Short-term Response in Molecular and Biochemical Adaptation of White Shrimp (*Litopenaeus vannamei*) Postlarvae Reared in A Biofloc System

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Abstract

Ngurah S. Yasa, Lutfi Anshory, Gemi Triastutik, Murwantoko, Alim Isnansetyo and Lusiana. 2019. Short-term response in molecular and biochemical adaptation of white shrimp (*Litopenaeus vannamei*) postlarvae reared in a biofloc system. *Aquacultura Indonesiana, 20(2) : 80- 92.*Up regulation of heat shock proteins (HSPs) in organisms can be detected in response to many kinds of stressor. Recently there were few studies have focused on the three kinds of heat shock proteins (HSPs) and antioxidant enzyme activity after biofloc stress. The objective of this study was to investigate the effect of different biofloc volume on the expressions of (LvHSP60, LvHSP70, and LvHSP90), the activity of superoxide dismutase (SOD) and phenoloxidase (PO) of the *L. vannamei* after biofloc addition. PL10 *L. vannamei* were divided into three groups: precondition, biofloc stress (5mL/L, 10mL/L, 15mL/L), and recovery. The gene expression results showed that the expressional levels of LvHSP60, LvHSP70, and LvHSP90 were increased significantly in 6h treatment and tend return to normal conditions after 48h treatment. Superoxide dismutase activity was reduced during treatment and phenoloxidase activity was elevated slightly after 12h to 24h and tend to pretreatment level at recovery periods. All of these HSPs expression reverted to normal levels 6h after the recovery period. The results indicated that different expression patterns of the three HSPs. HSP60 have a longer and higher protection expression after 12h treatment than HSP70 and HSP90. HSP90 was more sensitive in 6h at all treatments than HSP60 and HSP70. It is concluded that supplementation of biofloc with the volume of 5-15mL/L caused Hsp protection in *L. vannamei* PLs at the first 6h to 48h treatment and increased phenoloxidase activity at 24-48h treatment and reduced survival rate of the white shrimp.

Keywords: L. vannamei, biofloc stress, suspended solid, HSPs, superoxide dismutase, phenoloxidase

Introduction

The consequence of intensification shrimp farming system in Indonesia added production inputs and crops will lead to produce large quantities of waste that contain solids (e.g. feces and uneaten feed) and nutrients (e.g. nitrogen and phosphorus) and biofloc waste. It can be detrimental to the environment if managed improperly. These solids and nutrients originate from the uneaten feed, feces, and animal urea/ammonia (Maillard *et al* 2005; Sharrer *et al* 2007). If release directly to the environment, these solids and nutrients can be polluted and resulting in environmental issues such as eutrophication (Wetzel, 2001) or could be directly toxic to aquatic fauna (Timmons *et al* 2002; Boardman *et al* 2004).

Biofloc culture system has been widely applied worldwide to reduce environmental impact and the production cost through well manage heterogeneous mixture of heterotrophic bacteria, macroalgae, food, faecal, remnants, exoskeletons, invertebrates into grown flocs and its ability to maintain good water quality (Emerenciano *et al* 2013; Jatoba *et al* 2014). It also improve for disease resistance as a whole can increase the production of various aquaculture species such as white shrimp (*L. vannamei*) and fish (*Tilapia nilotica*) (Avnimelech 2006; Kuhn *et al* 2010; Crac *et al* 2012; Khatoon *et al* 2016).

Suspended solid from biofloc of shrimp (*L. vannamei*) farming that is truncated to the surrounding waters after the harvesting periods can affect water quality by increasing turbidity in the coastal waters closed to farming area. It may slightly affect the survival and physiologi

cal conditions of various marine species (Sanders *et al* 1991). The reuse of biofloc from the first crop to the second crop could prevent coastal deterioration and beneficial for shortened preparation period of the BFT culture for the next cycle crop.

HSPs are ubiquitously present in all living organisms including both vertebrates and invertebrates (Chaurasia *et al* 2016). Members of HSPs family are well known molecular chaperones (or chaperonins) which prevent the gathering of newly produced polypeptides manage protein abasement and help miss fold proteins to obtain their original states (Ellis 1994). Generally HSPs are found in the molecular weight ranging between 27 and 110 kDa. Based on their size, molecular weight and functions in eukaryotes, HSPs are broadly classified into six major groups namely HSP60, HSP70, HSP90, HSP100, HSP110 and small HSPs (Parsell and Lindquist 1993). Among the different families of HSPs, HSP60, HSP70 and HSP90 are highly conserved and most extensively studied. The members of HSP60, HSP70 and HSP90 family are stress inducible, multigenic, and are present in all organisms studied till date (Lindquist and Craig 1988). It has been observed by Parsell and Lindquist (1993) that HSP60, HSP70 and HSP90 family play a significant role in cell survival, stress and thermal tolerance in response to various heat shocks. HSP60 is also known as phage growth E large (GroEL) in prokaryotes such as bacteria (Georgopoulos and Welch 1993). HSP70 which is constitutively expressed cognate stress protein, thus denoted as HSC70 protein in eukaryotes (Lindquist and Craig 1988). The expression of stress-inducible HSP70 is hardly detectable under normal growth conditions, but greatly elevated after exposure to environmental or physiological stresses (Brown *et al* 1993; Chen *et al* 1996). HSP70 is involved in cellular repair and protective mechanisms (Mosser *et al* 2000).

