Fish & Shellfish Immunology 44 (2015) 172–179

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Improvement in non-specific immunity and disease resistance of barramundi, *Lates calcarifer* (Bloch), by diets containing *Daphnia similis* meal

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ARTICLE INFO

Article history:
Received 5 December 2014
Received in revised form
22 January 2015
Accepted 3 February 2015
Available online 11 February 2015

Keywords:
Barramundi
Daphnia meal
Aeromonas hydrophila
Immune response

ABSTRACT

A 42-day study was conducted with barramundi, *Lates calcarifer*, to evaluate the effects of *Daphnia* meal derived from *Daphnia similis* on fish growth, immune response, and disease resistance to *Aeromonas hydrophila*. Three isonitrogenous (45%) and isolipid (10%) experimental diets were formulated to contain 0% (control), 5% (D5), and 10% (D10) *Daphnia* meal. Growth was depressed when fish were fed with the D10 diet for 42 days compared to control. However, the growth in fish fed with control and D5 diets for 42 days was not significantly different. By day 42, the leukocyte phagocytic activity and respiratory burst activity were significantly increased in D5 and D10 groups compared to control. Mx gene expression in the spleen and head kidney of fish after being injected with nerve necrosis virus was also significantly up-regulated in both groups compared to control. In an increased immune response, D5 and D10 fish had significantly higher survival rates than control after being challenged by *A. hydrophila*. Therefore, we suggest that a 5% *Daphnia*-meal diet could improve the barramundi immune response and disease resistance without a negative impact on growth.

1. Introduction

Barramundi, *Lates calcarifer*, also known as the Asian seabass or giant sea perch, is a euryhaline species that is widespread in the western Indo-Pacific region from the Arabian Gulf to China, Taiwan, Philippines, Indonesian Archipelago, Papua New Guinea, and northern Australia. The barramundi supports important commercial and recreational fisheries throughout its range, and has developed into a mature aquaculture industry in Australia and some Asian countries including Taiwan.

Barramundi larvae production was first developed in Thailand [1] and subsequently applied to Australia, Singapore, and Taiwan. Taiwan is not presently totally self-sufficient in barramundi fry production, especially in the cold season, the demand for which is made up by imports from Thailand. These fry (up to two weeks after hatching) are usually reared at an initial stocking density of ~5000 per tonne of brackish water (~10‰), the salinity then being decreased at a rate of 2–3‰ per day to freshwater levels. Cladocerans such as *Daphnia* are used as a supplemental prey food for rearing barramundi larvae.

Zooplankton are an important food item of omnivorous and carnivorous fishes [2,3]. The larger fry and adults of some fish species often selectively prey on crustaceans [4]. Cladocerans are often known as “water fleas” because of their shape and “hop-sink” type of locomotion, and are the major group of zooplankton available in freshwater ponds. *Daphnia* is considered a nutrient-rich food for aquatic animals, as its protein content ranges 30.8–61% [5]. *Daphnia* bodies are enclosed by an uncalcified shell known as the carapace. The carapace is largely made of chitin, poly-β-1, and 4-D-glucosamine [6], and its deacetylated derivative, chitosan, has recently found numerous applications in various fields including the cosmetic, pharmaceutical, and biomedical sectors and wastewater treatment [7]. Cauchie et al. [8] reported that *Daphnia magna* contains significant amounts of chitin (3–7% of body dry weight), and that the basic constituents of chitin isolated from *D. magna* are similar to those of commercial polymers. Kaya et al. [9] also showed that the resting eggs of *Daphnia longispina* are 23–25% chitin and the chitosan yield of the chitin is 76–77%.

Chitosan is a de-acetylated product of chitin. Chitin and chitosan are both potential immunostimulants for aquaculture animals,

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http://dx.doi.org/10.1016/j.fsi.2015.02.002
1050-4648/

being used to protect salmonids from bacterial disease [10] and enhance fish immune responses such as respiratory burst and phagocytic activities [11,12]. Kledmanee [13] found that barramundi fed a chitosan-coated diet had increased innate immune activity, but no pathogen challenges were done in that study. On the other hand, Ranjan et al. [14] found that chitosan at 10 g kg^{-1} in the diet not only increased barramudi innate immunity but also increased disease resistance against *Vibrio anguillarum*. However, chitin or chitosan purification required extra processing and costs. Therefore, the present study aimed to evaluate whether *Daphnia* meal directly incorporated in the barramundi diet would improve the immunity and disease resistance of juveniles by tracking innate immune parameters and disease resistance against *Aeromonas hydrophila*.

2. Materials and methods

2.1. Fish and husbandry

Barramundi larvae (total length: ~0.5 cm) were imported from a private fish seed farm in Thailand. Upon arrival at the airport, fish were immediately transported to the farm at the Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung, Taiwan. Larvae were reared at a stock density of ~5000 larvae per tonne of water in a 2.5-tonne cement tank (1.2 × 1.9 × 1.2 m) with 2 tonnes of 10‰ brackish water. After five days, rearing water salinity was decreased to freshwater levels at a rate of 2–3‰ daily. Living *Daphnia* were fed to barramundi larvae twice daily to apparent satiation for a week. Fish were then trained to accept formulated diets by initially feeding them *ad libitum* with a semi-moist mixture of frozen *Daphnia* and formulated diet followed by formulated meal only. Cannibalism is frequently observed at this weaning stage, so grading was done once a week to ensure that fish in the tank were of similar size. Fish that reached an average size of ~0.2 g were selected for the experiment.

