity in the serum and skin mucus was measured with spectrophotometry based on lysis of freezer-dried particles of *Micrococcus lysodeikticus* (Sigma Chemical Co., USA) (Ellis, 1990). One unit of enzyme activity was defined as the amount of enzyme causing a decrease in absorbance of 0.001 $min^{-1}\cdot ml^{-1}$ serum or skin mucus. Phenoloxidase (PO) activity was measured according to Ashida and Säderhäll (1984) with some modifications. A 0.5 ml sample serum, 0.5 ml phosphate buffer solution (0.1 mol/L, pH=6.0), and 0.5 ml L-DOPA (L-dihydroxyphenylalanine) (0.01 mol/L) were added into a 1.5-ml centrifuge tube. The mixture was kept at 25 °C in water bath for 10 min, and then the OD value was measured at 2-min intervals for 10 min at 490 nm. One unit of enzyme activity was defined as an increase in absorbance of 0.001 $min^{-1}\cdot ml^{-1}$ serum. Acid phosphatase (ACP) activity in serum was measured with spectrophotometry, wherein sodium nitropheny-phosphate was used as substrate (Pipe, 1990). One unit of ACP activity was defined as the amount of enzyme in 100 ml serum necessary to produce 1 mg nitrophenol for 30 min at 37 °C. Levels of total IgM in serum and mucus of body surface were analyzed by enzyme-linked immunosorbent assays (ELISA) according to Bowden *et al*.(2003). The optical density (OD) was measured at 560 nm. The OD-value was directly related to the amount of total IgM.

Statistical analysis

Rank-sum test was used to determine the significant variation in growth ($P\leq 0.05$). One-way analysis of variance (ANOVA) was used to determine the significant variation between the treatments in the humoral immune responses (SYSTAT 10.0, SPSS, USA). The least significant difference (LSD) was used for multiple comparisons, and all tests used a significance level of $P\leq 0.05$.

RESULTS

Growth performance

Table 2 and Table 3 of the results show that *C. butyricum* supplemented to the diet could increase the TWD, RWG, FUE and SGR, decrease the FCR at dose of 10^9 CFU/g ($P<0.05$); and that, the growth

showed increasing tendency with *C. butyricum* dose.

Lysozyme activity of the serum and skin mucus in *Miichthys miiuy*

The lysozyme activity of the serum and the skin mucus in *Miichthys miiuy* are given in Table 4 showing similar increasing tendency with supplementation of the bacteria, which significantly increased at dose of 10^7 and 10^9 CFU/g *C. butyricum* in comparison with the control (P<0.05). Although the lysozyme activity in serum of CB1 and CB2 treatments was higher than those in the mucus, they reached almost the same level in CB3 and CB4.

Activity of phenoloxidase and acid phosphatase in serum of *Miichthys miiuy*

The activities of acid phosphatase and phenoloxidase in serum of *Miichthys miiuy* are given in Table 5. Acid phosphatase activity of serum in *Miichthys miiuy* was significantly higher than control at dose of 10^9 CFU/g (P<0.05) showed significant increase by dietary supplementation of the bacteria from 10^3 CFU/g to 10^9 CFU/g compared with control for phenoloxidase activity (P<0.05).

Total IgM level of serum and the skin mucus in *Miichthys miiuy*

The total IgM level of serum and skin mucus in *Miichthys miiuy* is given in Table 6. Total IgM level of serum peaked at (0.65±0.1) mg/ml for CB3 treat-

Table 2 Effect of *Clostridium butyricum* on RWG, FUE and SGR in *Miichthys miiuy*

	RWG (%)	*FUE* (%)	*SGR* (%)
Control	18.0±0.34^{b}	28.00±2.10^{b}	0.31±0.01^{b}
CB1	26.9±2.2^{ab}	37.80±1.60^{ab}	0.43±0.03^{ab}
CB2	29.1±8.1^{ab}	40.87±10.6^{ab}	0.45±0.10^{ab}
CB3	36.3±1.3^{ab}	49.20±16.4^{ab}	0.55±0.17^{ab}
CB4	39.2±7.1^{a}	54.20±12.2^{a}	0.58±0.09^{a}