HSP60, 70 and 90 play an important role as molecular chaperones in intracellular organelles and stabilize unstable proteins through regulating the correct folding by gathering of proteins into oligomeric configuration, and thereby inhibiting the natural qualities of proteins (Nakamura *et al* 1991). These proteins are also involved in many cellular processes including signal transduction, DNA replication, protein synthesis and protein trafficking (Otaka *et al* 1994). The extracellular role of HSPs was proposed by a number of observations but it still demands more clarification. Such HSPs have been found to be released from various types of cells which include human neuroblastoma cell line, vascular smooth muscle cells, rat embryo cells, rat glial cells and human islet cells (Hightower and Guidon 1989). However, the definite secretary mechanism of HSPs is still unknown.

This study aimed to evaluate the performance of *L. vannamei* adaptation ability at the early stocking period to various volumes of biofloc in terms of molecular responses such as Hsp60, Hsp70, and Hsp90 gene expression and antioxidant enzyme activity such as Phenoloxidase (PO) and Superoxide Dismutase (SOD). However, no comprehensive information about the changes in the mRNA expression of HSP genes in the response to different volumes of biofloc system as stressors is available in this organism up to now. To better understand the biological process by which shrimp cope with various environmental stresses, a comparative study on relative mRNA expression patterns of Lvhsp60, Lvhsp70, and Lvhsp90 in response to a range of environmental stresses was carried out here. Meanwhile, a biomarker candidate of suspended solid stress in white shrimp was suggested based on the obtained results.

Material and Method

Experimental animal sampling and maintenance

Postlarvae of *L. vannamei* with a total length of 2.14 ± 2.14 cm and weight 0.048 ± 0.05 g were obtained from the National Broodstock Center for Shrimps and Mollusk (NBCSM) in desa bugbug, Karangasem Bali. The PLs were adjusted to laboratory conditions for 1 week before trial. For the experiment, the test and control group consisted of 100 PLs in triplicate. For the test examination, the tests were performed on three repeat test groups consisting of one hundred shrimp in a 100L glass aquarium. In all tests, the PLs were not fed during the experiment. During the experiment, salinity was maintained at 34 ‰, pH 7.8 to 8.3 while the temperature was maintained at 29-30°C.

Suspended solid source

Suspended solid used in this study was collected from *L. vannamei* small scale farm that utilized biofloc technology (BFT) before harvest (DOC100). To check the volume of suspended solids, the water from the shrimp waste pond containing biofloc was taken and poured into Imhoff Cone and the suspended solid was allowed to settle for 20 minutes. Harvesting biofloc was kept in 1000L conical fiberglass tank and transported to laboratory conditions for this experiment.

Suspended solid challenge experiment

One hundred PLs were distributed into 12 units of 100 L glasses aquarium for the experiment. After acclimation of seawater, ten PLs were randomly selected as 0 h samples before biofloc treatment for gene expression and biochemical analysis. The volume of suspended solid was 0, 5, 10 and 15 mL/L. During suspended solid supplementation all of the treatment were observed for stress response, dead and survive PLs were counted until the end of the test. Vanname PLs which were randomly selected kept separately into a different 1500 µl microtube and immediately used for RNA extraction and antioxidant enzyme assay.

Body weight and survival rate of L.vannamei

The dead shrimps were collected from each aquarium, after which the number of surviving PLs in each aquarium was recorded. These results represented the survivorship at 6, 12, 24, 48, R6, and R12 h. To measure the body weight of PLs, we used a analytical measuring unit as a measuring tool from 0 to 96 h. After that PLs were transferred again into each biofloc treatment aquarium.

Total suspended solids (TSS) and settleable solid analysis

The TSS was determined through gravimetry by filtering aliquots of 20 mL of water through GF 50-A glass fibre filters, according to Strickland and Parsons (1972) and AOAC (2000). Settleable solids were analyzed using Imhoff cone and the volume of floc on the bottom of the cone was measured after 20 minutes of sedimentation (Avnimelech 2009).

