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Effects of dietary supplementation of *Bacillus subtilis* and fructooligosaccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, *Larimichthys crocea*

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ABSTRACT

A feeding experiment was conducted to examine the effects of dietary administration of *Bacillus subtilis* and fructooligosaccharide (FOS) on growth performance, survival, immune responses and disease resistance of juvenile large yellow croaker, *Larimichthys crocea* (mean initial body weight 7.82 g ± 0.68). Nine practical diets were formulated to contain three levels of *B. subtilis* (0.0, 0.42×10^7 cfu g^{-1} and 1.35×10^7 cfu g^{-1}), each with three FOS levels (0, 0.2% and 0.4% of dry weight). Each diet was randomly assigned to triplicate groups of 60 juveniles. The experiment was conducted in floating sea cages ($1.0\times1.0\times1.5$ m) for 10 weeks. At the termination of the feeding trial, alternative complement pathway (ACP), superoxide dismutase (SOD) and lysozyme activity of serum and respiratory burst activity of head kidney macrophage were determined and fishes were challenged intraperitoneally with *Vibrio harveyi*. The results showed that at each dietary FOS level, dietary supplementation of 1.35×10^7 cfu g^{-1} *B. subtilis* significantly increased the specific growth rate (SGR) ($P<0.01$) and feed efficiency ratio (FER) ($P<0.05$) compared with the groups without *B. subtilis* supplementation. The immune assay showed that at each FOS level, compared to the groups without *B. subtilis* supplementation significantly enhanced serum lysozyme was observed in fish fed the *B. subtilis*-supplemented diets ($P<0.05$) and significantly enhanced serum SOD activity was observed in fish fed the diet with 1.35×10^7 cfu g^{-1} *B. subtilis* ($P<0.05$), while the serum ACP activity and the respiratory burst activity of head kidney macrophage were independent of dietary treatments. The challenge experiment showed that compared to the groups without *B. subtilis* supplementation the cumulative mortality after infection with *V. harveyi* was significantly lower in fish fed the diet with 1.35×10^7 cfu g^{-1} *B. subtilis* ($P<0.05$), at each FOS level. However, at each dietary *B. subtilis* level, addition of FOS in diets did not significantly affect the growth performance, immune response and disease resistance of large yellow croaker. No significant interactions were observed between dietary *B. subtilis* and FOS. These results showed that dietary supplementation of *B. subtilis* at a dose of 1.35×10^7 cfu g^{-1} improved growth, feed efficiency ratio, non-specific immune responses and disease resistance of juvenile large yellow croaker, *L. crocea*.

1. Introduction

Over the years as intensive aquaculture expanded and culture density increased, diseases occurred more frequently. Moreover, the application of antibiotics caused many other problems such as the spread of drug resistant pathogens, environmental hazards and food safety problems. This situation resulted in increasing interests in the alternatives for the antibiotics. Probiotics and prebiotics, which have various health promoting properties and minor adverse side effects, are gaining increasing scientific and commercial interest in aquaculture practice.

In aquaculture, probiotics are usually live microorganisms which confer health benefits on host when administered via the feed or to the rearing water. The beneficial effects of probiotics, such as improvement of feed utilization, modulation of intestinal microflora, enhancement of immune responses and antagonism to pathogens, have been demonstrated in a number of previous studies (Balcázar et al., 2006; Irianto and Austin, 2002; Kesarcodi-Watson et al., 2008; Merrifield et al., 2010; Nayak, 2010; Wang et al., 2008b). Among the various benefits of probiotics, immunomodulatory activity is noteworthy in improving the overall health status of the host. However, there is limited research available for immunomodulatory activity of probiotics, especially for the long-term use of probiotics in fish diets. The most commonly used probiotics in aquaculture practices belong to lactic acid bacteria and *Bacillus* spp. (Wang et al., 2008b). When applied as probiotics, the spore-forming ability of the *Bacillus* species allows greater viability after pelleting and high resistance to gastric

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conditions (Casula and Cutting, 2002; Hong et al., 2005; Hyronimus et al., 2000). The probiont bacterium *Bacillus subtilis* has been reported to have various beneficial attributes when supplemented in fish diets (Aly et al., 2008; Kumar et al., 2008; Nayak et al., 2007; Newaj-Fyzul et al., 2007; Salinas et al., 2005, 2008).

Prebiotics have been defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the intestinal tract, and thus improves host health" (Gibson and Roberfroid, 1995). Contrary to the wide use of prebiotics in human and terrestrial animals, less information is available about the effects of prebiotics on aquatic animals (Merrifield et al., 2010; Ringø et al., 2010), especially on the immune responses of fish. Fructooligosaccharide (FOS) is one of the most common prebiotics studied in humans and terrestrial animals (Kapiki et al., 2007; Kelly-Quagliana et al., 2003; Reid, 2008; Swanson et al., 2002; Trevisi et al., 2008). However, to date, few studies concerning the use of FOS in aquatic animals, especially in fish are available (Mahious et al., 2006). The limited amount of work performed in turbot larvae (*Psetta maxima*) (Mahious et al., 2006), white shrimp (*Litopenaeus vannamei*) (Li et al., 2007; Zhou et al., 2007) and sea cucumber (*Apostichopus japonicus*) (Zhang et al., 2010) suggested that FOS beneficially affected the host by improving growth, regulating gastrointestinal microbiota composition and enhancing immune responses.

