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Transcriptome signatures of the Pacific white shrimp *Litopenaeus vannamei* hepatopancreas in response to stress in biofloc culture systems

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ABSTRACT

Comparative transcriptome analysis via high throughput sequencing was applied to gain knowledge on the immune response in *Litopenaeus vannamei* reared in biofloc technology systems (BFT). Two types of carbon sources, namely, traditional carbon sources (molasses) and biodegradable polymers [hydroxybutyric acid-co-3-hydroxyvaleric acid (PHBV)] were used in BFT systems. Clear water systems without the addition of carbon sources were treated as the control. Water quality assays showed that the average concentrations of several stress factors, including nitrite, nitrate and TSS, were the highest in molasses-based BFT systems. After sequencing and comparing the transcriptome profiles of the *L. vannamei* hepatopancreas, 743 and 201 genes were significantly differentially expressed in molasses- and PHBV-based BFT systems, respectively. GO enrichment analysis, which was performed using the differentially expressed genes, revealed seven significantly over-represented GO terms in molasses-based BFT systems, including catabolic process, hydrolase activity, cellular localization, organic substance metabolic process, cellular metabolic process, establishment of localization and response to stress. The captured key genes were mainly involved in the pathways including cellular stress response, immune response and pathogen recognition. However, no GO terms were significantly over-represented in PHBV-based BFT systems compared with control. This study indicates that shrimp are subject to stress in BFT systems when molasses serves as the carbon source. Thus, PHBV may be a better alternative.

1. Introduction

Pacific white shrimp, *Litopenaeus vannamei*, a member of the Penaeidae family of decapods crustaceans, is one of the most economically valuable cultured shrimp species worldwide [1,2]. In recent years, culture systems for *L. vannamei* have become more intensive resulting in total ammonia nitrogen (TAN) and nitrite accumulation within grow-out systems [3,4]. Therefore, attention should be given to the elevated TAN and nitrite concentrations because they can cause stress and even kill shrimp, fish and other aquaculture animals [5–7].

Biofloc technology (BFT), which includes the addition of carbon sources to promote the growth of heterotrophic bacteria, is a recently developed environmentally friendly aquaculture technique. In BFT systems, aquaculture species are grown in an intensive way with zero or minimum water exchange [8–12]. Moreover, the TAN can be effectively controlled by heterotrophic bacteria [13]. Other advantages of BFT systems include: the availability of food sources and the competition of micro–organisms with pathogens [14]. The carbon sources used to produce biofloc can be divided into two categories. Traditional carbon sources, such as molasses and glucose, need to be applied frequently (i.e., once or several times a day) with constant supervision to prevent overdosing and starving the bacterial floc [15]. By contrast, solid carbon sources, such as biodegradable polymers (BDPs) e. g. hydroxybutyric acid-co-3-hydroxyvaleric acid (PHBV), which are simply added into cultures once or twice, rely on the enzymes to release organic carbon [15,16]. Moreover, the use of different carbon sources may lead to differences in water quality [11,17]. Luo et al. used poly-β-hydroxybutyric (PHB), glucose (GLU) and PHB + GLU as carbon sources and found that the water quality, growth performance and survival of *L. vannamei* were significantly different among the carbon sources [18].

Penaeid shrimp, such as *L. vannamei*, can be affected by various

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environmental stress factors that include TAN, nitrite, nitrate and pH, thereby to problems in growth, survival, osmotic capacity, immune response, and the homeostatic regulation of metabolic processes [19–21]. Increasing the C/N ratio in biofloc systems can accelerate the uptake of TAN by heterotrophic bacteria, but negative effects such as high nitrite and nitrate concentrations during culture can be deadly to shrimp [21–24]. Furthermore, stress in biofloc systems is a poorly defined condition that is difficult to measure, and it has many causes that are not well understood [22]. Previous studies have reported that the antioxidant response of aquatic species can be effectively enhanced under biofloc stress, indicating that the immune response of shrimp is affected in biofloc systems [25,26].

Whole-transcriptome shotgun sequencing, which is known as RNA sequencing (RNA-seq), has been employed to reveal a snapshot of the transcriptome. The method can be used to capture, annotate and compare the transcriptome and to analyze digital gene expression in hemocytes in order to gain knowledge on the immune response [20,27–29]. To understand the complex molecular mechanism of a specific physiological process, RNA-seq is a practical and efficient method of identifying nearly all of the genes and pathways involved in a corresponding physiological function [30–32]. However, only few studies have used comparative transcriptome analysis to analyze differences in the immune response of shrimp exposed to multiple environmental stress factors in biofloc culture systems.