RNA extraction and cDNA synthesis

Total RNA was extracted from ten selected PLs using Quick-RNA™ MiniPrep Plus Kit (R1058) (Zymo Research) following manufacturer protocol. The integrity of RNA was assessed by electrophoresis on 1.2% agarose gel. RNA purity was verified by measuring absorbances at 260 and 280 nm with NDD 2000 (NanoDrop Technologies, USA) and cDNA spectrophotometers for each sample synthesized from the same amount of RNA (500 ng) by ReverTra Ace® qPCR RT Master Mix with gDNA removal (FSQ-301) (Toyobo, Japan) in accordance with the manufacturer protocol with oligo-dT and 3 random LvHsp primers based on Qian *et al* (2012).

Quantification of mRNA expression of HSP genes by real-time PCR

HSP gene expression in hemolymph after biofloc treatment was measured by reverse real-time reverse PCR transcription in Applied Biosystem (ABI, AS). Generally, total RNA is treated with DNaseI (Toyobo) to remove genomic DNA before reverse transcription. The cDNA obtained as described above is stored at -20 °C until used as a template for PCR reactions. The stress genes HSP60, HSP70 and HSP90 are adopted based on Gene Bank Access number FJ710169.1, AY645906.1, and HQ008268.1. The primary sequence is shown in Table 1. The reference genes EF1α (access number GenBank GU136229) are used as internal controls to calibrate the cDNA template.

The real-time PCR reaction was performed on a 20 µL reaction system with a mixture of 2 µL Thunderbird SYBR® qPCR, 2 µL forward primary (10 µM), 2 µL reverse primer (10 µM), 2 µL cDNA template equivalent to total RNA total 50 ng, and 4 µL free water nuclease. Each reaction is executed in triplicate. The thermal cycling condition was 95 °C for 30 seconds, followed by 40 cycles 95 °C for 5 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds. Melt curve analysis was added (65 °C to 95 °C, with 0.5 °C / s addition) to show PCR product specimens, as indicated by a single peak. The nuclease-free water (Promega, USA) is used instead of the cDNA template as the PCR negative control. Meanwhile, the NRT (non-reverse

transcription) control reaction using the template-treated with gDNA removal RNA

was performed to ensure there is no genomic DNA contamination in the reaction.

Table 1.
Primers used in the present study

Primer	Sequence (5'-3')	Gene bank Accession number	Product (bp)
LvHSP60 F	TGCCAACAACACCAACGAAG	FJ710169.1	138
LvHSP60 R	GCCAACATAACTCCACGCCT		138
LvHSP70 F	CTCCTGCGTGGGTGTGTT	AY645906.1	120
LvHSP70 R	GCGGCGTCACCAATCAGA		120
LvHSP90 F	TGGGCTTCTACTCCGCCTACC	HQ008268.1	109
LvHSP90 R	ACGGTGAAAGAGCCTCCAGCA		109
EF1α F	TGGCTACTCACCTGTGCTTG	GU136229	98
EF1α R	CCAGCTCCTTACCAGTACGC		98

SOD and PO enzyme activities

Ten PLs were collected from the experimental aquarium before and after biofloc exposure to measure antioxidant enzyme activity. The PLs were then minced in 500µl 1x PBS and centrifuged in (10^4 x g;10s;4°C).The supernatant were then ready for SOD analysis. SOD activity was determined by measuring the ability to inhibit the reduction of photochemical nitroblue tetrazolium chloride (NBT), as described previously Beauchamp & Fridovich (1971). SOD activity in this experiment was measured at 450 nm with SOD Kit-WST (water-soluble tetrazolium salt) Access (www.dojindo.com). The rate of the reduction of WST-1 with O_2 is linearly related to the xanthine oxidase (XO) activity, and this reduction is inhibited by SOD. A unit of SOD activity is defined as the amount of enzyme needed to induce 50% inhibition at the rate of NBT reduction under certain conditions. The result is expressed as unit activity (U) U / mg protein extract.
Phenoloxidase (PO) activity was measured using the indirect method at 20°C according to Hooper et al (2014). Briefly, 100 µL of 2 mM L-3,4-dihydroxyphenylalanine in 0.2 M Tris-HCl-1% SDS (v/v), pH 8 was added to 100 µL of PLs supernatant after centrifuged in (10^4 x g;10s;4°C). The absorbance at 490nm was read continuously for 30 min. The activity was given in PO unit which corresponds to the oxidation of 1 µmol of L-DOPA. Both of the assays were measured by Microplate reader Heales® MB-580, Shenzhen Huisong Technology Development Co.Ltd. China.

Data Analysis

The data for Hsp gene expression, SOD and PO enzyme activity were analyzed statistically by one-way ANOVA analysis followed by Tukey *post-hoc* test using SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). Values were significantly different at $P<0.05$.

