Aquaculture 468 (2017) 501–507

Contents lists available at ScienceDirect

Aquaculture

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

Dietary microbial floc potentiates immune response, immune relevant gene expression and disease resistance in rohu, *Labeo rohita* (Hamilton, 1822) fingerlings

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ARTICLE INFO

Article history:
Received 22 September 2016
Received in revised form 2 November 2016
Accepted 9 November 2016
Available online 12 November 2016

Keywords:
Biofloc
Feed supplement
Rohu (*Labeo rohita*)
Immune response
Immune gene expression
Disease resistance

ABSTRACT

Effects of dietary administration of microbial floc on immune response, immune relevant gene expression and resistance of rohu (*Labeo rohita*) against *Edwardsiella tarda* were investigated in the present study. Microbial flocs were collected from a biofloc system, dried and ground into fine powder. Four isonitrogenous diets were prepared with the addition of graded level of floc powder at 0% (control), 4% (B4), 8% (B8) and 12% (B12) inclusion. Fingerlings of rohu (10.36 ± 0.71 g) were fed with these diets and various immune responses, immune relevant gene expression and disease resistance were measured after 4 weeks of feeding. Different immunological parameters viz. oxygen radical production and lysozyme activity showed significant enhancement in fish fed with the B4 diet. However, myeloperoxidase and alkaline phosphatase activity did not show any significant difference. Immune relevant genes viz. IL-1β, IFN-γ, TNF-α, C3, iNOS and IL-10 showed significantly higher expression in liver and head-kidney tissues in most of the cases for the B4 and B8 diets. The B4 diet enhanced the resistance of rohu substantially against *E. tarda* challenge. In conclusion, the dietary supplementation of microbial floc meal had a health ameliorating effect in rohu particularly, at 4% inclusion level. The results collectively suggest the potential of applying microbial floc meal as immunostimulant in aquaculture.

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1. Introduction

Biofloc technology is a novel farming technique that efficiently manages water quality parameters in aquaculture through balancing carbon and nitrogen in the system. Currently, biofloc technology has received considerable attention because of its potential to generate high production, feed protein recycling, water quality management and a possible alternative measure for disease control (Crab et al., 2007; Avnimelech, 2012). The system operates via addition of carbon source as organic matter substrate and constant aeration of the water column to allow aerobic decomposition and maintenance of high levels of microbial floc in suspended condition (Avnimelech, 1999; Hargreaves, 2006). Theoretically, addition of carbon to the system increases C/N ratio which, in turn, enhance conversion of inorganic nitrogenous compounds to microbial biomass (Azim and Little, 2008). Apart from nitrogen, bacteria and other micro-organisms use carbohydrate as a food, to generate energy and to grow i.e. to produce protein and new cells. Thus, carbohydrate is microbially utilized which is accompanied by the immobilization of inorganic nitrogen (Avnimelech, 1999). Even though the biofloc technology is considered to be a sustainable technique, it is not without drawbacks. For large scale aquaculture, the major concerns lies in implementing a system with mixing and aeration that involves added cost of energy, removal of excessive floc materials, proper monitoring of the system and farmers' acceptability (Avnimelech, 2012; Crab et al., 2012).

Immunostimulatory substances make fish more resistant to infectious micro-organisms by enhancing the immune responses (Raa, 1996). A diverse group of biological and chemical substances and various other microbial derivatives, plant and animal products have been reported to function as immunostimulant in aquaculture (Raa, 1996; Sakai, 1999). Biofloc is rich in natural heterotrophic microorganisms and associated bioactive compounds (Ju et al., 2008a). All these microbes, their cellular components, metabolites and other microbial derivatives are expected to have immunostimulatory potential, thereby can contribute to a healthy status of cultured fish (Linan-Cabello et al., 2002; Babin et al., 2010; Crab et al., 2012). For example, poly-β-hydroxybutyrate (PHB) and PHB accumulating bacteria present in

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http://dx.doi.org/10.1016/j.aquaculture.2016.11.018
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biofloc based aquaculture system have been reported to enhance resistance of animals from bacterial infections (Defoirdt et al., 2007; Halet et al., 2007; De Schryver et al., 2010).

In general, most studies report water quality management, growth performance, nutritional contribution and survival of cultured animal in biofloc system (De Schryver et al., 2008; Crab et al., 2012). In some studies, the floc meal has been used as feed ingredients in shrimp and growth, digestive enzyme activity as well as survival of shrimp have been documented (Ju et al., 2008b; Bauer et al., 2012; Anand et al., 2014). Very few studies have been conducted to investigate the immunostimulatory potential of the biofloc system where the researchers studied disease resistance and immunological effects of growing cultured animals in biofloc based cultured system (Ekasari et al., 2014; Long et al., 2015; Ahmad et al., 2016). However, it remains unknown if the microbial floc collected from a biofloc based cultured system can be used as an immunostimulant. Moreover, study of immunity at molecular level has not been documented from finfish either grown in biofloc system or fed with floc meal supplemented diet.

Thus, the aim of the present study was to investigate the effects of different levels of dietary inclusion of dried microbial floc meal on immune response, immune gene expression in liver and head-kidney (HK) tissues and resistance of rohu (*Labeo rohita*) against experimental *Edwardsiella tarda* infection.