2.2. Daphnia meal preparation

Daphnia similis was obtained from a private live food culture farm of Da Li Feng Biotechnology Co., Pingtung, Taiwan. *D. similis* were collected with a phytoplankton net and spread smoothly over ~3 cm thick plates and dried in an oven at 60 °C to a moisture content <10%. Subsequently, the dried *Daphnia* meal was placed in plastic bags and stored at 4 °C until used in experiments.

2.3. Experimental diets and growth trials

The composition of all food ingredients was analyzed prior to experimental diet preparation following the methods of the Association of Official Analytical Chemists (AOAC) [15]. For the analysis of hydrolyzed amino acids, a 19.5 mg sample was hydrolyzed under nitrogen gas with 1 ml of 6 N HCl in an autoclave at 110 °C for 24 h and neutralized to pH 7.0 with 4 N NaOH. The hydrolyzate was filtered through a 0.22-μm nylon syringe filter and collected in a sample vial for high-performance liquid chromatographic (HPLC) analysis. Thereafter, OPA (ortho-phthalaldehyde) amino acids were injected into a reversed-phase HPLC (PU- 2089 plus, JASCO, Japan) with autosampler, a column of Gemini-NX 5u C18 110A 25 cm × 4.6 mm with 5 μm particle size (Phenomenex; Torrance, California, USA), and a fluorescence detector (FP-2020 plus, JASCO, Japan) (excitation wavelength = 340 nm; emission wavelength = 455 nm). Chromatographic conditions used for this study were as the description in the OPA (P0532, Sigma) product information with some modifications. Additionally, prior to analysis for methionine and cysteine in both mixtures, ingredients were pretreated by using formic acid (nine parts of 88% formic acid plus one part 30% hydrogen peroxide) for protection prior to acid hydrolysis (6 N hydrochloric acid for 24 h at 110 °C). OPA amino acids were also analyzed by a reversed-phase HPLC (PU- 2089 plus, JASCO, Japan) as described above.

After baseline composition analysis of ingredients, experimental diets (Table 1) were prepared based on the protein (45%) and lipid (10%) requirements of barramundi [16]. Three experimental diets, including control without *Daphnia* meal and two *Daphnia* meal concentrations of 5% (D5) and 10% (D10), were used in this study. Fish meal in the experimental diets decreased as the amount of *Daphnia* meal added into experimental diets increased. This was based on the protein content of *Daphnia* meal in order to prepare appropriate iso-nitrogen and iso-lipid diets. The amount of fish oil added to diets is based on the lipid content of fish meal, which decreased in experimental diets compared to control. Briefly, ingredients were ground up in a hammer mill to pass through a 60-mesh screen. Experimental diets were prepared by mixing the dry ingredients with fish oil and then adding water until a stiff dough resulted. Each diet was then passed through a die, and the resulting pellets were dried in a drying cabinet using an air blower at 40 °C to a moisture level <10%. After drying, pellets were stored in plastic bins at 4 °C until being used. The initial compositions of the three experimental diets were not significantly different. Crude protein, crude lipids, moisture, crude fiber and ash in the experimental diets ranged 45.05–45.64%, 9.29–10.13%, 7.05–8.42%, 1.29–1.31% and 11.18–12.13%, respectively. The amino acid composition of *Daphnia* meal, fish meal, and soybean meal are shown in Table 2.

Growth trials were carried out in 2.5 tonne cement tanks (1.2 × 1.9 × 1.2 m) that were continually supplemented and allowed to overflow at a volume of ~0.5 L h^{-1} and had continuous aeration to maintain dissolved oxygen at >5 mg L^{-1}. The three experimental diets (D5, D10, and control) were randomly assigned to triplicate floating cages (1 × 0.4 × 0.4 m) containing 80 fish (mean weight 0.2 ± 0.02 g) per cage. Fish were fed during the experiment twice daily at a rate of 10% of body weight. Any uneaten portions were collected after feeding and immediately dried in an oven at 80 °C. The amounts of all diets eaten were calculated by subtracting uneaten portions from the amounts fed, and recorded daily. Fish feces were removed by siphon once daily during culturing. Fish weights were measured at the start of the experiment and at one-week intervals until the end of the experiment. Fish size grading was

Table 1
Ingredients of experimental diets (g kg^{-1}) used in this study.

Ingredient	Control	D5	D10
Fish meal[a]	600	551	502
Soybean meal[b]	100	100	100
Daphnia meal[c]	0	50	100
Gluten	30	30	30
Starch	100	100	100
α-Cellulose	55	56.6	58.2
Fish oil	50	47.4	44.8
Vit Mix[d]	15	15	15
Min Mix[d]	40	40	40
Proximate composition (% dry matter)			
Crude protein	45.64 ± 0.64	45.32 ± 0.11	45.05 ± 0.53
Crude lipid	10.02 ± 0.24	9.29 ± 0.59	10.13 ± 0.48
Crude fiber	1.29 ± 0.19	1.3 ± 0.1	1.31 ± 0.11
Moisture	7.05 ± 0.11	8.42 ± 0.27	7.93 ± 0.72
Ash	12.13 ± 0.67	11.27 ± 1.25	11.18 ± 0.94

[a] Fish meal contains 63% of crude protein and 8.1% of crude lipid (imported from Peru).
[b] SBM contains 42.1% of crude protein and 2% of crude lipid (dehull SBM).
[c] *Daphnia* meal contains of 62% crude protein and 12.9% of crude lipid (obtained from Da Li Feng Biotechnology Co. Ltd., Pingtung County, Taiwan.).
[d] Vitamin and mineral premix mortified from Shiu et al. [37].