Results and Discussion.

Growth and survival rate of *L. vannamei*

Shrimp kept at different concentrations of biofloc exhibited no significant different ($P > 0.05$) in total length during the experiment (data not shown), otherwise, bodyweight of the PLs presented significant differences between treatments ($P < 0.05$) on weights as indicated in Table 2.

Table 2. Body weight of *L. vannamei* during the experiment

Biofloc volume	0	6	12	24	48	R6	R12
0 (control)	0.05 ± 0.00	0.05± 0.00	0.1± 0.10[b]	0.08± 0.08	0.08± 0.05	0.12± 0.12 [b]	0.1± 0.00
5ml/L	0.05 ± 0.05	0.05± 0.01	0.05± 0.05	0.06± 0.06	0.06± 0.06	0.08± 0.08 [a]	0.08± 0.01
10ml/L	0.05 ± 0.05	0.07± 0.02	0.07± 0.07	0.11± 0.11	0.08± 0.07	0.1± 0.10 [ab]	0.08± 0.02
15ml/L	0.05 ± 0.05	0.08± 0.01	0.07± 0.07	0.07± 0.07	0.1± 0.03	0.1± 0.10 [ab]	0.12± 0.04

The survival for the biofloc treatments was similar ($P > 0.05$), for the first 6h, but significantly decreased ($P < 0.05$) afterward until recovery phage R12.

Nevertheless, all of the biofloc treatments exhibited significance differences ($P < 0.05$) at the end of the trial than that of the control.
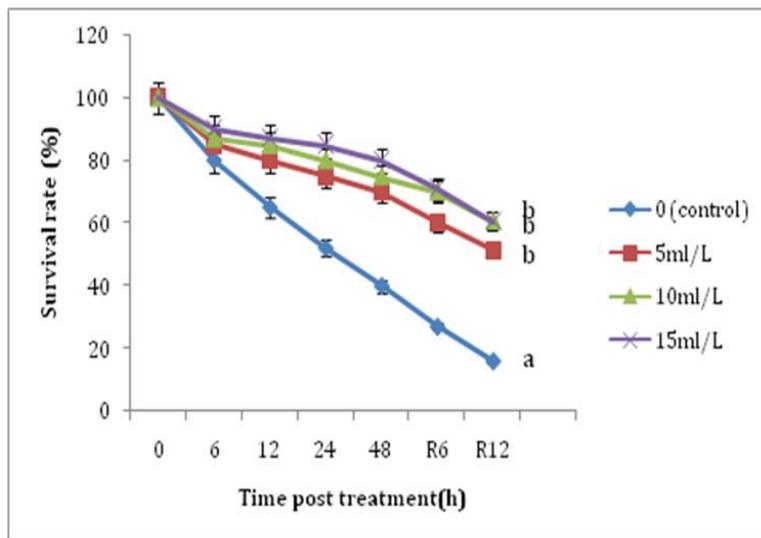

Figure 1. Survival rate of *L. vannamei* during suspended solid challenge in *L. vannamei*.

Total Suspended Solid

The volumes of total suspended solids (TSS) of the treatments were recorded daily during the biofloc treatments. Significant differences ($P<0.05$) in total suspended solids volumes were found between the treatments throughout the study, from 6 to 24 h (Table 4).

However, after 24 hours of the experiment, the TSS concentrations did not present a significant difference ($P > 0.05$) between all of the treatments during recovery phage (R6-R12) because all of them were transferred to fresh seawater.

Table 3. Total suspended solid measured during treatment

| Time (h)	0 (control)	5 ml/L	10 ml/L	15 ml/L
0	0	0	0	0
6	0[a]	39.33±10.12[b]	120.67±12.42[c]	136.67±9.81[c]
12	0[a]	40.32±5.19[b]	112.33±8.74[c]	126.67±6.43[c]
24	0[a]	42.67±11.02[b]	113.67±6.43[c]	133.00±9.64[c]
48	0[a]	43.38±10.17[b]	114.47±4.42[c]	136.33±4.64[c]
R6	0	0	0	0
R12	0	0	0	0

Responses of LvHsp60, LvHsp70 and LvHsp90 genes to different suspended solid exposure

The expression profile of the three HSP genes in the *L. vannamei* PLs after biofloc treatment is shown in Figure 2A, B and C. Although three genes were induced under similar suspended solid pressure, quite different expression patterns were observed. The relative expression of HSP60, HSP70, and HSP90 increased rapidly in the first 6 hours, after suspended solid exposure, and then gradually decreasing to pretreatment levels after 24 h (Figure 2A, B, C). The transcription rate of HSP60 was increased by suspended solid exposure, at 12-24 hours after treatment, whose value reaches (8-17 times over control) after that, the expression level decreased gradually. The transcription rate of HSP70 was significantly increased (*P*<0.05) by suspended solid exposure, on 15mL at 12 hours, peaking (7 fold control) however, the HSP70 expression compared to inducible HSP60, HSP90 did not show such a great variety of expression. The relative expression of HSP90 reached the highest point (13 times control) at 15mL treatment in 6 hours treatment. After that, the expression level of HSP90 decreased after 12 hours post suspended solid exposure closed to the control level, and remained at this level during the recovery period, (Figure 2C).

