Aquaculture 442 (2015) 29–36

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Effects of heat killed *Lactobacillus plantarum* (LP20) supplemental diets on growth performance, stress resistance and immune response of red sea bream, *Pagrus major*

Mahmoud A.O. Dawood [a,c], Shunsuke Koshio [b,*], Manabu Ishikawa [b], Saichiro Yokoyama [b]

[a] The United Graduate School of Agriculture Sciences, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0056, Japan
[b] Laboratory of Aquatic Animal Nutrition, Faculty of Fisheries, Kagoshima University, 4-50-20, Kagoshima 890-0056, Japan
[c] Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, 33516 Kafrelsheikh, Egypt

ARTICLE INFO

Article history:
Received 29 September 2014
Received in revised form 3 February 2015
Accepted 4 February 2015
Available online 11 February 2015

Keywords:
Heat killed bacteria
Lactobacillus plantarum
Red sea bream
Growth performance
Blood parameters
Immune response

ABSTRACT

A feeding trial was carried out to determine effects of heat-killed *Lactobacillus plantarum* (HK-LP) for red sea bream (*Pagrus major*). Five dietary levels of a commercial product containing 20% HK-LP at 1, 10, 100, 1000, and 2000 mg kg^{-1} diets were supplemented to the basal diet (control), respectively. Triplicate groups of fish (initial weight: 11 g) were stocked in 100-L polycarbonate circular tanks at a density of 12 fish per tank under the flow-through system, and were fed the respective test diets for 56 days. At the end of a feeding trial, the results showed that the fish fed the diet at 10, 100, 1000 and 2000 mg kg^{-1} HK-LP significantly grew faster than control group. Similarly, significantly improved feed intake, feed efficiency ratio, protein retention, and apparent digestibility coefficients were also found at 1000 mg kg^{-1} HK-LP group than those in HK-LP free group. Some parameters such as serum lysozyme activity, total serum protein, mucus secretion, and the tolerance against low salinity stress were improved in fish fed 1000 mg kg^{-1} HK-LP compared to those in HK-LP free group. This study demonstrated that HK-LP enhanced non-specific immune defense system of red sea bream, providing them with higher resistance to the stress and better immune response.

1. Introduction

The success of modern aquaculture requires good management practices. In aquaculture, antibiotics and chemotherapy have been applied to prevent disease outbreaks and control proliferation of pathogens, causing proliferation of bacteria resistant to drugs for a long time (Miranda and Zemelman, 2002). More recently, the administration of probiotics to fish seems to be a very promising control measure for the fish farms (Kesarcodi-Watson et al., 2008). Some concerns may arise in aquaculture due to the oral delivery of probiotics that may introduce live bacteria into the environment. Thus, the use of inactivated bacteria shed a light on such a safety-related issue since they can no longer interact with other aquatic organisms (Díaz-Rosales et al., 2006). Currently, the definition of probiotics includes the metabolites of live or dead bacterial cells, which function as immunostimulants for modification of enzyme activity or microflora in gastrointestinal tracts that have beneficial effects on host health (Naidu et al., 1999; Salminen et al., 1999). Several works reported increased disease resistance (Giri et al., 2014; Son et al., 2009) and stronger immune responses (Giri et al., 2013; Salinas et al., 2005) in fish fed live bacteria supplemented diets. On the other hand, dietary supplementation of inactivated bacteria stimulated fish innate immune parameters (Biswas et al., 2013a; Cerezuela et al., 2012; Díaz-Rosales et al., 2006) and increased disease resistance (Biswas et al., 2013b; Pan et al., 2008). Inactivated probiotic preparations appear as an interesting alternative to live probiotics, which could potentially cause safety problems in open aquatic environments. A comparative studies between killed and live bacteria on immune responses showed that heat killed *Clostridium butyrium* retain interesting immunomodulating properties on Chinese drum, *Miichthys miiuy* (Pan et al., 2008), and heat killed bacterins of *V. vulnificus* induced a better antibody response than that induced by formalinised bacterins in flounder, *P. olivaceus* (Park et al., 2001).

Lactobacillus plantarum is a gram-positive, heterofermentative lactic acid bacterium. The bacterium has a high adapting capacity to many environmental conditions, and is effective in suppressing the growth of pathogenic and spoilage microorganisms by secreting bacteriocin in food. In aquaculture, administration of live *L. plantarum* induced immune modulation, enhanced the growth performance, immune ability, and increased disease resistance in fish (Giri et al., 2013, 2014; Son et al., 2009).

Heat-killed *L. plantarum* (HK-LP) was used as an immunostimulant to induce interleukin-12 production and antitumor effect in mice, and to enhance gamma interferon production, which stimulates a substance that suppresses virus reproduction and other T-cells and activates macrophages cells (Murosaki et al., 1998, 2000). Daily intake of HK-LP as an immunostimulant enhanced immunity response in healthy adults

* Corresponding author.
E-mail address: koshio@fish.kagoshima-u.ac.jp (S. Koshio).

http://dx.doi.org/10.1016/j.aquaculture.2015.02.005
0044-8486/

(Hirose et al., 2006) and broiler chickens (Khonyoung and Yamauchi, 2012). Moreover, daily intake of HK L-137 stimulates innate immunity for production of type I interferon (IFN) in humans and pigs (Arimori et al., 2012). In aquatic animals, Tung et al. (2010) concluded that the stress resistance is higher in larval and post larval kuruma shrimp, *Marsupenaeus japonicas* fed a diet containing HK-LP. Recently, Biswas et al. (2013a) reported the potential effects of HK-LP (strain 06CC2) isolated from the Mangolian dairy products as novel immunostimulant to fish. In the light of these observations, it could be hypothesized that HK-LP might be effective in responses of growth and biological defense systems of fish species. At the same time, the collective data on the effects of HK-LP should be investigated to find the effective use of HK-LP for marine species.

