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Short communication

Effects of stocking density of the white shrimp *Litopenaeus vannamei* (Boone) on immunities, antioxidant status, and resistance against *Vibrio harveyi* in a biofloc system

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ABSTRACT

Determining optimum stocking density of the white shrimp *Litopenaeus vannamei* (Boone) is a big concern for shrimp farmers. However, few studies have assessed the influence of stocking density on the antioxidant status, immunology, digestive enzyme activities, and growth performance of white shrimp in biofloc systems. In this study, these parameters of white shrimp in a biofloc system were compared at three stocking densities: 300 orgs m^{-3} as low stocking density (LD), 400 orgs m^{-3} as medium stocking density (MD), and 500 orgs m^{-3} as high stocking density (HD). The feed conversion ratio in the LD group was significantly lower than that in the MD and HD groups ($P < 0.05$), and the ultimate individual weight in the LD group was significantly higher than that in the other two groups ($P < 0.05$). The antioxidant status and immunology parameters, including complement 3, complement 4, lysozyme, superoxide dismutase, glutathione peroxidase and malondialdehyde were all depressed in the HD groups. Furthermore, activities of the digestive enzymes, amylase, trypsin, and lipase were lower in the MD and HD groups than that in the LD group. The highest relative percentage survival was observed in the LD group 10 days after challenge with the pathogen *Vibrio harveyi*. Results of this study indicated that the immune status and welfare of white shrimp can be seriously impaired in the HD condition (i.e., $\geq$500 m^{-3}) in biofloc systems. These findings can be used to determine suitable stocking densities in the white shrimp farming industry using the biofloc system.

1. Introduction

The white shrimp *Litopenaeus vannamei* (Boone) is widely cultured in many parts of the world. Rapid growth, good survival in high-density culture and high disease tolerance make it a good choice for intensive and/or bio-secure closed grow-out systems [1]. Over the past decade, production of this species in high-density, biofloc-based no water exchange systems had achieved success [2]. Super-intensive production of shrimp is gaining increased attention worldwide as a potential means to improve aquaculture production, Survival, growth and stocking density are of primary concern in this endeavor [3]. For example, white shrimp reared at stocking densities ranging from 150 to 600 orgs m^{-3} achieve rapid growth and high survival rates [4]. Some research indicated that growth performance and welfare of aquaculture animals were not influenced in high stocking density tanks containing probiotics [5]. Some study deduced that *in situ* biofloc are available 24 h a day as supplemental food source for the cultured shrimp, meanwhile it was also a kind of probiotic for the water quality and the health of aquatic animal [6,7].

The bioflocs or its attached microorganisms could exert a positive effect on the digestive enzyme activity of shrimp [8–11]. The immune and antioxidant systems in physiological role are critical to shrimp to control their health and satisfactory growth performance under the environmental stresses [12]. A wide variety of microorganisms and their cell components or metabolic products have been investigated and applied as probiotics and/or immunostimulants to improve innate immunity and/or antioxidant capacity of shrimp, thereby enhancing their resistance against pathogens [13–15]. Xu and Pan [16] reported that the total haemocyte count and phagocytic activity of the haemocyte of the shrimp from biofloc groups were significantly higher than those of the shrimp in the non-biofloc control group. Furthermore, the authors also noted that

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shrimp grown in a biofloc environment harbored a higher total antioxidant capacity. A study showed that the expression of six selected genes (prophenoloxidase [ProPO1 and ProPO2], serine protease [SP1], prophenoloxidase activating enzyme [PPAE1], masquerade-like serine protease [mas] and Rat-sarcoma-related nuclear protein), directly and indirectly related to the shrimp immune response, were significantly upregulated in biofloc groups [17].

However, little or nothing is known about how the stress of stocking density affects the digestive enzyme activities, antioxidant status, immune responses, and growth performance of shrimp in zero-water exchange biofloc systems. Further, stocking density may affect shrimp susceptibility to *Vibrio harveyi*, which is the most frequently detected species that infects the shrimp hepatopancreas to cause acute or chronic infections known as vibriosis, this bacterial was deemed as the main dangerous factor in the industry of white shrimp in nursery and grow-out phase in China.

The objectives of this study were to investigate the effects of stocking density on water quality, physiology, and production performance of *L. vannamei* cultured at different densities and to determine optimal stocking density to support the economic viability of shrimp production. We also conducted a bacterial challenge test as an indicator of shrimp immune status after the stress trial. The approach and framework used in this study can be adapted by shrimp producers to develop crucial management strategies regarding stocking density in BFT systems. Our results also offer a potential practical approach for health management and a novel alternative for disease prevention in *L. vannamei* aquaculture.