HSP60, HSP70, and HSP90 are chaperonin families whose expression is caused by various stress factors (Farcy et al 2007; Qian et al 2012). After 6 hours, its expression induced by biofloc treatment (Figure 2A, B, C). It has been shown that three HSP genes expression can be regulated by the biofloc exposure which depends on suspended solid volumes. HSP70 induction may modulate cytosolic redox status in cells for protection against oxidative stress (Kalmar and Greensmith, 2009). HSP90 influences the folding of newly synthesized polypeptides by providing an environmental companion coupled with translation (Frydman, 2001). The up-regulation of HSP70 is caused by many denaturation proteins caused by the accumulation of toxic ROS and is then used to refold and reassemble this protein (Zang et al 2009).

After the shrimp were challenged with different suspended solid volumes, the relative mRNA expression of LvHSP genes in the whole body of white shrimp was observed for 48 h. Different genes possessed different expression profiles in response to biofloc stress, and the relative mRNA expression for a particular gene was shown to be time and biofloc volume dependent (Figure 2A, B, C). The relative expressions of Lvhsp60 at biofloc volumes of 10mL and 15mL were induced at 6-24h compared with that of the control, reaching the highest levels (12 times and 18 times that of the control at volume 10mL and 15mL, respectively) at 12 and 24h post-challenge. At a volume of 15mL, the Lvhsp70 transcript reached its peak (7 fold that of the control) at 12 h post-challenge, reverting to the level of the control at 24 h and 48 h post-challenge. At a suspended solid volume of 15mL, however, the maximal relative expression (13-fold that of the control) of Lvhsp90 was observed at 6 h post-challenge. The relative expression of Lvhsp60 was still higher than that of the control at 12 h post-challenge. However, it became level with the control at 24 h post-challenge. Compared with that of the control, no apparent difference was observed in the relative expression of Lvhsp90 for the shrimp held at the suspended solid volume of 10mL and 15mL at 24 h post-challenge (Figure 2C)
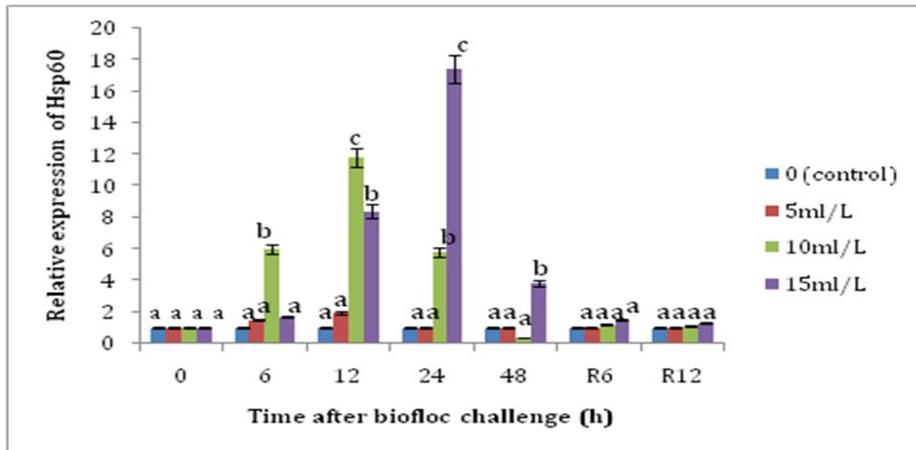
(a)
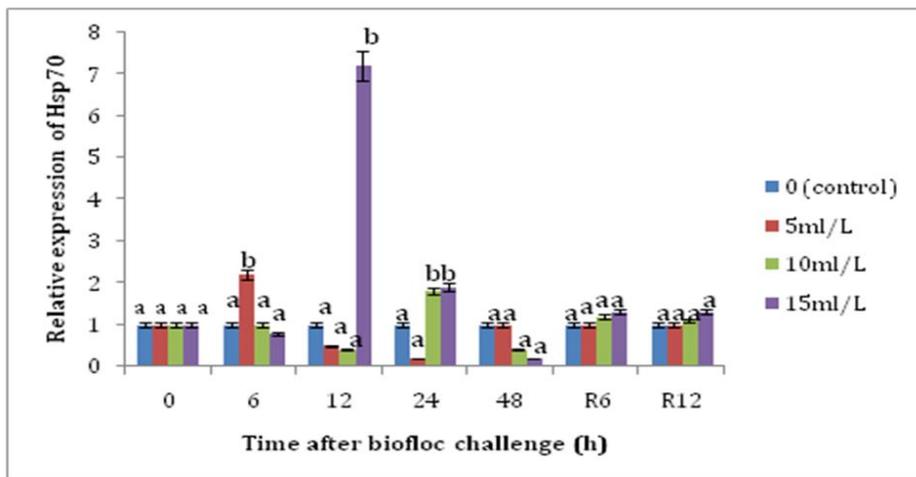
(b)
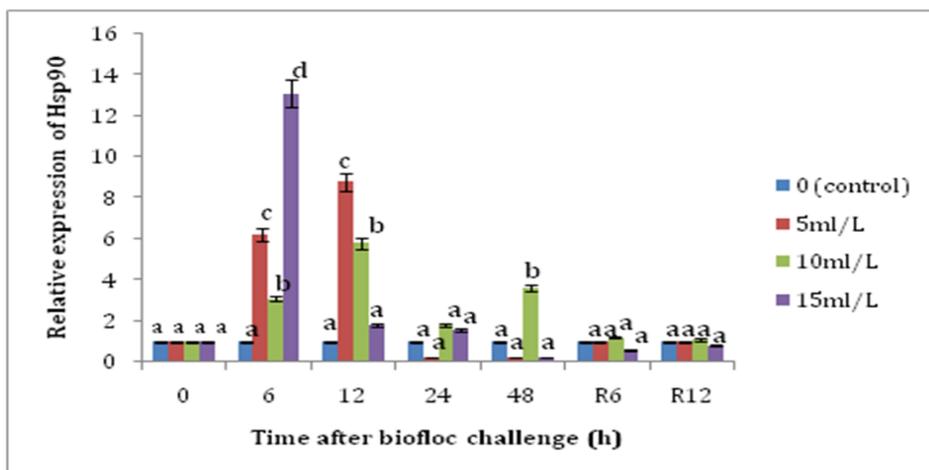
(c)