In the present study, Pacific white shrimp were cultured in biofloc systems, where molasses or PHBV were added as carbon sources. In order to investigate whether the addition of an organic source into a biofloc culture system can cause stress response, we conducted comparative transcriptome analysis of the white shrimp *L. vannamei* hepatopancreas. Significantly differentially expressed genes that were mainly involved in stress-response related pathways were captured. An in-depth investigation into the molecular response of organisms under stress will advance our knowledge of biofloc culture system and suggest strategies for culturing Pacific white shrimp.

2. Materials and methods

2.1. Experimental shrimp and acclimation

The *L. vannamei* juveniles used in this experiment were supplied by Baorong Aquaculture Corporation, Qingdao, China and the experiment was conducted at Ruizi Marine Food Product Co., Ltd, Qingdao, China. Shrimp were allowed to acclimate for 10 days in 11 polyethylene fiber tanks (400 L) in which 50% of water was renewed daily. And shrimp were fed a commercial feed (crude protein, 40%; moisture, 12%; ash, 16%; crude fat, 4%; NaCl, 3%) produced by Yuehai Feed Co., Ltd, Guangdong, China twice daily (at 7:00 a.m. and 6:00 p.m.) based on a feed amount equivalent to 5% of the shrimp body weight.

2.2. Experimental design

After acclimation, the shrimp were randomly distributed into 500 L circular tanks and sixty heathy shrimp (2.70 ± 0.02 g) were stocked into each tank. Three treatments with three replicates were tested: clear water systems (CW), molasses adding biofloc systems (M-BF) and PHBV adding biofloc systems (P-BF). In M-BF group, the amount of molasses added was calculated based on the formula proposed by Avnimelech [33]. The molasses with a total organic carbon concentration of 26.5% was diluted in tanks and splashed uniformly into the tanks twice a day. The PHBV (633 g), which was activated for 7d in acclimation tanks [31], was placed in a PVC tube with an inner diameter of 15 cm and operating depth of 30 cm. An air stone was placed inside the tube to sufficiently stir PHBV. The PVC tube was wrapped with filter screen and it was cleared every five days. No molasses or PHBV were added in the CW treatments.

One-fifth of the water in the tanks was replaced daily in CW treatment and one-fifth of the water in the tanks was replaced every five days in M-BF and P-BF treatments. Each tank was continuously aerated with four air stones. Tanks were covered with a shade net to control the sun light. The feeding regime was the same as in the acclimation period. All the tanks were located in a greenhouse with clear plastic sheeting. The experiment was conducted from August 20 to September 24, 2017, and lasted for 34 days.

2.3. Water quality

During the 34-day experimental period, temperature, dissolved oxygen (DO) and pH were measured on 8:00 a.m. every day using a portable DO meter (RDB20, Yuanmu, Shanghai, China) and pH meter (pH-100, LICHEN, Shanghai, China). Water samples were collected from each tank every five day to analyze total ammonia nitrogen (TAN), Nitrite nitrogen (NO_2^--N), Nitrate nitrogen (NO_3^--N) and total suspended solids (TSS) following the method by the Eaton, Clesceri, Greenberg, Grenberg and Trussel [34].

2.4. Sampling, RNA isolation and sequencing

At the end of the study, all the tanks were drained and shrimp in the tanks were weighted. The hepatopancreas from 10 individuals were collected from each tank and pooled into one tube. All samples were placed in RNA keeper tissue stabilizer (Vazyme, Nanjing, China), and kept at −80 °C until RNA extraction. Total RNA were isolated using TRIzol (Invitrogen) with DNase I following the manufacturer's instructions. The quality of RNA was assessed by electrophoresis in 1% agarose gels and verified using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). High quality RNA was sequenced on an Illumina platform.

2.5. De novo assembly of reference sequences and annotation

All raw sequencing data were filtered by removing adaptor sequences, low quality reads (Q < 20), ambiguous nucleotides and short reads with length less than 30 bp by using CLC Genomics Workbench (CLC bio, Aarhus, Denmark). All cleaned reads were assembled by using Trinity software (version 2.5.1) with default parameters. CD-hit program was used to reduce redundancy of the resulting assembled contigs. Contigs longer than 200 bp were considered as the final non-redundant transcripts and were used as the reference sequences.

