Fish & Shellfish Immunology 46 (2015) 225–231

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effects of dietary *Bacillus licheniformis* on growth performance, immunological parameters, intestinal morphology and resistance of juvenile Nile tilapia (*Oreochromis niloticus*) to challenge infections

Biao Han [a, b], Wei-qing Long [c], Ju-yun He [a, b], Yong-jian Liu [a, b], Yu-qi Si [a, b], Li-xia Tian [a, b, *]

[a] Nutrition Laboratory, Institute of Aquatic Economic Animals, School of Life Science, Sun Yat-sen University, No. 135, Xin-gang Xi Road, Guangzhou 510275, PR China
[b] South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, No. 135, Xin-gang Xi Road, Guangzhou 510275, PR China
[c] Department of Clinical Laboratory, The First Affiliated Hospital of Sun Yat-sen University, No. 58, Zhongshan Er Road, Guangzhou 510080, PR China

ARTICLE INFO

Article history:
Received 27 November 2014
Received in revised form
19 May 2015
Accepted 15 June 2015
Available online 21 June 2015

Keywords:
Bacillus licheniformis
Growth
Immunity
Intestinal morphology
Streptococcus iniae
Oreochromis niloticus

ABSTRACT

The effects of oral administration of *Bacillus licheniformis* on growth performance, immunity, intestinal morphology and disease resistance of juvenile tilapia were investigated. Six experimental diets supplemented with different concentrations of *B. licheniformis* (0%, 0.02%, 0.04%, 0.06%, 0.08% and 0.1% of AlCare®, containing live germ 2×10^{10} CFU/g) were formulated, viz. control, T_1, T_2, T_3, T_4 and T_5. Each diet was randomly assigned to triplicate groups of 30 fishes (3.83 ± 0.03 g). After 10 weeks of feeding trial, weight gain (WG), final body wet weight (FBW) and specific growth rate (SGR) increased significantly in groups T_2, T_3, T_4 and T_5 compared with control and T_1 ($p < 0.05$). However, survival rate and feed conversion ratio (FCR) were not found to be significantly affected ($P > 0.05$). Compared with control, dietary *B. licheniformis* supplementation increased the content of complement C3 in serum significantly ($P < 0.05$). The lysozyme activity was observed to be highest in T_2 ($P < 0.05$) without differences among other groups. However, SOD activity was not affected by *B. licheniformis* supplementation ($P > 0.05$). When tilapia were challenged against *Streptococcus iniae*, survival rate improved significantly when tilapia fed with T_2, T_3, T_4 and T_5 ($P < 0.05$). Although there was no significant differences in villi length and muscular layer thickness of anterior intestinal among the treatments, intestinal villi of fish fed with higher concentrations of *B. licheniformis* (T_2, T_3, T_4, T_5) tended to be regularly arranged and exhibited less exfoliation, twist and fusion. These results indicated that dietary supplementation of *B. licheniformis* not only increased the growth, immune response and disease resistance of juvenile tilapia, but also influenced anterior intestinal development and integrity. Furthermore, in our study, the optimal concentration of *B. licheniformis* in diets for tilapia was greater than or equal to 4.4×10^6 CFU/g.

1. Introduction

Tilapia is one of the most important cultured fish species around the globe with high economic value. After being introduced to China in 1987, Nile tilapia (*Oreochromis niloticus*) is popularly farmed on a big scale due to its fast-growing rate, short feeding cycle, improved disease resistance and delicious taste [1]. However, diseases caused by *Streptococcus iniae*, which had been identified by the American Tilapia Association as one of the most important pathogen in tilapia, bring out massive mortality and economic losses in aquaculture [2–4]. During recent decades, prevention and control of diseases have led to a substantial increase in the use of antibiotics and chemical disinfectants. However, the abuse of antibiotics resulted in antimicrobial resistance among pathogenic bacteria and environmental damage [5–7]. The use of probiotics or beneficial bacteria, which confer a health effect on the host, is increasingly viewed as an alternative to antibiotic treatment in aquaculture [8–11].

As one of the commonly studied probiotics, *Bacillus* spp. have been shown to possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immunostimulation [12–14]. *B.* spp. hold added interest in probiotics as they can be kept in the

* Corresponding author. Nutrition Laboratory, Institute of Aquatic Economic Animals, School of Life Science, Sun Yat-sen University, No. 135, Xin-gang Xi Road, Guangzhou 510275, PR China.
E-mail address: lixiatian2002@163.com (L.-x. Tian).

http://dx.doi.org/10.1016/j.fsi.2015.06.018
1050-4648/

spore form and therefore stored indefinitely on the shelf [15]. Balcázar et al. [16] demonstrated that the administration of a mixture of bacterial strains (*Bacillus* and *Vibrios* spp.) positively influenced the growth and survival of juvenile white shrimp, presented a protective effect against the pathogens *Vibrio harveyi* and white spot syndrome virus. In addition, many researches have demonstrated that *B.* spp. used as dietary supplementation or water additive could significantly increase growth rate, enhance the disease resistance of shrimp/fish by suppressing the pathogens, enhancing immunity and improving water quality [17–23]. *Bacillus B. licheniformis* is a gram-positive, oxidase-positive and catalase-positive endospore forming non-pathogenic bacterium belonging to the genus *Bacillus* [24]. It has shown to act as an antiviral and immunoregulatory agent and has been reported as a probiotic bacterium in terrestrial animals [25]. However, to the best of our knowledge, there is limited information concerning the application of *Bacillus licheniformis* in freshwater fish. Moreover, Merrifield at al [26]. revealed that *Bacillus B. subtilis* could improve intestinal microvilli structure, absorptive surface area and improve gut healthy in rainbow trout. Therefore, the present study was carried out to evaluate the effect of *B. licheniformis* on growth, immune responses, intestinal morphology and disease resistance against *Streptococcus iniae* of juvenile tilapia (*O. niloticus*).

