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Growth, non-specific immunity and disease resistance of *Labeo rohita* against *Aeromonas hydrophila* in biofloc systems using different carbon sources

Irshad Ahmad.H, A.K. Verma *, A.M. Babitha Rani, G. Rathore, Neelam Saharan, Adnan Hussain Gora

Central Institute of Fisheries Education, Panch Marg, Yari Road, Versova, Andheri (W), Mumbai 400061, India

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ABSTRACT

A 60 day growth trial was carried out to evaluate biofloc based rearing system for *Labeo rohita* fingerlings. The C/N ratio of 15 was maintained in four biofloc treatments (T1, T2, T3 and T4) using indirect and long-lasting carbon sources (tapioca, wheat, corn and sugar bagasse) along with one control (C). Each group consisted of triplicate tanks and each tank was stocked with 50 fingerlings (4.80 g ± 0.12) of *L. rohita*. Fishes were challenged with *A. hydrophila* 60 days post rearing and relative percentage survival (RPS) was recorded over 14 days post challenge. Serum samples were collected on 20, 40 and 60 days interval of sampling for the evaluation of non-specific immune and stress parameters. The results revealed that fishes reared in tapioca based biofloc system showed significantly ($p < 0.05$) higher serum protein, serum albumin, total immunoglobulin, respiratory burst activity, myeloperoxidase activity and lower serum glucose and serum cortisol when compared to wheat, corn, sugar bagasse based biofloc system and control. Growth, feed conversion ratio (FCR), feed efficiency ratio (FER), and specific growth rate (SGR) of fishes in the biofloc treatments were significantly better ($p < 0.05$) than control. Tapioca based biofloc treatment showed significantly ($p < 0.05$) higher RPS (66.66%) than other treatments. The study suggests that the fishes reared using *in-situ* biofloc produced by tapioca can enhance growth and non-specific immune responses under zero water exchange system and hence ensures sustainability.

Statement of relevance

- Zero water exchange reduces water scarcity and pollution problems
- Improves welfare of the animals by stimulating immunity and protection against diseases
- *In-situ* production of valuable microbial protein thus reducing dependence on fishmeal.

1. Introduction

Aquaculture intensification is one of the prime requirements to cope with the present demand for fish protein. Increase in productivity per unit space is performed by increasing the rearing density of fishes. Health management and biosecurity are major challenges in production intensification due to limited control over pathogens (Kautsky et al., 2000; Moss et al., 2012; Xie and Yu, 2007). Attaining sustainability in feed management is also an important aspect in production intensification of any aquatic organism. In addition to that there is a need to establish the culture system which uses limited natural resources, non-polluting and economically sustainable. Biofloc technology (BFT) based farming system has proved to be a limited water exchange, biosecure system which can ensure sustainable feeding management and production intensification. The benefits include the uptake of ammonium by the microbial community by maintaining a high C/N ratio (Avnimelech et al., 1994) and improve health management and biosecurity through zero water exchange and possible probiotics effect (Crab et al., 2010; Haslun et al., 2012; Zhao et al., 2012).

Biofloc is a heterogeneous aggregate of suspended organic particles and useful microorganism associated with extracellular polymeric substances (De Schryver et al., 2008; Ray et al., 2010). Different types of organic carbon sources (e.g. glucose, acetate, tapioca, corn, wheat, glycerol, molasses etc.) can be additionally added to the culture system or changing the feed composition by increasing its carbon content (Avnimelech, 1999). The source of organic carbon determines to a large extent the degree of composition of flocs produced, mainly regarding

* Corresponding author.
E-mail address: akverma45@yahoo.com (A.K. Verma).

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the type and amount of storage polymers (Hollender et al., 2002; Oehmen et al., 2004). Biofloc technology was proved to be better in water and feed usage efficiency compared to conventional practices for farming of Tilapia and *Litopenaeus vannamei* (Avnimelech, 1999; Milstein et al., 2001).

A wide range of microorganisms and their cell components have been applied as probiotics or immunostimulants in order to improve the innate immunity, antioxidant status and disease resistance of aquatic organisms (Smith et al., 2003; Vazquez et al., 2009). Bioflocs are rich in various bioactive compounds like carotenoids, chlorophylls, polysaccharides, phytosterols, taurine and fat-soluble vitamins (Jang et al., 2011). However, little effort was made to study the effect of bioflocs on physiological health and non-specific immunity of cultured fishes.

Aeromonas hydrophila is the most common and frequently encountered bacterial pathogen in India which causes severe damage to carp production (Karunasagar et al., 1991). It is an important pathogen in causing stress related diseases in fish with the common symptoms of ulceration, exophthalmia and abdominal distension (Amin et al., 1985; Miyazaki and Jo, 1985; Rahman et al., 1997). Therefore the present study was designed to evaluate four kinds of bioflocs developed using different long lasting carbon sources (tapioca, wheat, corn and sugar bagasse) for their effect on growth, non-specific immune parameters and disease resistance of *Labeo rohita* fingerlings.

2. Materials and methods

2.1. Preparation of inocula

For initial floc formation, pond bottom soil was collected from Array Farm, Goregaon, and Mumbai, India. Inocula were formed in glass tanks (5 L) by adding 20 g of pond bottom soil in well aerated water (1 L) containing 10 mg L^{-1} ammonium sulfate $(NH)_4SO_4$ and 400 mg L^{-1} of different carbon sources (tapioca, wheat, corn and sugar bagasse). The suspension was incubated for 24 h for development of microbial growth.