The assembled reference sequences were used as query sequences to search against the UniProt Database by using BlastX program with an E-value cutoff of 1e-10. Gene ontology (GO) was performed by using Blast2GO software (https://www.blast2go.com/). The annotation output was categorized by cellular component, biological process and molecular function.

2.6. Differential gene expression analysis

CW group was used as control, to investigate whether the organic carbon addition induced the immune response of *L. vannamei*, we conducted a comparative transcriptome analysis on hepatopancreas tissue of M-BF group and P-BF group comparing with CW group. At first, clean reads from each group were aligned to the reference sequences using Bowtie2 [35]. Then, RNA-Seq by Expectation-Maximization (RSEM) was used to calculate transcript abundance. The expression level of each transcript in each sample was normalized using edgeR [36]. Only the transcripts with *P* value lower than 0.05 and |fold change| larger than 2 were considered as significantly differentially expressed genes and used for further analysis.

2.7. GO enrichment and pathway analysis

The web-based tool WEGO2 [37] was used for the statistical

analysis of GO term over-representation among the differentially expressed genes, with default parameters. The population set represented the GO terms in whole reference gene set, while the study set represented the GO terms in the differentially expressed gene set. All genes in the GO term related to stress response were further used for gene pathway analysis. Downstream pathway analysis was conducted using KEGG pathway analysis combined with manual literature searches.

2.8. Statistical analysis

The statistical analyses were performed using SAS v9.4 for windows (Cary, NC, USA). The normality of distribution of data was checked using the Kolmogorov-Smirnov test. The Levene test was applied for assessing the homogeneity of variance. All the data met the assumption of normal distribution and equal variance with $P > 0.05$. One-way analysis of variance (ANOVA) was used to compare the discrepancy of water quality index and the average final weight of shrimp among different systems. Differences were considered significant at $P < 0.05$. When significant differences were found, Turkey's test was used to identify differences between treatments.

3. Results

3.1. Growth performance and water quality parameters

After 35 days of culture, the final weight of the shrimp in CW, M-BF and P-BF were 5.17 g, 6.94 g, 8.04 g, respectively. The final weight of shrimp in P-BF was significantly higher than CW ($P < 0.05$). There was no significantly difference of the final weight of shrimp between CW and M-BF. Descriptive statistics of water parameters are given in Table 1. The temperature in all the treatments ranged from 22.6 °C to 28.6 °C and there were no significant differences on average temperature between different treatments ($P > 0.05$). DO remained fairly constant between 4.8 and 5.3 mg/L during the study and there were no significant differences on average DO among treatments. The pH in CW was significantly higher than other treatments and pH in M-BF was significantly higher than P-BF ($P < 0.05$). The average TAN concentration in the three systems maintained a relative low concentration and showed no significant difference ($P > 0.05$). The average NO_2^--N concentration in CW and P-BF groups were significantly lower than M-BF system ($P < 0.05$), and the NO_2^--N concentration in CW was lower than P-BF system. The average NO_3^--N concentration in CW was significantly lower than M-BF and P-BF systems, and the NO_3^--N concentration in P-BF was significantly lower than M-BF system ($P < 0.05$). TSS in M-BF and P-BF systems were higher than CW system, but there were no significant differences among them ($P > 0.05$). The TSS in M-BF was the highest, which was 395.72 mg/L (Table 1).

Table 1
The mean and standard deviations of water quality parameters in the three treatments during the experiment (n = 3).

Parameters	CW	M-BF	P-BF
Temperature (°C)	25.5 ± 1.56[a]	25.5 ± 1.56[a]	25.5 ± 1.56[a]
DO (mg L^{-1})	5.27 ± 0.74[a]	4.83 ± 0.88[a]	4.95 ± 0.84[a]
pH	7.7 ± 0.03[a]	7.1 ± 0.03[c]	7.4 ± 0.02[b]
TAN (mg L^{-1})	0.19 ± 0.16[a]	0.17 ± 0.04[a]	0.23 ± 0.05[a]
NO_2^--N (mg L^{-1})	0.82 ± 0.26[b]	6.44 ± 1.70[a]	3.36 ± 0.49[b]
NO_3^--N (mg L^{-1})	0.94 ± 0.10[c]	4.54 ± 0.85[a]	2.79 ± 0.20[b]
TSS (mg L^{-1})	308.48 ± 50.72[a]	395.72 ± 13.54[a]	346.75 ± 64.28[a]

CW, M-BF and P-BF represented the three treatments of this study. TAN: total ammonia nitrogen. TSS: total suspended solids. In each row, different superscript letters indicate significant differences at the $P < 0.05$ level (one way ANOVA and Tukey test).