Figure 2. HSP60 (a), HSP70 (b) and HSP90 (c) relative expression levels after biofloc challenge in *L. vannamei*. The 0, 6, 12, 24 and 48 h denote 0 (untreated), exposed to the biofloc 0, 5, 10 and 15mL/L exposure condition. Different letters between treatments and the 0mL control were considered significant differences (*P*<0.05) in relative expression.

Biochemical responses of *L. vanamei*

The antioxidant enzyme activity of SOD, and PO was investigated to identify changes in biochemical activity that occurred in response to biofloc exposure for 0, 6, 12, 24, 48, and recovery stage (6-12 hours) (Figure 3 and 4). The results showed that superoxide dismutase (SOD) activity was decreased in *L. vannamei* exposed to a different volume of suspended solid ($P<0.05$) compared with control of 0 mL in 6 hours (Figure 3) and tend to increase at 5mL in 24h but still lower than that the control group. The most significant decrease of SOD activity was observed in suspended solid at 5 mL treatment after 48 hours post transferred to clear water (recovery period). After the next 6 hours, SOD activity increased significantly ($P>0.05$) at all treatment to the normal condition. The pattern of SOD enzyme activity is affected in time depending and biofloc volume in the culture medium of *L. vannamei*. Decrease antioxidant response might indicate an accumulation of superoxide anion radical and consequent oxidative stress in cells and susceptibility to pathogens (Mercier et al 2006; Mohankumar & Ramasamy, 2006).