2. Material and methods

2.1. Production of microbial floc meal

Despite the fact that biofloc can be produced even without culturing of fish in tanks, for the present study, however, the floc materials were collected from three indoor fibreglass reinforced plastic (FRP) tanks (1000 L) already in vogue, with simultaneous culturing of fish therein, as part of a separate research. Briefly, the tanks were filled with dechlorinated freshwater with a water volume of 500 L. Continuous aeration and agitation of the water column in each tank were maintained by using an air pump connected to air stones. Prior to stocking of fish, each tank was inoculated with 250 mL of concentrated biofloc developed in a separate indoor tank. Fingerlings of rohu with a mean weight of 7.43 ± 0.54 g were purchased from a local market, acclimatized for two weeks, randomly distributed in each tank (20 fish tank^{-1}) and cultured for a period of four months with regular addition of molasses as carbon source to maintain the C/N ratio at 15:1. The amount of molasses to be added was determined following the method as described previously (Crab et al., 2012). The fish were fed daily with a pelleted feed (6.84 ± 0.22% moisture, 22.39 ± 0.72% crude protein, 7.30 ± 0.41% crude lipid, 12.21 ± 0.68% ash, 13.87 ± 0.49% crude fibre and 37.39 ± 1.14% nitrogen free extract) at 3% of body weight. The aeration was stopped and the microbial flocs were collected by passing the floc water through nylon filter of 10 μm mesh size at weekly interval. The collected floc was centrifuged at 600*g* for 15 min, washed with distilled water, spread on a polythene sheet and sun dried for 11 h. The dried flocs were ground into fine powder using a mixer grinder and kept in air tight containers at 4 °C until further experiment. The proximate composition of the microbial floc meal, thus obtained, was determined using standard methods of AOAC (1995) (Table 1).

Table 1
Proximate composition (%) of dried floc meal (mean ± SD).

Nutrients	Proximate composition
Moisture	8.57 ± 0.26
Crude protein	14.37 ± 0.40
Crude lipid	3.13 ± 0.32
Ash	40.55 ± 0.77
Crude fibre	4.52 ± 0.03
Nitrogen free extract	28.98 ± 0.16

2.2. Experimental diets

Four isonitrogenous diets (designated as control, B4, B8 and B12) were formulated. The control diet was without the addition of microbial floc meal. Three other diets were formulated with addition of graded level of microbial floc meal at 4% (B4), 8% (B8) and 12% (B12) by manipulating the wheat flour level. The feed ingredients were pulverized before mixing. The ingredients were mixed in correct proportion and pressed through a pelletizer to obtain a pellet size with 4 mm diameter. Pellets were then sun dried for 5 h followed by drying at 40 °C for 24 h using a drying cabinet. The pelleted feeds were stored in air tight containers at 4 °C until use. The ingredients and proximate composition of the diets are given in Tables 2 and 3, respectively.

2.3. Experimental fish, feeding and rearing

A fresh lot of fingerlings of rohu (10.36 ± 0.71 g) were purchased from a local fish farm and acclimatized for two weeks with the control pelleted diet. After acclimatization, fish were distributed randomly into twelve 200 L plastic tanks (15 fish in each tank). The tanks were allocated following a completely randomized design. Four treatments comprising control and microbial floc meal supplemented diets (B4, B8 and B12) were replicated 3 times each. The fish were fed twice daily (at 9:00 h and 17:00 h) at a rate of 3% of the body weight. The fish were reared for 4 weeks with aeration and the basic water quality parameters were measured periodically to maintain the desired levels (dissolved oxygen: 5.21 ± 1.16 mg mL^{-1}; pH: 7.25 ± 0.20; ammonia: 0.07 ± 0.013 mg mL^{-1}; nitrite: 0.033 ± 0.013 mg mL^{-1}). Daily water exchange (up to 50%) was done and the excess feed and faecal materials were removed by stopping aeration, allowing them to settle and siphoning them off.

2.4. Sampling of fish, collection of serum and tissue samples

After the end of 4 weeks of feeding, five fish were randomly sampled from each tank. The fish were euthanized (using clove oil @ 100 μL L^{-1}) and bled with the help of a sterilized 1 mL hypodermal syringe and 24 gauge needles directly from the caudal vein. Pooled blood was allowed to clot at room temperature for 30 min and then kept at 4 °C for 3 h. The clotted blood was centrifuged at 5000*g* for 5 min to collect serum which was then stored at −20 °C in sterilized vial until further use (Dash et al., 2014). Aliquot of blood was also kept with EDTA as anticoagulant for nitroblue tetrazolium (NBT) assay. Liver and HK tissues were immediately excised aseptically from three fish and were stored in TRIzol solution (Invitrogen, USA) at −20 °C for RNA isolation.

Table 2
Ingredient composition of the isonitrogenous diets on dry matter basis (kg).

	Experimental diets			
Ingredient	Control	B4	B8	B12
Fish meal[a]	0.16	0.16	0.16	0.16
Soya meal[a]	0.21	0.21	0.21	0.21
Rice bran[a]	0.08	0.08	0.08	0.08
Mustard oil cake[a]	0.15	0.15	0.15	0.15
Corn[a]	0.12	0.12	0.12	0.12
Wheat flour[a]	0.24	0.2	0.16	0.12
Floc meal	0	0.04	0.08	0.12
Vegetable oil[a]	0.02	0.02	0.02	0.02
Vitamin-mineral mix[b]	0.02	0.02	0.02	0.02
Total	1	1	1	1

[a] Purchased from local dealers, Agartala, India.
[b] Vitamin mineral mixture (Kalvimin Forte, India) (quantity per 2.5 kg). Vitamin-A: 50,00,000 IU; vitamin-B2: 2.0 g; vitamin-B12: 6.0 mg; vitamin-D3: 10,00,000 IU; calcium pantothenate: 4.0 g; calcium: 800 g; phosphorus: 150 g; manganese: 27.5 g; iodine: 1.0 g; iron: 7.5 g; zinc: 15.0 g; copper: 2.0 g.

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