Fish and Shellfish Immunology 72 (2018) 143–152

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Dietary supplementation of biofloc influences growth performance, physiological stress, antioxidant status and immune response of juvenile sea cucumber *Apostichopus japonicus* (Selenka)

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ARTICLE INFO

Keywords:
Apostichopus japonicus
Microbial biofloc
Physiological stress
Non-specific immune response
Disease resistance

ABSTRACT

Bioflocs are rich in various probiotics and bioactive compounds, which play an important role in improving growth and health status of aquatic organisms. A 60-day experiment was conducted to investigate the effects of dietary supplementation of biofloc on growth performance, digestive enzyme activity, physiological stress, antioxidant status, expression of immune-related genes and disease resistance of sea cucumber *Apostichopus japonicus*. Juvenile sea cucumbers were fed five experimental diets containing graded levels of biofloc from 0% to 20% (referred as B0, B5, B10, B15 and B20, respectively). The results showed that the sea cucumbers at dietary supplementation levels of 10%–15% biofloc had significantly higher specific growth rate (SGR) compared to control group (diet B0). Digestive enzyme activity increased with the increasing of dietary biofloc level, while no significant difference was found between diets B15 and B20. Dietary supplementation of biofloc also had significant influences on physiological stress parameters except for lactate. There was no significant discrepancy in total coelomocytes counts (TCC) in coelomic fluid of sea cucumber between the treatments. Phagocytosis and respiratory burst of cellular immune at 15% and 20% biofloc levels were significantly higher than those of control group. Significant increases in superoxide dismutase (SOD), total nitric oxide synthase (T-NOS), lysozyme (LSZ), acid phosphatase (ACP) and alkaline phosphatase (AKP) activities of sea cucumber were found at highest dietary supplementation level of 20% biofloc. The expression patterns of immune-related genes (i.e., Hsp90, Hsp70, p105, Rel, NOS and LSZ) in tissues of sea cucumber were analyzed between the experimental diets, and a general trend of up-regulation was observed at higher biofloc levels. Furthermore, dietary 10%–20% biofloc significantly reduced cumulative mortality of sea cucumber after being challenged with *Vibrio splendidus*. In conclusion, dietary supplementation of biofloc could improve growth performance of *A. japonicus*, by increasing digestive enzyme activity, releasing physiological stress, enhancing immune response and disease resistance of sea cucumber. The suitable supplemental level of approximately 15% biofloc was recommended in the present study.

1. Introduction

Sea cucumber *Apostichopus japonicus* has been used as a traditional remedy for wound healing, and extensively believed to be aphrodisiac and curative effects [1]. With the overfishing of natural resources and increasing market demand, the farming scale of sea cucumber has been rapidly expanded in the last decades [2]. The total production of this species in China has reached 206,000 t in 2015, with 101% increase compared to that in 2009 [3,4]. However, it resulted in serious diseases such as skin ulceration and peristome tumescence, which caused enormous economic losses [5,6]. Due to the restriction of antibiotics and chemicals, dietary supplements are widely used to enhance growth performance and health status of sea cucumber, e.g., probiotics [6–8], prebiotics [9], immunostimulants [10], nutritional additives [11,12], etc.

Biofloc technology (BFT) has proved to be a limited water exchange and biosecure system, which ensures sustainable feeding management and production intensification [13]. Briefly, these systems depend on the promotion of microbial proliferation through the addition of extra carbohydrates to improve water quality. The microbes are expected to use, recycle and transform the excess nutrients from farming wastes (i.e., uneaten feed, feces and excretion) into bacterial biomass, which would be further consumed by the cultured organisms [14]. Apart from being a protein source, a wide range of microorganisms and their cell

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http://dx.doi.org/10.1016/j.fsi.2017.10.061
Received 18 July 2017; Received in revised form 18 October 2017; Accepted 31 October 2017

components in biofloc have been applied as probiotics or immunostimulants, to improve growth performance, non-specific immunity and disease resistance of fish and crustacean [15,16]. Moreover, bioflocs are rich in various bioactive compounds which can enhance digestive enzyme activity and physiological health of organisms [17]. Sea cucumber, as an obligate deposit-feeding species, might take up organic matter in sediment as food sources, e.g., bacteria, prozotoa, benthic microalgae, detritus of macroalgae and sea grass [18–20]. Dietary supplementation of biofloc might have a beneficial effect in consideration of the advantages of biofloc and feeding habits of sea cucumber.