Shrimp rely on innate immunity to detect and respond to foreign particles like microbial components which are hugely available in biofloc system, including LPS, bG, and peptidoglycan (PG), known as pathogen-associated molecular patterns (PAMPs) (Soderhall and Cerenius, 1998). PAMP recognition by pattern-recognition protein (PRP) initiates and activates the innate immune response in which the proPO activation system and phagocytosis are the two important processes (Carroll & Jeneway 1999; Cerenius & Shoderhall 2008). Several studies indicated that shrimp hemocytes incubated with bG, fucoidan, and carrageenan exhibit degranulation of granules, change in cell size and increased PO activity and RB (Smith et al 1984; Kitikiew et al 2013; Chen et al 2014). In the present study, PO activity after the shrimp were treated with biofloc shown lower activity at 6 to 12h. After that, the activity was increased from 24 to 48h. Therefore, the activation of innate immunity in shrimp encountering foreign particles is greatly affected by time and concentration of biofloc.
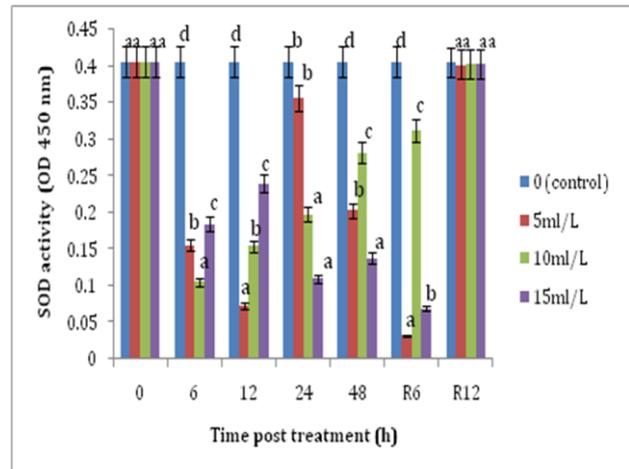
Figure 3. SOD enzyme activity in the *L. vannamei* exposed to biofloc gradient for 0, 6, 12, 24, 48h and recovery periods. The experiments were conducted in triplicate, and data represent the mean ± SD. Differences in letters between treatments and the 0 mL control were considered significant differences at ($P<0.05$).
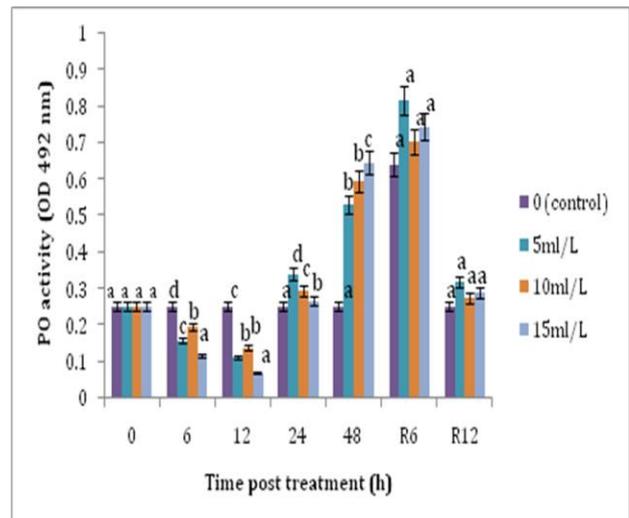
Figure 4. PO enzyme activity in the *L. vannamei* exposed to biofloc gradient for 0, 6, 12, 24, 48h and recovery periods. The experiments were conducted in triplicate, and data represent the mean ± SD. Differences in letters between treatments and the 0 mL control were considered significant differences at ($P<0.05$).

L. vannamei exposed to different biofloc treatments in 6 to 12 h had significantly lower ($P<0.05$) phenoloxidase (PO) activities than the control group. After 12 h phenoloxidase activities were increased and significantly higher ($P<0.05$) than control until 48h. The PO activity returned to normal conditions at the recovery periods.

Discussion

In a Biofloc Technology System (BFT), there is constant biofloc formation and suspended solids accumulation, leading to effects on water quality parameters that may affect the body weight and survival of cultured shrimp because of total suspended solid production. This study aimed to analyze during biofloc formation the effect of different total suspended solids (TSS) levels on the molecular and biochemical adaptation of *Litopenaeus vannamei* shrimp culture in an early BFT system. The distinguished protection mechanisms developed by animals in response to environmental pressure is the induction of HSPs and antioxidant enzyme activities. Hepatopancreas is the major metabolic center in crustaceans that plays important roles in the production of reactive oxygen species, the detoxification of xenobiotics, immune defenses, digestion, absorption, and secretion of nutrients (Brunet et al 1994; Soderhall and Cerenius, 1998). Because of its metabolic activity, hepatopancreas may be more sensitive to environmental changes. Our study is the first report to explore the difference mRNA expression patterns of LvHSPs genes in response to different biofloc exposure and to evaluate the potential of each LvHSP gene as a molecular biomarker for specific environmental stress in this shrimp.

HSP gene involvement in the heat shock response has been well documented in many aquatic animals. The results indicate that LvHSP60 and LvHSP70 may play important roles in mediating the immune responses of *L. vannamei* to bacterial challenge and that the Ca^{2+} signaling transduction pathway may be involved in the initiation of the shrimp's immune responses in early stages of infection (Zhou et al 2010). It has been shown that the expression levels of HSP70 and HSP90 on disc *H. discus* hemolymph were significantly induced within the first 24 hours after exposure to heat (Park et al 2015). Another case was observed in Chinese shrimp *F. chinensis*, where HSP70 and HSP90 were dramatically induced under heat stings, while hsc70 cognate was slightly affected (Li et al 2009; Luan et al 2009). Similar results were obtained in *M. rosenbergii* (Liu et al 2004)., HSP gene exhibits a very strong response to acute thermal stress for the *Crassostrea gigas*, with mRNA levels changing up to 40-fold (Farcy et al 2008). In other aquatic animals such as abalone *H. tuberculata* (Farcy et al 2007), Zhikong *Chlamys farreri* (Gao et al 2007).

