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*Research Article*

# Effects of bacilli, molasses, and reducing feeding rate on biofloc formation, growth, and gene expression in *Litopenaeus vannamei* cultured with zero water exchange

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**ABSTRACT.** The effects of reducing feeding rate and molasses on water quality, growth, and gene expression of *Litopenaeus vannamei* were evaluated in a super intensive system with zero water exchange. A bioassay of 35 days (0,75 ± 0,22 g, 36 shrimp tank$^{-1}$, 450 shrimp m$^{-3}$) with treatments in triplicate was performed: I) 100% commercial feed (control group) with water exchange; II) 100% commercial feed without water exchange; III) 100% commercial feed + *Bacillus licheniformis* BCR 4-3 + molasses without water exchange; IV) 90% commercial feed + *B. licheniformis* BCR 4-3 + molasses without water exchange, and V) 80% commercial feed + *B. licheniformis* BCR 4-3 + molasses without water exchange. Shrimp survival, weight, nitrogenous wastes, settleable solids (SS), and the relative expression (RT-qPCR) of stress and digestive genes were determined. Shrimp survival was 100% in all treatments. The reduced diet, bioflocs, and bacilli did not affect shrimp growth. The culture conditions reduced nitrogenous wastes and modulated mRNA expression of trypsin, chymotrypsin, and heat shock protein 70. Reduced feed ration can help to maintain water quality and lower costs in intensive shrimp cultures with bioflocs.

**Keywords:** *Bacillus licheniformis*, *Litopenaeus vannamei*, bioflocs, gene expression, molasses, water quality.

## INTRODUCTION

Reduction of conventional feeding rate can be achieved in shrimp culture when animals fed bioflocs (Panjaitan, 2010). In the systems with zero-water exchange, feed intake and nutrients are controlled and the accumulation of flocculated particles (bioflocs) formed by aggregates of algae, bacteria, protozoa, feces, and uneaten feed is favored (Hargreaves, 2013). Bioflocs prevent the proliferation of pathogenic microorganisms and recycle waste nutrients (Ray *et al.*, 2010; Vinatea *et al.*, 2010). Furthermore, the microbial biomass contained in the flocs can be eaten as a protein source by the cultured animals and, therefore, decreases production costs (Wasielesky *et al.*, 2006; Crab *et al.*, 2010; Xu & Pan, 2014). *Bacillus* spp. and external carbon source such as molasses can be inoculated (Lalloo *et al.*, 2007; Ekasari *et al.*, 2014) to stimulate the heterotrophic community and to improve water quality.

Bioflocs can stimulate the production of digestive enzymes (protease, amylase, cellulase, and lipase) in the shrimp and/or enhance their activity (Xu *et al.*, 2012a, 2012b). Digestive enzymes are produced in the hepatopancreas that has three main functions: synthesis and secretion of digestive enzymes, temporary and cyclical retention of reserves (lipids, glycogen), and absorption of nutrients (free fatty acids, aminoacids, glucose) from digestion (Johnston *et al.*, 1998; Verri *et al.*, 2001; Wang *et al.*, 2014). Trypsin and chymotrypsin are two of the most abundant proteolytic enzymes in decapod's hepatopancreas (Muhlia-Almazán *et al.*, 2002, 2003). However, food is one of the determinant factors in the synthesis of these enzymes since biochemical properties (protein, lipid, and carbohydrate concentration) of food may affect their activity (Ezquerra *et al.*, 1997, 1999). Nowadays, research is focused on shrimp responses (*e.g.*, metabolic, physiological) to dietary changes, as well as

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on the expression of genes responsible for such responses (Sánchez-Paz *et al.*, 2003; Chávez-Calvillo *et al.*, 2010; Flores-Miranda *et al.*, 2015).