2. Materials and methods

2.1. Experimental design

The experiment was carried out in the plant (800 m^2) of an aquaculture company in Tianjin, China. Each cement tank (6 m × 3.5 m × 1.8 m) had a water volume of 25 m^3 (i. e. the depth of water is 1.2 m). The experiment included nine tanks, with three tanks for each of three shrimp stocking densities (see section 2.2).

No water was exchanged in the biofloc tanks. All tanks were aerated and agitated continuously using air-stones connected to a Rootes blower (11kw, Zhangyi Machinery Co., Ltd., Shandong, China), and seawater was added regularly to compensate evaporation loss.

2.2. Shrimp stocking and system management

Five-day-old postlarvae (PL5) of *L. vannamei* were obtained from a commercial farm (Haiyi, Hainan province, China). Nursery period experienced 40 days, with no water exchange. Molasses was added to the culture water when measured total ammonia nitrogen (TAN) concentrations were above 1 mg L^{-1}. During the nursery phase carbon supplementation was based on the actual level of TAN in the culture water; requiring the addition of 6 g of carbon for each 1 g of TAN found in the water [18]. After the 40 days nursery period, juvenile shrimp were graded for similar weight (1.58 ± 0.10 g). During the intermoult period shrimp were randomly selected and stocked into 9 tanks. Three tanks as one group, 300 orgs m^{-3} as the low density (LD), 400 orgs m^{-3} as the medium density (MD), and 500 orgs m^{-3} as the high density (HD).

The shrimp were fed with a commercial feed (36% crude protein, Tongwei, Group Co. Ltd., Chengdu, China), and cultured for a period of 30 days. Feeding was done manually to apparent satiation three times per day at 7:00, 15:00 and 23:00, respectively. The daily feeding rate was adjusted according to the feeding trays to make sure that the feed was fully consumed, and the quantity of the food was recorded to calculate the growth parameters. The pre-weighed carbon source (molasses) was also added daily at 15:30 to maintain an optimum C/N ratio.

2.3. Assessment of water quality parameters and carbohydrate addition

Throughout the 30-day experimental period, water temperature, salinity, dissolved oxygen (DO) and pH were measured daily around 9:00. Whenever the pH of the water in a tank dropped below 7.4, $NaHCO_3$ was carefully added into the tank water to raise the pH slowly [19]. Water samples (50 mL each) were collected weekly from each tank. Half of the water sample was analyzed spectrophotometrically for total ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen following 'Standard methods for the examination of water and Wastewater' [20]; the remaining half was filtered under vacuum pressure through predried and preweighed filter paper. The filter paper containing suspended materials was dried at 105 °C in an oven until constant weight was reached, and the dried sample was weighed afterwards. The weight difference and the amount of total suspended solids (TSS) were calculated. Biofloc volume (BFV) was determined weekly by using Imhoff cones, by registering the volume sedimented from 1000 mL of water sample in 30 min [21].

The amount of carbon resource (molasses) used per treatment was determined by the content of protein (%) in commercial feed during cultivation, assuming that protein is 16% nitrogen and that shrimp will excrete 65% of protein nitrogen. If 1000 g of feed contains 36% protein (16% nitrogen), then, to obtain 57.6 g of nitrogen, of which 35% is the fraction that would be digested and transformed to muscle; 37.44 g nitrogen will be excreted. To maintain a 15:1 C:N ratio, 561.6 g carbon were required, and that was provided by 1404 g molasses because molasses contains 40% carbon, which was determined in the laboratory by proximal chemical analysis. The experiment treatments were determined with the same calculation and adjusted, based on the protein content of the diet and the amount of feed supplied in each treatment, not taking into account the amount of carbon contained in the feed [22].

2.4. Shrimp sampling

At the end of the experiment, live shrimps from each tank were counted and the final body weight (wet weight) of each individual was recorded. Then, 10 shrimps were randomly collected from each tank. The hepatopancreas of each shrimp were taken out and homogenized three times, using an electric blender operating at 8000 rpm. The homogenate was then centrifuged at 10 000×*g*, 4 °C for 20 min to eliminate tissue debris and lipids. The supernatant (enzyme extract) was dispensed into 1.5 mL Eppendorf tubes and kept at −80 °C used for until enzymatic assays.

2.5. Enzyme activities

The fish digestive enzyme activities, immunological parameters and oxidative enzymes were determined by using commercial assay kits (Nanjing Jiancheng Institute, Nanjing, China) according to the instructions given by the manufacturer.

Superoxide dismutase (SOD) activity was determined by the xanthine oxidase method, based on its ability to inhibit the oxidation of hydroxylamine by the xanthine-xanthine oxidase system. Glutathione peroxidase (GSH-Px) activity was measured by quantifying the rate of H_2O_2-induced oxidation of GSH to oxidized glutathione (GSSG), catalyzed by GSH-Px. The extent of lipid peroxidation in the hepatopancreas was estimated by