The objective of this study was to investigate the effects of dietary supplementation of biofloc on growth performance, digestive enzyme activity, physiological stress, antioxidant status and immune response of *A. japonicus*, providing valuable information on the health management and intensive culturing of sea cucumber.

2. Materials and methods

2.1. Diet preparation

Biofloc production was carried out in three indoor cylinder fiberglass tanks (1500 L; φ = 1.4 m) in six batches, and then used as a dietary supplement in artificial feed of sea cucumber. As an inoculum, 10 L water with microbial floc developed from an intensive aquaculture system that utilizes BFT was added to each tank. The C:N ratio was maintained at 10:1 using ammonium sulphate as nitrogen source and wheat flour as carbon source in the first three days. All tanks were aerated and mixed continuously using an air pump to meet the oxygen demand. On the 5th day, biofloc was harvested by passing culture water though a 10-μm mesh size nylon filter bag [17]. The collected flocs were centrifuged at 1000 × *g*, and then air-dried in a greenhouse until moisture levels were less than 12% [21]. The dried floc material was subsequently ground into fine powder and stored at −80 °C until the experimental diets were made.

Five isonitrogenous and isolipidic diets were formulated at average crude protein level of 19.00% and crude lipid of 2.45% (Table 1). The experimental diets used fish meal, soybean meal, *Sargassum thunbergii* and dried biofloc powder as protein sources and squid liver oil as a lipid source respectively, which contained graded biofloc levels from 0% to 20% (referred as B0, B5, B10, B15 and B20, respectively). To comply with feeding habit of sea cucumber, sea mud was collected from an intertidal zone, dried and ground to powder. Then, it was combusted at 550 °C for 6 h to remove any organic material [19]. Dry ingredients were mixed sufficiently with 30% distilled water, and pellets were prepared using a moist pelletizer. The pellets were dried at 60 °C for 24 h and ground into desirable particle size (180 mesh). All diets were packed and stored at −20 °C until used.

2.2. Experimental design and sample collection

Juvenile sea cucumbers of similar wet weight (~6 g) were collected from a local farm in Dongying City, China. All animals were transported to the laboratory immediately and acclimated for 3 weeks at 20 ± 0.5 °C, salinity was 30–32 PSU, dissolved oxygen was above 6.5 mL L^{-1} and a 14 h light: 10 h dark photoperiod, which were same with the experimental conditions. After acclimation, the sea cucumbers were randomly divided into 5 groups. Each group contained 120 sea cucumbers that were randomly allocated into 3 cylinder aquaria, i.e., 40 individuals per aquarium. The experiment lasted for 60 days. During the acclimation and experimental period, the sea cucumbers were fed twice at 08:00 and 16:00, and up to 5% of their total biomass per day. Feeding ration was adjusted once every ten days based on its growth performance [22]. Uneaten feed residues and feces were removed by siphoning before next feeding [23]. Sufficient continuous water circulation and aeration ensured homogenous environmental conditions for each aquarium.

Table 1
Formulation and proximate composition (dry matter, %) of the experimental diets for *A. japonicus*.