In intensive shrimp culture systems, high density, the variation in environmental factors (temperature, salinity, dissolved oxygen, and pH), excess feed, and toxic chemicals are considered as stressors (Hickey & Singer, 2004; Schock *et al.*, 2013). Heat shock proteins (Hsp) are highly conserved proteins well known for their quick responses to stress (Qian *et al.*, 2012). When induced by environmental stress, Hsp70s work to repair partially denatured proteins, facilitate the degradation of irreversibly denatured proteins, and inhibit protein aggregation (Parsell & Lindquist, 1993). Hsp70s are also implicated in eliciting immune responses against many bacterial diseases (Tsan & Gao, 2009; Chen & Cao, 2010).

This study was conducted to determine the effects of *Bacillus licheniformis* BCR 4-3, molasses, and reducing feeding rate on biofloc formation, water quality, growth, and digestive and stress gene expression in *Litopenaeus vannamei* cultured in a super intensive system with zero water exchange.

## MATERIALS AND METHODS

### Shrimp culture

Shrimp collected from a commercial farm (Acuícola Cuate Machado, Guasave, and Sinaloa, Mexico) were acclimated to culture conditions for three days in an outdoor culture system covered with a shadow mesh. The culture system consisted of 120 L plastic tanks containing 80 L of filtered (20 µm) seawater (30 salinity) and continuous aeration. Thirty-six shrimp were placed per tank and they were fed twice daily at 09:00 and 17:00 h with 35% protein commercial feed (Nutrimentos Acuícolas Azteca, S.A. de C.V., Guadalajara, Jalisco, Mexico). Feeding ratio was according to the average body weight, starting with 17%. Half of the water was changed at day three, before starting the bioassay. Uneaten food and waste material were removed daily before feeding. Animals were free of WSSV and IHHNV (data not shown).

In the 35-d bioassay, animals (initial mean weight = 0.75 ± 0.22 g, stocking density = 36 shrimp/tank = 450 shrimp $m^{-3}$) and culture system from the acclimation period were used. Animals were fed twice daily at 09:00 and 17:00 h with 35% protein commercial feed (Nutrimentos Acuícolas Azteca, S.A. de C.V.), according to the average body weight, starting with 17% and ending with 7%. Probiotic bacteria, grown in tryptic soy broth at 35ºC for 24 h (Escamilla-Montes *et al.*, 2015), was added to the water ($1\times10^6$ CFU $L^{-1}$, $8\times10^7$ CFU $tank^{-1}$) every four days (Ávila-Leal *et al.*, unpublished data). On the other hand, molasses (Ávila-Leal *et al.*, *unpublish. data*) were added daily until day 17 (1 g $feed^{-1}$: 0.8 g molasses), always maintaining a C/N ratio of 5.7:1 (Ávila-Leal *et al.*, *unpublish. data*). The bioassay was conducted as a randomized design with five treatments, each one in triplicate: I) 100% feed ration (control group) with water exchange (50%); II) 100% feed ration without water exchange; III) 100% feed ration + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses without water exchange; IV) 90% feed ration + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses without water exchange; V) 80% feed ration + *B. licheniformis* BCR 4-3 ($1\times10^6$ CF $mL^{-1}$) + molasses without water exchange. In treatments II, III, IV, and V no water exchange was done, but freshwater was regularly added to recover the water lost due to evaporation. In treatment I, uneaten food and waste matter were removed every four days before feeding and 50% of the water was exchanged.

Temperature, pH, salinity, and dissolved oxygen were determined every three days. Ammonium, nitrite, and nitrate were determined at weekly basis (Strickland & Parsons, 1972).

At the end of the bioassay, survival, weight, settleable solids (SS), and expression of stress and digestive genes were determined.

SS was determined every three days and at the end of the bioassay using Imhoff cones (Avnimelech & Kochba, 2009). A water sample (1 L) was taken and placed in the cone, allowing to sediment during 30 min. The SS is defined as the volume of bioflocs in milliliters per liter of water after 30 min of sedimentation (Avnimelech & Kochba, 2009).

### Specific growth rate

Specific growth rate (SGR) was determined using the following formula:

$$SGR\ (\%\ d^{-1}) = 100\ (\ln W2 - \ln W1)/t$$

where t is the culture period (days), ln is the natural logarithm, $W_1$ and $W_2$ are the initial and final mean weights of the shrimp, respectively.