Values with different superscript letters are significantly different (P<0.05); Values are means±*SD* (n=3)

Table 3 Effect of *Clostridium butyricum* on the TWG, DWG and FCR in *Miichthys miiuy*

	W_i (g)	W_t (g)	*TWG* (g)	*DWG* (g)	*FCR*
Control	258±17	306.5±18	48.5±3.5^{b}	0.86±0.06^{b}	3.40±0.25^{b}
CB1	238±12	301.5±20	63.5±2.7^{ab}	1.13±0.05^{ab}	2.64±0.11^{ab}
CB2	236±11	304.7±16	68.7±17.8^{ab}	1.20±0.30^{ab}	2.93±0.93^{ab}
CB3	228±14	310.1±14	82.1±11.0^{ab}	1.47±0.49^{ab}	2.18±0.68^{ab}
CB4	231±12	322.3±13	97.3±20.0^{a}	1.62±0.36^{a}	1.89±0.39^{a}

Values with different superscript letters are significantly different (P<0.05); Values are means±*SD* (n=3)

Table 4 Effect of *Clostridium butyricum* on lysozyme activity of the serum and skin mucus in *Miichthys miiuy* (U/ml)

	Control	CB1	CB2	CB3	CB4
Serum	333±35^{b}	475±28^{b}	526±33^{b}	813±45^{a}	782±36^{a}
Skin mucus	140±38^{b}	156±46^{b}	317±29^{b}	716±35^{a}	786±35^{a}

Values with different superscript letters are significantly different (P<0.05); Values are means±*SD* (n=3)

Table 5 Effect of *Clostridium butyricum* on activity of phenoloxidase and acid phosphatase in serum of *Miichthys miiuy* (U/ml)

	Control	CB1	CB2	CB3	CB4
Acid phosphatase	6.90±1.4^{b}	6.6±0.4^{b}	7.1±1.2^{b}	7.4±0.4^{b}	8.9±0.8^{a}
Phenoloxidase	3.90±0.4^{d}	13.6±1.2^{c}	18.9±0.7^{b}	25.9±1.8^{a}	26.6±0.7^{a}

Values with different superscript letters are significantly different (P<0.05); Values are means±*SD* (n=3)

Table 6 Effect of *Clostridium butyricum* on total IgM levels in serum and skin mucus in *Miichthys miiuy* (mg/ml)

	Control	CB1	CB2	CB3	CB4
Serum	0.16±0.06^{c}	0.29±0.03^{bc}	0.62±0.05^{a}	0.65±0.10^{a}	0.42±0.07^{b}
Skin mucus	0.11±0.01^{c}	0.20±0.04^{b}	0.20±0.05^{ab}	0.26±0.04^{a}	0.17±0.02^{c}

Values with different superscript letters are significantly different (P<0.05); Values are means±*SD* (n=3)

ment and was significantly different from the control ($P<0.05$), while there were no significant differences among CB2, CB3 and CB4 treatment. Although there was similar variation tendency of the serum and skin mucus IgM level, obviously the total IgM level in serum was always higher than that in skin mucus.

DISCUSSION

Probiotics used as the alternative of antibiotics to promote fishes and shrimp growth was shown in (Wang and Xu, 2006; Ziaei-Nejad *et al*., 2006; Lara-Flores *et al*., 2003). In the present experiment, *C. butyricum* could decrease the FCR (feed conversion ratio) and improve *Miichthys miiuy* growth. The results were consistent with the results of previous researches. Effects of *C. butyricum* to promote the growth have been demonstrated early in chicken (Han *et al*., 1984). A compound additive containing protease, lipase, lactic acid and *C. butyricum* was proved to increase the protein and fat absorption, decrease feed conversion ratio and reduce disease in fish (Yang, 1998). Therefore, *C. butyricum* used singly as the additive or mixed with other additives could improve animal growth.