2. Materials and methods

2.1. Experimental design and diets

In this study, 0%, 0.02%, 0.04%, 0.06%, 0.08% and 0.1% *Bacillus B. licheniformis* (AlCare®, Zoetis, Shanghai, China, containing live germ 2×10^{10} CFU/g) were supplemented respectively to formulate six experimental diets, viz. Control, T_1, T_2, T_3, T_4 and T_5. Proximate composition analysis were given in Table 1. All ingredients were ground through a 320-um mesh, weighed precisely and mixed thoroughly with the oils. The pellets (2.5 × 5.0 mm) were prepared using pelletizer (Institute of Chemical Engineering, South China University of Technology, Guangzhou, PR China) and dried naturally. The diets were labeled, packed in bags accordingly and stored at −20 °C until use. Crude protein, crude lipid, moisture and crude ash in diets were determined by standard methods (AOAC 1995) [27].

2.2. Detection of the viable cell count

The total live germ counting in the diets was assessed by spread plate method [28]. In brief, the samples (diets and AlCare®) were weigh out 1 g to be tested (accurate to 0.01 g), placed in a flask with glass beads and 99 mL sterile water, then mixed it by turbine mixer for 10 min, ultrasound for 10 min in the ultrasonic cleaner, and the solution is 1:10 diluted bacteria suspension. Transfer 1 ml of the above cell suspension into 9 ml sterile water using sterile pipette gun, mix thoroughly and obtain $1{:}10^3$ diluted bacteria suspension. Follow this method to obtain $1{:}10^7$, $1{:}10^8$ and $1{:}10^9$ dilution gradient of the bacteria suspension. For each diet, take $1{:}10^5$, $1{:}10^6$ and $1{:}10^7$ consecutive dilutions and for AlCare®, take $1{:}10^8$, $1{:}10^9$ and $1{:}10^{10}$ consecutive dilutions, three petri dishes each dilution, culture at 37 °C for 24–48 h, the results were given in Table 2. Calculating the average colony number of the triplicates of the same dilution using the following formulas.

$$\text{Total live germ counting (cfu/g)} = \text{average number of colonies} \times \text{dilution factor}$$

2.3. Fish

The study was carried out at Marine Aquarium, Sun Yat-Sen University, Zhuhai, China. Approximate one thousand apparently healthy juvenile tilapias were collected from a commercial hatchery (Panyu, China) and acclimated for two weeks with commercial

Table 2
Concentrations of *B. licheniformis* in the diets and AlCare®.

(CFU/g)	Theoretical values	Measured values
AlCar®	2.0×10^{10}	2.1×10^{10}
Control	nd	$\leq 1.0 \times 10^3$
T_1	2.0×10^6	2.1×10^6
T_2	4.0×10^6	4.4×10^6
T_3	8.0×10^6	5.0×10^6
T_4	1.0×10^7	1.3×10^7
T_5	2.0×10^7	2.1×10^7

nd: not detected.

Table 1
Composition and proximate analysis of the experimental diets.

Ingredients (g kg^{-1} diet)	Experimental diets					
	Control	T_1	T_2	T_3	T_4	T_5
Fish meal	20	20	20	20	20	20
Soybean meal	200	200	200	200	200	200
Cottonseed meal	250	250	250	250	250	250
Canada rapeseed meal	200	200	200	200	200	200
Wheat flour	199.9	199.7	199.5	199.3	199.1	198.9
Rice bran	70	70	70	70	70	70
Soy oil	20	20	20	20	20	20
Soy lecithin	10	10	10	10	10	10
Choline chloride	20	20	20	20	20	20
[a]Vitamin and Mineral mix	10	10	10	10	10	10
Yttrium (III)-Oxide (Y_2O_3)	0.1	0.1	0.1	0.1	0.1	0.1
B.licheniformis (2×10^{10} CFU/g)	0	0.2	0.4	0.6	0.8	1
Proximate analysis (g kg^{-1} diet, %dry weight)						
Moisture (%)	92.4	92.4	92.7	93.3	93.0	93.2
Crude protein (%)	340	337	335	334	335	337
Crude lipid (%)	29.8	30.1	29.8	29.5	29.6	29.6
Ash (%)	73.6	73.0	73.3	72.8	74.0	73.9

[a] Vitamin and Mineral mix (IU or mg g^{-1} of diet): vitamin A, 6000 IU; vitamin D3, 5600 IU; vitamin E, 0.04; vitamin K3, 10; vitamin B1, 9; vitamin B2, 18; vitamin B6, 12; vitamin B12, 0.04; vitamin C, 140; niacin, 70; biotin, 0.16; folic acid, 3.2; D-calcium pantothenate, 40; Magnesium, 100; iron, 70; manganese, 13.3; iodine, 2.24; copper, 10.5; zinc, 56; selenium, 0.3; cobalt, 1.75.