Red sea bream (*Pagrus major*) is one of the most economically cultured warm water marine fish in Japan as well in the world. Until now, there are no studies on the application of probiotics for red sea bream. Therefore, this study aims to investigate the effect of the oral administration of HK-LP on the growth, survival, immune response, and stress resistance of juvenile red sea bream.

2. Materials and methods

2.1. Preparation of HK-LP

HK-LP Prep (LP20) was made by House Wellness Foods Corp. (Itami, Japan), and it contains 20% HK-LP and 80% dextrin in dried-weight basis. The concentration of HK-LP in the dry product is 2×10^{11} cfu/g. HK-LP Prep was prepared based on the method previously described (Murosaki et al., 1998). The product was stored at −20 °C until use.

2.2. Test fish and experimental system

The feeding trial was carried out at the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The juvenile red sea bream, *Pagrus major*, were purchased from a private fish hatchery (Ogata Suisan Co, Kumamoto, Japan). The fish were acclimatized for two weeks in the laboratory condition and reared in a 500-L tank with flow-thru system. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. The feeding trial was conducted in 100-L polycarbonate tanks (filled with 80 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period. During the experimental period, the monitored water quality parameters (mean ± S.D.) were: water temperature 26.1 ± 1.7 °C; pH 8.1 ± 0.5 and salinity 33.1 ± 0.5. These ranges are considered within optimal values for juvenile red sea bream.

2.3. Test diets

The formulation and chemical composition of the experimental diets are shown in Tables 1 and 2 respectively, which followed (Yokoyama et al., 2005; Ren et al., 2008) with slight modification. Protein source was brown fishmeal which contained 67% of crude protein and 8% of crude lipid and casein which contained 87% of crude protein. The lipid sources were Pollack liver oil and soybean lecithin. Activated gluten was used as a binder to produce pellet diet. α-starch and dextrin was the carbohydrate source in the diets. HK-LP Prep was supplied in the diets with six levels: 0 (as control diet), 1, 10, 100, 1000, and 2000 mg kg^{-1} diet. Cellulose powder was used to adjust to 100% total proportion. HK-LP Prep was thoroughly mixed with lipid before adding other ingredients. Pollack liver oil, soybean lecithin and HK-LP Prep were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 15 min.

Table 1
Basal diet composition.

Ingredients	g/kg dry diet
Brown fish meal[1]	280
Casein[2]	280
Dextrin[3]	70
α-Starch[4]	60
Soybean lecithin[5]	50
Pollack liver oil[6]	60
Vitamin mixture[7]	30
Mineral mixture[8]	30
Stay-C[9]	0.8
Activated gluten[10]	50
α-Cellulose + HK-LP Prep.[11]	89.2

[1]Nippon Suisan Co. Ltd (Tokyo, Japan), [2]Wako Pure Chemicals Industries, Inc. (Osaka, Japan), [3]Kanto Chemicals (Tokyo, Japan), [4]Asahi Chemicals (Wakayama, Japan), [5, 6]Riken Vitamin, Tokyo, Japan, [7]Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01; Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92, [8]Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04, [9] L-Ascrobyl-2-monophosphate-Ca/Na, [10]A-glu SS: Glico Nutrition Company Ltd. Osaka, Japan, [11]HK-LP: Heat-killed *Lactobacillus plantarum* (LP20), House Wellness Foods Corp., Itami, Japan.

The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 15 min. the required amount was mixed with water (35–40% of the dry ingredients), and then added to the premixed ingredients and mixed for another 15 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 50 °C for about 120 min. to approximately 11% moisture, The test diets were stored at −28 °C in a freezer until use.

2.4. Feeding protocol

At the beginning of the feeding trial, juveniles (average body weight, 11 ± 0.03 g) (mean ± S.D.), were randomly stocked in previously prepared eighteen tanks at a stocking density of 12 fish per tank with triplicates per dietary treatment. All fish were fed the respective test diets visually near satiation level (at 7 to 8% of body weight) by hand twice a day, 7 days per week for 56 days. Any uneaten feed left was removed 1 h after feeding and dried using a freeze drier, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. The water quality was checked regularly. All fish were weighed in bulk at 2 weeks interval to determine growth, check their health condition and ration was adjusted according to mean fish weight.

Table 2
Chemical analysis of the experimental diets (% dry matter basis).

Proximate composition	HK-LP supplemented (mg/kg)					
	0	1	10	100	1000	2000
Crude protein	50.8	50.6	51.3	50.9	51.3	51.1
Total lipid	14	14.4	14	14.2	14.4	14.2
Ash	10.7	10.7	10.7	10.8	10.9	10.8
Gross energy (KJ g^{-1})[a]	20.2	20.2	20.1	20.1	20.2	20.2

Carbohydrate was calculated by the difference: 100-(protein + lipid + ash + moisture).
[a] Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ g^{-1}, respectively.