3.2. Sequencing, assembly and annotation

The genome and transcriptome resource of *L. vannamei* are scarce due to its complicated genome. Therefore, in the present study, we first generated a valuable reference sequence for further analysis. As shown in Table 2, a total of 519,254,306 paired end reads with length of 150 bp were obtained. Ambiguous nucleotides, short reads with length less than 30 bp, and low quality reads with $Q < 20$ were removed from raw sequencing reads. 514,544,142 reads with a total length around 72,627 Mbp were retained and used for further assembly (Table 2). All sequencing data were deposited in NCBI Sequence Read Archive (SRA) under accession number SRR8755079-SRR8755081.

De novo assembly for generating a reference sequences was performed by trinity software. After removing redundant sequences, a total of 275,887 contigs were obtained, of which, 56,379 contigs were longer than 1000 bp (Table 3). The N50 of all contigs was 1441 bp, and the maximum length was 19,299 bp. In order to assess the quality of the sequencing and *de novo* assembly, all clean reads were mapped back to the reference sequences. As shown in Table 2, the mapping ratios were higher than 97.45%. The high mapping ratio suggested that the assembled reference sequences was reliable.

To predict the protein-coding genes, the reference sequences was used as query to search against the UniProt database by BlastX searches, with an E-value cutoff of 1e-5. As shown in Table 3, there were 159,559 contigs with significant hit, which corresponded to 79,582 unique genes. Of which, 77,811 contigs corresponding to 32,193 unique genes were assigned to at least one GO term. A total of 66 GO terms were assigned at the 2nd level, of which, 18 were from cellular component category, 16 were from molecular function category and 32 were from biological process category. As shown in Fig. 1, binding was the most predominant GO term in the molecular function category, accounting for 13.6%, while cellular process was the most predominant GO term in the biological process category, accounting for 23.7%.

3.3. Identification of differentially expressed genes

Based on the criteria of $|\text{fold change}| \geqq 2$ and $P < 0.05$, compared with control (CW group), 743 and 201 genes were significantly differentially expressed in M-BF group and P-BF group, respectively. The number of genes with significantly differential expression in M-BF group was higher than that in P-BF group. As shown in Fig. 2, in M-BF group, there are 671 genes up-regulated and 72 genes down-regulated, while in P-BF group, there are 151 genes up-regulated and 50 genes down-regulated.

3.4. GO enrichment and pathway analysis

GO annotation was performed using all differentially expressed genes, which were classified into different cellular, biological and functional gene ontologies according to their likely functions. The results revealed 7 significantly over-represented GO terms at the 3rd level in M-BF group with $P < 0.05$ (Table 4), including catabolic process (GO:0009056), hydrolase activity (GO:0016787), cellular localization (GO:0051641), organic substance metabolic process (GO:0071704), cellular metabolic process (GO:0044237), establishment of localization (GO:0051234), response to stress (GO:0006950). However, no significantly over-represented GO terms at the 3rd level was found in P-BF group.

Since the primary interest in this study was to detect whether adding carbon sources in biofloc culture system cause stress response on shrimp, all genes in the GO term response to stress were further used for gene pathway analysis. Representative key genes such as superoxide dismutase [Fe], C-type lectin, hepatic lectin and lysozyme were found that may play important roles in host response under stress. Pathway analysis results showed that all these genes were mainly involved in three pathways, including cellular stress response, immune response

Table 2
Summary of the sequencing data from CW, M–BF and P-BF groups.

	CW			M-BF			P-BF		
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
No. of reads	63,277,210	65,026,654	51,341,958	55,509,964	53,784,358	52,745,082	50,486,926	66,378,794	60,703,360
Average length of reads (bp)	150	150	150	150	150	150	150	150	150
No. of clean reads	63,256,744	65,003,630	50,284,798	54,575,665	52,877,206	51,884,903	49,616,005	66,359,515	60,685,676
Average length of clean reads (bp)	141	141	140	141	141	141	141	142	142
Percentage of reads retained	99.97%	99.96%	97.94%	98.32%	98.31%	98.37%	98.27%	99.97%	99.97%
Mapped reads	61,824,307	63,585,192	48,502,512	52,840,443	51,244,178	49,984,372	47,974,834	64,838,515	59,486,335
Mapping ratio (%)	97.77%	97.85%	97.86%	97.96%	98.06%	97.45%	97.87%	97.74%	98.05%

CW, M-BF and P-BF represented the three treatments of this study.