Ingredients	Diet treatment				
	B0	B5	B10	B15	B20
Fish meal[a]	8.0	8.0	8.0	8.0	8.0
Soybean meal[a]	17.0	16.0	15.0	14.0	13.0
Sargassum thunbergii[a]	30.0	26.0	22.0	18.0	14.0
Biofloc powder	0.0	5.0	10.0	15.0	20.0
Wheat starch[a]	6.0	6.9	7.8	8.7	9.6
Squid liver oil[a]	1.0	0.9	0.8	0.7	0.6
Lecithin[a]	0.5	0.5	0.5	0.5	0.5
$Ca(H_2PO_4)_2$	1.0	1.0	1.0	1.0	1.0
Mineral premix[b]	1.0	1.0	1.0	1.0	1.0
Vitamin premix[c]	1.0	1.0	1.0	1.0	1.0
Sea mud	34.5	33.7	32.9	32.1	31.3
Proximate analysis					
Crude protein	19.08	18.91	19.13	19.06	18.85
Crude lipid	2.52	2.39	2.40	2.51	2.41
Ash	42.79	43.75	43.16	43.02	43.45
Carbohydrate[d]	35.61	34.95	35.31	35.41	35.29
Energy (kJ g^{-1})	10.51	10.58	10.31	10.77	10.64

[a] Fish meal (dry matter, %): crude protein 60.00, crude lipid 4.91, ash 12.80; soybean meal (dry matter, %): crude protein 44.10, crude lipid 1.92, ash 6.10; *Sargassum thunbergii* (dry matter, %): crude protein 19.20, crude lipid 1.21, ash 16.33; These ingredients were supplied by Great Seven Bio-Tech (Qingdao, China).

[b] Mineral premix (g kg^{-1}): $MgSO_4{\cdot}7H_2O$, 90.0; ferric citrate, 18.0; $ZnSO_4{\cdot}7H_2O$, 3.0; $MnSO_4{\cdot}H_2O$, 2.5; $CuCl_2$, 0.8; $AlCl_3{\cdot}6H_2O$, 0.18; NaCl, 2.0; KIO_3, 0.04; $CoCl_2{\cdot}6H_2O$, 0.07.

[c] Vitamin premix (g kg^{-1}): L-ascorbic acid, 150.0; DL-α-tocopherol acetate, 10.0; thiamin hydrochloride, 6.0; riboflavin, 8.0; pyridoxine hydrochloride, 5.0; niacin, 40.0; myo-inositol, 100.0; D-biotin, 0.3; folic acid, 1.5; *p*-amino benzoic acid, 10.0; menadione, 4.0; retinyl acetate, 1.5; cholecalciferol, 0.005; cyanocobalamin, 0.005.

[d] Carbohydrate = 100 − crude protein − crude lipid − ash.

At the end of the experiment, the sea cucumbers were starved for 24 h prior to sampling, and then collected from each aquarium, weighted and counted for calculation of growth performance. Ten sea cucumbers sampled randomly from the same aquarium were pooled as one replicate and those from 3 different aquaria were used as three replicates for further parameter analysis. After individuals were dissected by incision at the esophagus and cloaca, the coelomic fluid was sampled immediately by puncturing the abdomen with a 1 mL disposable syringe, and then thoroughly mixed with an equal volume of anticoagulant [6]. The intestine tract, respiratory tree and muscle were separated and put into 1.5 mL tubes (RNAase-Free, Axygen), respectively. After an aliquot of coelomic fluid sample was taken for cellular immune assay, the left separated coelomocytes by centrifugation (3000 × *g*) for 10 min at 4 °C. Coelomocytes were resuspended in 600 μL cold 0.85% saline, and then sonicated at 22 kHz for 25 s at 0 °C followed by centrifugation at 4000 × *g* for 10 min at 4 °C, to obtain the cells lysate supernatant for determination of antioxidant and immune enzyme activities [24]. All the samples were frozen in liquid nitrogen and stored at −80 °C for further analysis. All sampling tools were pretreated at 180 °C for 6 h to eliminate RNAase.

2.3. Estimation of biofloc microbial community

During the biofloc production cycle, total heterotrophic bacterial count, *Bacillus*, *Lactobacillus* and *Vibrio* counts were estimated by the method of Anand et al. [15] (Table 2). Total heterotrophic bacterial count increased from 0.36 × 10^8 to 180.33 × 10^8 cfu mL^{-1}. The colony ranged from 30 to 300 were counted and expressed as colony forming unit (cfu).

2.4. Biochemical composition

Proximate compositions of the experimental diets and biofloc