### Hepatopancreas collection

Individual hepatopancreas samples (150-200 mg) from nine inter-molt shrimp per treatment (three per tank) were placed in microfuge tubes with 300 µL of precooled Trizol reagent (Invitrogen, Carlsbad, CA, USA) and stored at -70°C until RNA extraction.

### Gene expression

Total RNA was extracted from the hepatopancreas tissue using 1 mL of Trizol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's

protocol. The concentration and purity of RNA (low protein and phenol) was determined by measuring the absorbance at 260/280 nm on a Pearl (Implen®, Westlake Village, CA, USA) nanophotometer and stored at -70°C until cDNA construction. To transcribe the cDNA, the total RNA was treated with DNAse I (1 U $\mu L^{-1}$, Sigma-Aldrich, St. Louis, MO, USA). Aliquots of each sample were diluted at 100 ng $\mu L^{-1}$. Reverse transcription was performed to synthesize the first strand of cDNA with reverse transcriptase (Improm II, Promega, Madison, WI, USA) and the oligo ($dT_{20}$) primer using 1.0 µg of total RNA (10 µL). The cDNA was diluted five times with ultrapure water and stored at -70°C until qPCR analysis. Five microliters of this cDNA dilution was used as template in each qRT-PCR reaction.

Relative expression of stress (heat shock protein 70 [Hsp70]) and genes encoding for digestive enzymes (trypsin and chymotrypsin) (Stephens *et al.,* 2012; Flores-Miranda *et al.,* 2015) in the hepatopancreas of shrimp was measured by quantitative real-time PCR using a CFX96 system and the CFX Manager version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA). We analyzed the expression of reference genes 40S-S24, β-actin, EF1α, and L21 (Wang *et al.,* 2010; Stephens *et al.,* 2012; Flores-Miranda *et al.,* 2015) in order to determine their stability in the hepatopancreas tissue. The relative stability was analyzed with the algorithms GeNorm (Vandesompele *et al.,* 2002) and NormFinder (Andersen *et al.,* 2004), using the RefFinder web application (http://150.216.56.64/referencegene.php). Expression of the genes of interest was normalized with the most stable reference genes.

To diminish technical variations in the results, a PCR Master Mix 2X (1.5 µL of 10X reaction buffer, 0.75 µL of 50 mM $MgCl_2$, 0.3 µL of 10 mM dNTPs, 0.75 µL of EvaGreen® 20X [Biotium Inc., Hayward, CA, USA]; 0.1 µL of 5 U $\mu L^{-1}$ GoTaq DNA Polymerase (Promega, Madison, WI, USA), and 4.1 µL of ultrapure water was prepared for all the samples in one batch.

Amplifications were performed in triplicate in 96-well plates. PCR reactions contained 7.5 µL of 2X PCR Master Mix, 0.35 µL of each primer (10 µM, Sigma-Aldrich, St. Louis, MO, USA), 5 µL of cDNA, and 1.8 µL of ultrapure water to achieve a final volume of 15 µL. PCR conditions were: initial denaturation at 95°C for 3 min; followed by 40 cycles at 95°C for 10 s, 60°C for 15 s, 72°C for 30 s, and an additional step of 79°C for 5 s to acquire fluorescence. At the end, a dissociation curve from 65 to 90°C was recorded at increments of 0.5°C. All runs were examined for unique and specific products.

Amplification efficiency was determined by calculating a slope with five serial dilutions (dilution factor of 5 or 10) of a representative pool of cDNAs [$E = 10^{(-1/slope)} - 1$] (Bustin *et al.,* 2009). To calculate expression of target genes, Cq values were exported to Excel (Microsoft) and transformed to relative quantities (RQ) using the equation $RQij=E^{(Cq(mean)-Cq(ij))}$. Where E is the gene-specific efficiency, and [(Cq mean –Cq(ij)] is the absolute difference for each Cq sample against the mean Cq in the dataset for each gene. Relative expression was calculated with the equation (RQ-target)/Geometric mean of the most stable reference genes (RQ-reference genes) (Vandesompele *et al.,* 2002).