Lysozyme is one of the major components in the immune defense system in both invertebrates and vertebrates and can split peptidoglycan in bacterial cell walls especially of the gram-positive species and cause lysis of the cells (Chipman and Sharon, 1969). It exists in the mucus, lymphoid tissue, plasma and other body fluids of most fish (Grinde *et al*., 1988; Grinde, 1989; Yousif *et al*., 1994) and plays an important role in defense against pathogens invasion. Lysozyme concentrations in fish have been reported to be increased after injection of a bacterial product (Chen *et al*., 1996) and in response to bacterial infection (Møyner *et al*., 1993). Panigrahi *et al*.(2004) demonstrated that the *Lactobacillus rhamnosus* JCM 1136 could modulate the lysozyme activity in fish. In his study, both LAB (*Lactobacillus rhamnosus*) fed groups showed elevated level of lysozyme activity, but the group receiving the higher density was observed to have significantly higher lysozyme activity compared with that of the control, indicating activation of the immune system. The present study is the first report that lysozyme activity in marine fish was increased by dietary supplementation with *C. butyricum* and showed increasing tendency with supplemented dose. The mechanism that *C. butyricum* could increase lysozyme activity in *Miichthys miiuy* is not clear.

Phenoloxidase (PO) is known to be a defense enzyme, and serves as a non-self recognition system in the host defense reactions, and is usually an indicator of the immune response in shrimp and crab. The present study showed that phenoloxidase is detectable in fish serum and enhanced by *C. butyricum* supplementation. Phenoloxidase usually exists as an inactive precursor (proPO) (Smith and Säderhäll, 1991; Asokan *et al*., 1997), which can readily be activated to phenoloxidase (PO) by an endogenous activating system or exogenous elicitors such as trypsin, lipopolysaccharide and zymosan (β-1,3-glucan) (Asada *et al*., 1993; Sugumaran and Nellaiappan, 1991). Therefore, it could be assumed that *C. butyricum* might trigger proPO activation via a limited proteolytic cleavage of the proenzyme as exogenous elicitor.

Acid phosphatase, an enzyme localized within lysosomes, is important for intracellular digestion of phagocytized antigens, has been used as a marker of macrophage activation in mammalian models and invertebrates. In the present study, it was also observed that an increase in acid phosphatase activity occurred in *Miichthys miiuy* when they were fed with *C. butyricum* at the dose of 10^9 CFU/g ($P<0.05$), which indicated that *C. butyricum* could stimulate immune response.

Immunoglobulins are well recognized to provide protection in animals and human beings against various diseases. In teleost fish, two types of immunoglobulin (belonging to the IgM and IgD subpopulations) have been characterized (Watts *et al*., 2001), with IgM being the main immunoglobulin which resembles mammalian IgM in both structure and physiological characteristics. IgM is present in blood and other body fluids, and plays a role as an immune effector molecule (Ross *et al*., 1998). Some studies have shown the effects of probiotics on immunoglobulin secretion. Perdigon *et al*.(1990) reported an increase of IgA in the intestine via an oral administration of lactic acid bacteria which protects mice against *Salmonella typhmirium*. However the LAB feeding resulted in a higher total Ig level in

rainbow trout without significant differences in comparison with the control (Panigrahi *et al*., 2004). In this study, dietary supplementation with *C. butyricum* at higher dose treatment ($P<0.05$) caused secretion of IgM in the blood sera and the skin mucus in comparison with the control. Stimulatants, such as vitamin A, chitin, yeast cells or levamisole supplemented to the diet resulted in the increase of IgM level (Cuesta *et al*., 2004). Therefore, it was assumed that *C. butyricum* might be an effective immune stimulatant in *Miichthys miiuy*.

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