Fish & Shellfish Immunology 36 (2014) 187–193

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

The influence of various feeding patterns of emodin on growth, non-specific immune responses, and disease resistance to *Aeromonas hydrophila* in juvenile Wuchang bream (*Megalobrama amblycephala*)

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ARTICLE INFO

Article history:
Received 31 July 2013
Received in revised form
27 October 2013
Accepted 28 October 2013
Available online 7 November 2013

Keywords:
Megalobrama amblycephala
Emodin
Feeding patterns
Growth
Non-specific immunity

ABSTRACT

The present study was conducted to evaluate the effect of various feeding patterns of emodin on growth, non-specific immune response, and disease resistance to *Aeromonas hydrophila* in juvenile Wuchang bream. Healthy *Megalobrama amblycephala* (initial weight: 3.47 ± 0.032) were grown in a circulating water system for 8 weeks. Five groups were studied: one control group was fed with a basal diet for eight weeks (Pattern 1, P1), and three treatment groups were fed with a trial diet of 30 mg emodin kg^{-1} at one-week (Pattern 2, P2), two-week (Pattern 3, P3), four-week (Pattern 4, P4) intervals. The final treatment group maintained the trial diet for the entire eight-week study duration (Pattern 5, P5). Results indicated that different feeding patterns of emodin significantly influenced the weight gain rate of Wuchang bream ($P < 0.05$). Fish in the P4 treatment group had significantly higher rates of weight gain (WG) than those in other treatment groups. There were no significant differences in survival rates or feed conversion ratios (FCR) between treatment groups and the control group. White blood cell count (WBC), respiratory burst activity, superoxide dismutase (SOD) activity, myeloperoxidase (MPO) activity and tumor necrosis factor-α (TNF-α) activity were shown to increase at first and then decrease from P3 condition to P5 condition. Fish under P4 treatment showed the most significant improvement of all tested parameters compared to control. Significantly higher levels ($P < 0.05$) of plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were observed in P2 and P4 treatment groups when compared with the control group, while no significant differences were observed in the AST and ALT activity of fish in P2, P3, P4 and P5 treatment groups. In a bacterial challenge experiment with *A. hydrophila*, fish under P4 and P5 treatment showed lower cumulative mortality than the control group. The results of this study suggest that an initial 4-week feeding interval is recommended for the economic and practical culture of *M. amblycephala*.

1. Introduction

Wuchang bream (*M. amblycephala* Yih) is a major species of freshwater fish cultured in China. Due to the high stocking density prevalent in intensive aquaculture and the deterioration of local environmental conditions, fish often encounter high temperatures, overcrowding, and poor water quality. These conditions produce a poor physiologic environment for aquaculture, increasing the susceptibility of fish to infectious agents and paving the way for the disease outbreaks due to an increasing range of potential pathogens

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http://dx.doi.org/10.1016/j.fsi.2013.10.028

[1,2]. In recent years, an increasing correlation has been observed between cultured Wuchang bream infections and disease outbreak. This has the potential to increase stock mortality in China and in other areas of the world, especially during the summer months [3]. Thus far, disease control has consisted of immunostimulation by feeding the stocks probiotics and herbs found in traditional Chinese medicine, both of which have been used for the treatment of human and animal diseases for thousands of years [4]. Several plant-based products are being tested for potential immunomodualtory activity in fish.

Emodin (1, 3, 8-trihydroxy-6-methyl-anthraquinone), a medicinal extract present in many herbs such as rhubarb (*Rheum officinale* Baill), aloe (*Aloe barbadensis* Miller), senna (*Cassia angustifolia*), and thunberg (*Polygonum multiflorum*), has been widely used as a traditional medicine in many countries, especially in Eastern Asia, showing promise as a potential immunoenhancer. Several therapeutic properties have been reported for emodin extracted from the rhizome of rhubarb (*R. officinale* Baill): anti-bacterial and anti-inflammatory [5], antioxidation and free radical scavenging [6], reduction of blood lipid concentration [7], hepatoprotection [8], and immune regulation [9]. We previously reported that anthraquinone extract (a mixture of emodin, chrysophanol and rhein) and emodin [1] could promote the growth and enhance the non-specific immunity and high temperature tolerance of freshwater prawn (*Macrobrachium rosenbergii*) [10]and Wuchang bream (*M. amblycephala*) [11]. In this previous study, anthraquinone extract and emodin were fed continuously as additives to a regular diet. Notably, some studies have indicated that the efficacy of immunostimulants such as probiotics [12] and functional sugars [13, 14] was affected by feeding time; optimal efficacy could potentially be obtained by varying feeding time. Preliminary research suggests that appropriate dietary emodin supplementation could enhance the growth and immune responses of Wuchang bream and improve its resistance to infection by *Aeromonas hydrophila* [15].