A synbiotic is defined as a combination of a probiotic and a prebiotic. The synbiotics are presumed to impart the beneficial effects of both ingredients. Limited data is available regarding the application of synbiotics in aquaculture (Daniels et al., 2010; Li et al., 2009; Rodriguez-Estrada et al., 2009; Zhang et al., 2010). The aim of the present study was to study the effects of individual and combined supplementation of a potential probiotic *B. subtilis* and the prebiotic FOS on growth performance and immune responses of large yellow croaker (*Larimichthys crocea*) which is widely cultured in China. Large yellow croaker is an important species and its industry is being threatened by increased prevalence of disease.

2. Materials and methods

2.1. Experimental diets

The basal practical diet was formulated to contain approximately 44% crude protein and 14% lipid, which have been shown to be sufficient to support the optimal growth of large yellow croaker. A *B. subtilis* preparation (*B. subtilis* spores content, approximate 1.0×10^{10} cfu g^{-1}; Qingdao Master Bio-Tech Co., Ltd, China) was supplemented separately to the basal diet at the expense of wheat meal to obtain 0.00, 0.42×10^{7} and 1.35×10^{7} (cfu g^{-1}) *B. subtilis* respectively and at each *B. subtilis* level a fructooligosaccharide preparation (purity, 95.48%; kestose% + nystose% + fructofuranosyl nystose% > 95%; Shandong Baolingbao Biotechnology Co., Ltd, China) was supplemented separately at the expense of wheat meal to obtain 0, 0.2% and 0.4% (of dry weight) FOS, respectively (Table 1).

Ingredients were ground into fine powder through 200 μm mesh. All ingredients were thoroughly mixed with fish oil and soybean oil, and water was added to produce stiff dough. The dough was then pelleted with an experimental feed mill and dried for about 12 h in a ventilated oven at 60 °C. After drying, the diets were broken up and sieved into proper pellet size (1.5×5.0 mm, 2.5×5.0 mm), and were stored at −15 °C until used.

2.2. Experimental procedure

Large yellow croaker juveniles were obtained from a commercial farm in Ningbo, China. Prior to the start of the experiment, the juveniles were reared in floating sea cages ($3.0\times3.0\times3.0$ m), and fed Diet 1 for 2 weeks to acclimate to the experimental diet and conditions.

At the initiation of the experiment, the fish were fasted for 24 h and weighed after being anesthetized with eugenol (1:10,000) (Shanghai Reagent, China). Fish of similar sizes (7.82 g ± 0.68) were randomly distributed into 27 sea cages ($1.0\times1.0\times1.5$ m) and each cage was stocked with 60 fish. Each diet was randomly assigned to

Table 1
Formulation and chemical proximate composition of the experimental diets (% dry matter).

Ingredient	Diet NO. (*Bacillus subtilis*/fructooligosaccharide (FOS) supplementation level cfu g^{-1}/%)								
	Diet 1 (0/0)	Diet 2 (0.42×10^{7}/0)	Diet 3 (1.35×10^{7}/0)	Diet 4 (0/0.2)	Diet 5 (0.42×10^{7}/0.2)	Diet 6 (1.35×10^{7}/0.2)	Diet 7 (0/0.4)	Diet 8 (0.42×10^{7}/0.4)	Diet 9 (1.35×10^{7}/0.4)
Fish meal[1]	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00
Soybean meal[1]	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00
Wheat meal	24.56	24.46	24.36	24.35	24.25	24.15	24.14	24.04	23.94
Fish oil	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Soybean oil	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Lecithin	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Mineral premix[2]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix[3]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Attractant[4]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Mold inhibitor[5]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
B. subtilis preparation[6]	0.00	0.10	0.20	0.00	0.10	0.20	0.00	0.10	0.20
FOS preparation[7]	0.00	0.00	0.00	0.21	0.21	0.21	0.42	0.42	0.42
Proximate analysis									
Crude protein (%)	43.24	43.45	42.84	43.48	43.55	42.76	42.08	42.48	42.17
Crude lipid (%)	14.14	14.84	13.41	13.79	14.45	13.53	14.84	13.91	14.76
Ash (%)	12.86	12.93	12.90	14.05	14.05	13.45	14.17	14.10	13.87

[1] Fish meal: crude protein 69.7% dry matter, crude lipid 7.1% dry matter; soybean meal: crude protein 53.3% dry matter, crude lipid 1.9% dry matter.
[2] Mineral premix (mg or g/kg diet): NaF, 2 mg; KI, 0.8 mg; $CoCl_2\cdot6H_2O$ (1%), 50 mg; $CuSO_4\cdot5H_2O$, 10 mg; $FeSO_4\cdot H_2O$, 80 mg; $ZnSO_4\cdot H_2O$, 50 mg; $MnSO_4\cdot H_2O$, 60 mg; $MgSO_4\cdot7H_2O$, 1200 mg; $Ca(H_2PO_3)_2\cdot H_2O$, 3000 mg; zoelite, 15.55 g.
[3] Vitamin premix (mg or g/kg diet): thiamin, 25 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; vitamin B_{12}, 0.1 mg; vitamin K_3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin, 1.20 mg; retinol acetate, 32 mg; cholecalciferol, 5 mg; alpha-tocopherol, 120 mg; ascorbic acid, 2000 mg; choline chloride, 2500 mg; ethoxyquin, 150 mg; wheat middling, 18.52 g.
[4] Attractant: glycine and betaine.
[5] Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.
[6] *B. subtilis* preparation: *B. subtilis* spores content, approximate 1.0×10^{10} cfu g^{-1}; Qingdao Master Bio-Tech Co., Ltd, China.
[7] FOS preparation: purity, 95.48%; kestose% + nystose% + fructofuranosyl nystose% > 95%; Shandong Baolingbao Biotechnology Co., Ltd, China.