**Statistical analysis**

One-way variance analysis (ANOVA) using the *F*-test was performed among treatments to examine the differences in the water quality parameters, growth and survival of shrimp, and gene expression. Where significant ANOVA differences were found, a Tukey's *HSD* test was performed to determine the nature of these differences at ($P < 0.05$).

## RESULTS

Values of final weight were as follows: I) 5.14 ± 0.50 g, II) 5.06 ± 0.50 g, III) 5.58 ± 0.27, IV) 5.01 ± 0.40 and V) 4.61 ± 0.1. There were no significant differences in shrimp final growth. The following values of SGR (% $d^{-1}$) were obtained in treatments I (5.73 ± 0.2), II (5.57 ± 0.14), III (5.79 ± 0.14), IV (5.58 ± 0.39), and V (5.24 ± 0.51). There were no significant differences in shrimp growth. Survival of organisms was 100% in all treatments (Table 1).

Temperature was between 23.29 ± 2.13 and 23.71 ± 2.18°C, with a mean of 23.46 ± 2.16°C for all treatments. Salinity was maintained at 30.18 ± 0.32 to 30.9 ± 1.22 with a mean of 30.43 ± 0.72 for all treatments, dissolved oxygen concentrations were between 5.87 ± 0.56 and 6.46 ± 0.59 mg $L^{-1}$, with a mean of 6.18 ± 0.52 mg $L^{-1}$ for all treatments, and pH were within 8.31 ± 0.06 – 8.40 ± 0.05 with a mean of 8.35 ± 0.06 for all treatments (Table 2). The physical and chemical parameters were within optimal range according to Boyd & Tucker (1998), with the exception of the temperature that was below 23°C during the night.

Ammonium concentration was higher in treatment II as compared with treatment I ($P = 0.005$) and V ($P = 0.047$). Nitrite and nitrate were found in low concentrations, between optimal range (nitrite [< 0.5 mg $L^{-1}$], nitrate [0.4-0.8 mg $L^{-1}$]), with no significant differences among treatments. The SS was significantly higher in treatments II ($P = 0.001$), III ($P = 0.0002$), IV ($P = 0.0001$), and V ($P = 0.0007$) as compared with treatment I (Table 3).

**Table 1.** Growth and survival of *L. vannamei* cultured with reduced feeding and molasses. Treatments: I) 100% commercial feed (control group) with water exchange, II) 100% commercial feed without water exchange, III) 100% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange, IV) 90% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange, V) 80% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange. Values are mean ± SD. Different letters indicate significant differences ($P < 0.05$). SGR: specific growth rate.

| Treatments | Initial weight (g) | Final weight (g) | SGR (% $d^{-1}$) | Survival (%) |
|---|---|---|---|---|
| I | 0.69 ± 0.23 | 5.14 ± 0.50 | 5.73 ± 0.20 | 100 |
| II | 0.72 ± 0.22 | 5.06 ± 0.50 | 5.57 ± 0.14 | 100 |
| III | 0.74 ± 0.21 | 5.58 ± 0.27 | 5.79 ± 0.14 | 100 |
| IV | 0.71 ± 0.17 | 5.01 ± 0.40 | 5.58 ± 0.39 | 100 |
| V | 0.74 ± 0.19 | 4.61 ± 0.17 | 5.24 ± 0.51 | 100 |

**Table 2.** Temperature, salinity, dissolved oxygen, and pH in the culture system of *L. vannamei*. Treatments: I) 100% commercial feed (control group) with water exchange, II) 100% commercial feed without water exchange, III) 100% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange, IV) 90% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange, V) 80% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU mL) + molasses (80% regarding feed) without water exchange. Values are mean ± SD.

| Treatments | Temperature (ºC) | Salinity (‰) | DO (mg $L^{-1}$) | pH |
|---|---|---|---|---|
| I | 23.43 ± 2.45 | 30.9 ± 1.22 | 6.46 ± 0.59 | 8.34 ± 0.09 |
| II | 23.29 ± 2.13 | 30.45 ± 0.88 | 6.20 ± 0.61 | 8.37 ± 0.06 |
| III | 23.57 ± 2.00 | 30.18 ± 0.32 | 5.87 ± 0.56 | 8.31 ± 0.06 |
| IV | 23.30 ± 2.08 | 30.35 ± 0.46 | 6.24 ± 0.39 | 8.35 ± 0.05 |
| V | 23.71 ± 2.18 | 30.30 ± 0,75 | 6.13 ± 0.48 | 8.40 ± 0.05 |