2.2. Experimental design

The experiment was conducted for 60 days in 15 FRP circular tanks (300 L) with four treatments and one control *viz.*, C (clear water), T1 (tapioca as carbon source), T2 (wheat as carbon source), T3 (corn as carbon source) and T4 (sugar bagasse as carbon source). The prepared inocula were added to the respective experimental groups after 24 h of floc formation. The tanks were kept well aerated for 10 days to ensure optimum floc production. Nitrogen and respective organic carbon sources were added regularly to maintain a C/N ratio of 15 (De Schryver et al., 2008). The Fingerlings of *L. rohita* were collected from the State Fisheries Department, Array Farm, Goregaon, and Mumbai, India. The fishes were acclimatized in the FRP tanks (1000 L) for 25 days and were fed with pelleted feed having a protein content of 30% approximately. Also during acclimatization period, any fish showing apparent signs of disease or malnutrition were separated out. *L. rohita* fingerlings (n = 750) of mean weight (4.80 g ± 0.12) were randomly distributed into 15 tanks to form five experimental groups in triplicate following a completely randomized design (CRD). All the experimental fishes were fed at 2% body weight once a day at 11.00 h. The experimental diet was formulated with 28% protein using fishmeal (25%), soya bean flour (30%), groundnut oil cake (7%), wheat bran (14%), corn flour (14%), oil mix (6%), carboxymethyl cellulose (2%) and vitamin and mineral mix (2%).

Water quality parameters like Dissolved oxygen (DO), pH and temperature were measured *in-situ* on daily basis using multi parameter (water quality) [EUTECH (Multi 350i MPP-25)] analyzer. Total ammonia nitrogen (NH_3-N), Nitrite-N (NO_2-N) and Nitrate-N (NO_3-N) were determined every 10 day interval according to standard methods (APHA, 1998).

2.3. Growth parameters

Fishes (n = 10) of different treatment groups were weighed at bi-weekly interval and assessed for the growth parameters like weight gain (%), feed conversion ratio (FCR), feed efficiency ratio (FER), and specific growth rate (%) (SGR) as follows.

Weight gain(%) = (FW-IW) × 100/IW, FCR = Feed given (DW)/body weight gain (WW), FER = 1/FCR, SGR (%) = [ln (FW) − ln (IW) / N] × 100. Where FW = final weight, IW = initial weight, DW = dry weight, WW = wet weight, ln = natural log and N = number of culture days.

2.4. Proximate composition

The proximate composition of the whole body of fishes (n = 10) was determined after completion of the experiment, following the standard methods of (AOAC, 1995). The moisture content was determined by drying the fishes at 106 °C to a constant weight and, the difference in weight of the sample indicated the moisture content. Nitrogen content was estimated by Kjeldahl (Kelplus, DXVA, Pelican Equipments, and India) method and crude protein content was calculated by multiplying nitrogen percentage by 6.25. Crude lipid was determined by the solvent extraction method by Soxtec system (Soxtec system, SCS-6, Pelican Equipments, and India) using diethyl ether (boiling point, 40–60 °C) as a solvent. Ash content was determined by incinerating the samples in a muffle furnace at 600 °C for 8 h.

2.5. Non-specific immune and stress parameters

After every 20 days interval, fishes from each replicate (n = 5) were anesthetized using clove oil (Merck, Germany) and the blood was collected with and without anticoagulant for serum and plasma. The blood used for serum was allowed to clot for 4 h. Collected blood was then centrifuged at 5000 rpm for 5 min followed by collection of serum and plasma. Serum and plasma was stored at −80 °C for further analysis.

Serum protein was estimated by biuret and BCG dye binding method by Reinhold (1953) using total protein kit (Merck, India). Albumin was estimated by bromocresol green binding method developed by Doumas et al. (1971) using albumin kit (Merck, India). Globulin was calculated by subtracting albumin values from total serum protein values. Total Immunoglobulin in plasma was separated by precipitation with polyethylene glycol as described by Anderson and Siwicki (1995). The respiratory burst activity was measured by nitroblue tetrazolium (NBT) assay following the method of Secombes (1990). Myeloperoxidase content present in serum was measured according to Quade and Roth (1997). The lysozyme activity level was measured using the turbidimetric assay following Sankaran and Gurnani (1972) using hen egg white lysozyme (Sigma) as standard. Serum cortisol was estimated through the Caymans cortisol EIA kit (Caymans chemical, USA), while serum glucose level was determined using standard kit as per the manufacturers instruction (Merck, India).

2.6. Challenge study

After 60 days of rearing, 12 fishes from each treatment group were challenged with virulent strain of *A. hydrophila* (ATCC 7966) procured from Aquatic Animal Health Management department of CIFE, Mumbai. *A. hydrophila* was cultured in nutrient broth at 30 °C for 24 h. The cultures were centrifuged at 3000 rpm for 10 min. The supernatants were discarded and the pellets were suspended in phosphate buffered saline (PBS, pH 7.4) having an OD of 0.48 at 450 nm which corresponds to the final bacterial concentration of 1.8×10^7 cfu/mL (Kumar et al., 2013). A 20 μL of bacterial suspension was injected intra-peritoneally to 4 fishes per replicate in each treatment using 1 mL tuberculin syringe and mortality was observed for 14 days.