Biofloc residues, caused by insufficient completion maintenance of culture tanks, shrimp ponds effluent runoff, and human activity are common phenomena in waters with poor waste management. It has been known that suspended solid with concentration about 100-300 mg L^{-1} during biofloc formation is important for maintaining water quality particularly when the nitrification process is not well established (Gaona et al 2015). At a higher level, it can increase intracellular ROS (reactive oxygen species) which eventually leads to oxidative stress in the aquatic organism (Liu et al 2007; Zhang et al 2009). Biofloc can improve the immunity of aquatic animals as well as their growth performance (Ekasari et al 2014). Long et al (2015) have observed an increased immune response in tilapia, *Oreochromis niloticus* reared in biofloc. Regarding fish antibodies, Ig M, which is produced as part of a specific innate immune response, is the most abundant in both plasma and mucus and has various functions such as cell-mediated cytotoxic responses and complement pathway activity (Kim and Kang, 2016). Bakhshi et al (2017) also reported that the biofloc system is effective in improving fish immunity, and many authors have suggested that biofloc plays a role as an immunostimulant increasing innate immune system responses (Ekasari et al 2014; Kim et al 2015). In this study, survival rate of *L. vannamei* reared in all biofloc treatment were significantly higher than that of those reared in fresh seawater, which indicates that biofloc acted as an immunostimulant.

In this study, four suspended solid volume (0mL, 5mL, 10mL, and 15mL/L) were designed using seawater in the 100L aquarium to gene expression profiles. Our results show that the value of biofloc exposure may induce the expression of three HSP genes, suggesting HSP gene involvement in the mitigation of oxidative stress induced by suspended solids in the water medium.

Suspended solid from biofloc as aquaculture waste has become a serious problem in aquatic ecosystems for the aquatic organism. The presence of biofloc in the marine / estuarine environment in aquatic toxicology concern, as it may lead to resistance to pathogenic microorganisms, altering some

of the reproductive and physiological processes of aquatic organisms, such as osmoregulation, respiration, and growth. Exposure to elevated suspended solid levels can cause oxidative damage to cells with disruption of endocrine function with alteration of liver steroid metabolism (Sindermann, 1986). In this study, we examined the effects of suspended solids from biofloc on gene expression profiles of three known HSPs at relatively mRNA levels. Generally three of the Hsp gene were response in 6 h post treatment. The relative expression of HSP60 mRNA showed a stronger and longer response than HSP90 and HSP70. Maximum HSP60 mRNA levels 18 times were observed at 24 hours after biofloc exposure when compared to control. These results also show that HSP60 giving longer protection caused by suspended solid exposure than HSP70 and HSP90. In contrast, HSP90 expression rates (in all treatment) were faster in response to suspended solid exposure compared with HSP60 and HSP70. Different expression profiles of three genes under intervention of suspended solid demonstrate their different roles in overcoming cell damage by biofloc exposure.

The reconstituted HSP70 expression takes place until the protective and adaptive response of the cells to biofloc is almost complete at 48 hours post-exposure, while the relative expression of HSP90 is still much higher than the control in post-96 h biofloc exposure, indicating the different duration of HSP70 induction in response to exposure to biofloc and bacterial infections.

Conclusions

In conclusion, if we compared the expression profiles of HSP60, HSP70 and HSP90 in *L. vannamei* under suspended solid exposure in this study. Three HSP genes which were induced under the treatment of suspended solid shows the different way of expression. Commonly all of them showed significant response in protection started in 6 h post treatment. HSP60 exhibited stronger and longer protection in response to biofloc exposure, otherwise HSP90 on all of the treatment showed quicker protection in response to suspended solid exposure compared with HSP60 and HSP70. The HSP70 gene exhibited a lower response to biofloc exposure. HSP60 and HSP90 showed the

dominant role of HSP60 in overcoming cellular stress induced by suspended solid exposure and more potential to become a biomarker in the aquatic environment.

The profile of antioxidant enzyme activity such as SOD and PO also differs from different HSP genes and the presence of different volumes of suspended solid from biofloc shrimp culture. SOD activity showed lower activity than control during biofloc treatment otherwise PO showed increased activity at the end of the experiment before the recovery period. Our results presented here provide useful insights for investigating cellular stress-related responses and for identifying potential stress gene stress-causing environmental biomarkers in white shrimp, *L. vannamei*. More detailed investigations using other networks and development stages should be undertaken for a better understanding of the functioning of these genes and antioxidant enzymes.

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