**Table 3.** Concentration of ammonia, nitrite, nitrate, and settleable solids in the culture system of *L. vannamei*. Treatments: I) 100% commercial feed (control group) with water exchange, II) 100% commercial feed without water exchange, III) 100% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange, IV) 90% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange, V) 80% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange. Values are mean ± SD. Different letters indicate significant differences ($P < 0.05$). SS = Settleable solids.

| Treatments | Ammonium (mg $L^{-1}$) | Nitrite (mg $L^{-1}$) | Nitrate (mg $L^{-1}$) | SS (mL $L^{-1}$) |
|---|---|---|---|---|
| I | $0.34 \pm 0.16^{a}$ | 0.11 ± 0.05 | 0.22 ± 0.35 | $1.09 \pm 0.43^{b}$ |
| II | $1.65 \pm 1.25^{b}$ | 0.16 ± 0.15 | 0.50 ± 0.49 | $21.14 \pm 2.21^{a}$ |
| III | $1.30 \pm 1.34^{ab}$ | 0.26 ± 0.16 | 0.73 ± 0.40 | $20.08 \pm 3.77^{a}$ |
| IV | $1.01 \pm 1.15^{ab}$ | 0.21 ± 0.16 | 0.72 ± 0.41 | $21.50 \pm 3.23^{a}$ |
| V | $0.61 \pm 0.57^{a}$ | 0.22 ± 0.17 | 0.71 ± 0.41 | $18.34 \pm 3.12^{a}$ |

Results of mRNA expression of three genes are summarized in Table 4. The mRNA expression of trypsin in treatment I was significantly higher than in treatments IV ($P = 0.007$) and V ($P = 0.03$). The mRNA expression of chymotrypsin in treatament I was significantly higher than in treatments IV ($P = 0.001$) and V ($P = 0.002$). The mRNA expression of chymotrypsin in treatament II was significantly higher than in treatments IV ($P = 0.004$) and V ($P = 0.009$). In treatment II, mRNA expression of Hsp70 was significantly different from treatments III ($P = 0.049$), IV ($P = 0.001$), and V ($P = 0.048$).

## DISCUSSION

In this work, shrimp survival was 100% in all treatments and there were no significant differences in weight, despite the fact that feeding rate was reduced in

**Table 4.** Relative expression of stress and digestive genes in *L. vannamei* cultured with reduced feeding and molasses. Treatments: I) 100% commercial feed (control group) with water exchange (50%), II) 100% commercial feed without water exchange, III) 100% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange, IV) 90% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange, V) 80% commercial feed + *B. licheniformis* BCR 4-3 ($1\times10^6$ CFU $mL^{-1}$) + molasses (80% regarding feed) without water exchange. Values are mean ± SD. Different letters indicate significant differences ($P < 0.05$).

| Genes | Relative expression to β-actin | | | | |
|---|---|---|---|---|---|
| | I | II | III | IV | V |
| Trypsin | $1.47 \pm 0.39^{b}$ | $1.2 \pm 0.34^{ab}$ | $1.20 \pm 0.17^{ab}$ | 0.61± 0.13a | $0.75 \pm 0.28^{a}$ |
| Chymotrypsin | $1.56 \pm 0.37^{b}$ | $1.42 \pm 0.56^{b}$ | $1.10 \pm 0.25^{ab}$ | $0.53 \pm 0.12^{a}$ | $0.60 \pm 0.10^{a}$ |
| Hsp70 | $1.04 \pm 0.24^{ab}$ | $1.72 \pm 0.74^{b}$ | $0.93 \pm 0.35^{a}$ | $0.52 \pm 0.12^{a}$ | $0.92 \pm 0.11^{a}$ |

treatments IV (90% feed ration) and V (80% feed ration) as compared with treatments I, II, and III with 100% feed ration. The results are consistent with those obtained by Panjaitan (2010) who cultivated *Penaeus monodon* in an intensive system with zero-water exchange, molasses, and different percentages of feed, and found that it is possible to reduce feeding rate (formulated diets) up to 25% without affecting growth, because bioflocs served as natural food. Likewise, Becerra-Dorame *et al.* (2012) found that the hyper-intensive culture of *L. vannamei* with zero-water exchange, bioflocs, and formulated feed improved growth and survival.