The effect of oral immunostimulants on non-specific parameters of the immune response is often associated with the dose and duration of immunostimulants administered to aquatic animals. High oral doses of immunostimulants can improve the immune response of host organisms in a short time. On the contrary, low oral dose immunostimulants need a longer period of time to enhance the immune response of host organisms. In addition, long-term oral immunostimulation can lead to immunosuppression [16]. The previous study indicated that appropriate dietary emodin supplementation (especially 30 mg emodin kg^{-1} diet) could enhance the growth and immune responses of fish and improve its resistance to infection by *A. hydrophila* [15]. Therefore, the present study seeks to determine the effects of different temporal feeding patterns on the growth performance, non-specific immunity and disease resistance to *A. hydrophila* in juvenile Wuchang bream under appropriate dietary emodin supplementation. The outcomes of this study will improve our understanding of pathogen defense mechanisms in fish, explore means to enhance non-specific immunity through emodin supplementation, and also provide a theoretical basis for production practices to optimize bream cultures.

2. Materials and methods

2.1. Experimental diets

The formulation and proximate composition of the basal diet are shown in Table 1. Basal diet consisted of 2 mm pelleted feed based on fish-meal and soybean oil (protein 31.12%, fat 6.75% and ash 11.02%). Experimental feed was similarly composed, with the addition of 30 mg kg^{-1} emodin (Xi'an Feida Bio-Tech Co., Ltd, Xian, China). For preparation of experimental diets, feed ingredients were ground into a fine powder through a 60-mesh sieve. Each additional component was sequentially added to the mixture. The final formulations were thoroughly mixed with soybean oil, and water was added to produce a thick dough. The dough was then pelleted using a laboratory pellet machine and dried in a forced-air oven at room temperature. After drying, the resultant feed sheets were broken into smaller pieces and sieved into proper pellet size. All feed were stored at −20 °C until use.

Table 1
Formulation and proximate composition of the basal diet (%).

Ingredients	Percentage dry weight
Fish meal	8
Soybean meal	18
Rapeseed meal	17
Cotton meal	16.5
Rice bran	8
Wheat middling	22
Soybean oil	4
Lecithin	1
Choline chloride	0.5
Vitamin premix[a]	1
Mineral premix[b]	1
Powdered zeolite	1
Calcium dihydrogen phosphate	2
Proximate composition (%)	
Crude protein	31.27
Crude lipid	8.15
Crude ash	11.02
Gross energy (kJ g^{-1})[c]	16.32

[a] Vitamin premix (IU or mg per kg premix): Vitamin A, 900000 IU; Vitamin D, 250000 IU; Vitamin E, 100 mg ; Vitamin K_3, 220 mg; Vitamin C, 5000 mg; Vitamin B_1, 320 mg; Vitamin B_2, 1090 mg; Vitamin B_6, 5000 mg; Vitamin B_{12}, 116 mg; biotin, 50 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60,000 mg; Inositol, 15000 mg; Niacin acid, 2500 mg.
[b] Mineral premix (per kg premix): blue copperas, 2.5 g; green vitriol, 28 g; zinc sulfate heptahydrate, 22 g; Manganese sulfate tetrahydrate, 9 g; sodium selenate, 0.045 g; potassium iodide, 0.026 g; cobalt chloride hexahydrate, 0.1 g.
[c] Energy, calculated by using standard physiological fuel values of 17.15, 23.64 and 39.54 kJ/g for carbohydrate, protein and lipid, respectively.

2.2. Fish and animal husbandry

Wuchang bream were obtained from the Nanquan fish farm of the Fresh-water Fisheries Research Center, Chinese Academy of Fishery Sciences. Prior to experiments, fish were fed with a basal diet for an acclimation period of 15 days. After acclimation, all fish (average weight, 3.47 ± 0.032 g) were randomly distributed into five groups across 15 circular fiberglass tanks (300 L water/tank, 3 tanks/group). Five groups were studied. One control group was fed with a basal diet for eight weeks (Pattern 1, P1), and three treatment groups were fed with a trial diet of 30 mg emodin kg^{-1} at one-week (Pattern 2, P2), two-week (Pattern 3, P3), and four-week (Pattern 4, P4) intervals. The final treatment group maintained the fish on a trial diet for the entire eight week study duration (Pattern 5, P5). The flow rate of water in each tank was maintained at approximately 2 L min^{-1}. Each tank was stocked with 25 fish. Fish were hand-fed with the test diets four times (08:30, 11:00, 13:30 and 16:00) daily to apparent satiation for 8 weeks. A photoperiod was set at natural conditions. Water was maintained at 26 ± 1 °C, 7.4–7.8 pH, ≥6 mg dissolved oxygen L^{-1} and 0.08–0.09 mg NH_3–N L^{-1} over the experimental period.

2.3. Sample collection

At the end of the feeding period, fish were fasted for 24 h to empty the digestive tract, then anesthetized in diluted MS-222 (tricaine methanesulfonate, Sigma, USA) at a concentration of