The physicochemical parameters were within optimal range according to Boyd & Tucker (1998) with the exception of the temperature that was below 23°C during the night. The addition of molasses, bioflocs, and the reducing feeding rate were effective in maintaining nitrogenous wastes between the optimal ranges for shrimp (Boyd & Tucker, 1998). These results agree with those obtained by Arnold *et al.* (2009) and Ballester *et al.* (2010). Results denoted nitrification process in the culture system, due to the decrease of the ammonium and the increase of the nitrate, and the positive effect on growth (similar in all treatments) of reducing feeding rate. Likewise, Panjaitan (2010) reduced nitrogen wastes concentration with addition of molasses (C/N, 20:1) and decreasing feeding rate; although, he did not apply bacilli to the culture system. *Bacillus* strains can play an important role in aerobic nitrification-denitrification (Sakai *et al.*, 1996; Kim *et al.*, 2005; Lalloo *et al.*, 2007).

The pH remained at around 8.3 in all treatments, indicating that probably an autotrophic system prevailed, since molasses (treatments III, IV, and V) promoted the heterotrophic system, but it was stopped on the 17th day. Becerra-Dorame *et al.* (2012) found that the pH in heterotrophic systems decreases due to the release of $CO_2$ into the water column by hetero-trophic bacteria. In the same way, Ballester *et al.* (2010) mentioned that the pH decreases (6.4-7.7) in *Farfantepenaeus paulensis* cultured in a heterotrophic hyper intensive system with bioflocs.

SS revealed no significant difference among treatments without water exchange. However, the lowest SS (1.09 mL $L^{-1}$) was showed in treatment I with water exchange as compared with the rest of the treatments, indicating a lower biofloc development, although in this treatment growth and survival of shrimp showed no significant differences from the rest of the treatments. The final concentration of SS in the treatments without water exchange was higher (18-21.5 mL $L^{-1}$) than the optimal range (10-15 mL $L^{-1}$) reported by Hargreaves (2013); however, although Schveitzer *et al.* (2013) mention that in intensive culture systems, high solids concentration is related to mortality of shrimp by increased gill occlusion, in the present work the growth and survival of shrimp was not affected by the concentration of solids.

Messenger RNA expression of trypsin and chymotrypsin showed a significant decrease in treatments IV (90% feed ration) and V (80% feed ration) as compared to treatments I, II, and III with 100% feed ration. Conversely, at biochemical level, Becerra-Dorame *et al.* (2012) and Wang *et al.* (2015) found that digestive protease activity was higher in *L. vannamei* cultured in a biofloc system and fed 100% diet as compared to a control without bioflocs. Results of this work showed that a lower mRNA expression of digestive enzymes did not affect shrimp since growth was not significantly different among treatments.

Regarding Hsp, this kind of proteins are expressed not only when there is a heat stress, but also in the presence of free oxygen radicals, anti-inflammatory drugs, heavy metals, malnutrition, excess feed, high density, and viral and bacterial infections (Hickey & Singer, 2004; Multhoff, 2007; Asea *et al.*, 2008; Schock *et al.*, 2013). In this work, higher expression of

mRNA was found in treatment II without water exchange, without additives, and fed 100% commercial diet as compared with treatments III, IV, and V. No reports were found about the effect of reducing feeding rate, additives, and bioflocs on shrimp stress, but it is clear that they reduced it.

Results from this work show that reduced feed ration can help to maintain water quality and lower costs in intensive shrimp cultures with bioflocs.

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