Table 3
Statistics of reference sequence assembly.

Assembly	No. of contigs (> 200 bp)	275,887
	No. of larger contigs (> 1000 bp)	56,379
	Maximum length (bp)	19,299
	N50 (bp)	1441
	Average length (bp)	781
Annotation	No. of contigs with Blast hit to UniProt	159,559
	No. of contigs with GO terms	77,811
	No. of unique genes predicted	79,582
	No. of unique genes with GO terms	32,193

and pathogen recognition. As shown in Table 5, six heat shock genes, solute carrier family 35 member C2 and superoxide dismutase [Fe] were grouped into cellular stress response pathway. In immune response pathway, 15 significantly differentially expressed genes such as ferritin, lysozyme, lectins were found. In pathogen recognition pathway, 7 significantly differentially expressed genes were found such as macrophage mannose receptor 1 and NLRC3.

4. Discussion

Comparative transcriptome analysis via high throughput sequencing is a fully quantitative and truly unbiased approach, which can quickly and economically detect the systemic gene expression and regulatory mechanisms of various organisms under different conditions [20,21,38]. To better understand the molecular response mechanism against the multiple stresses caused by the addition of various carbon sources and to identify the key genes and pathways that play critical roles in *L. vannamei* exposed to those stresses, comparative transcriptome analysis was used to identify systemic gene expression

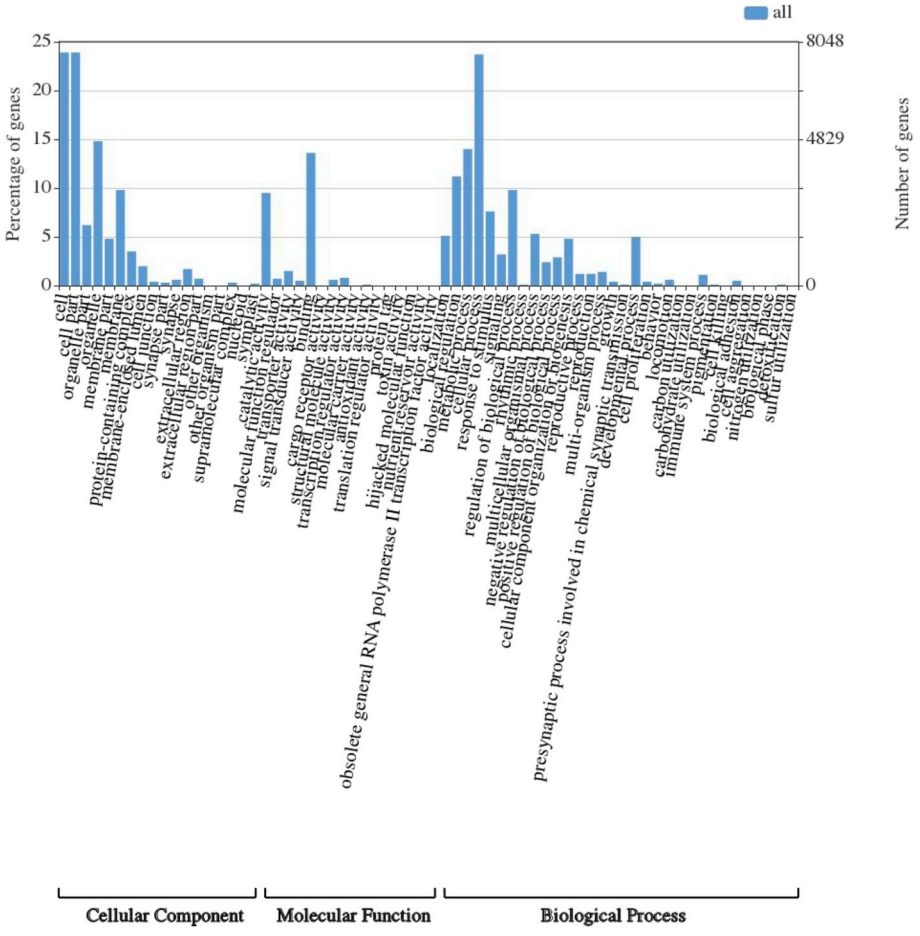

Fig. 1. Summary of GO annotation.

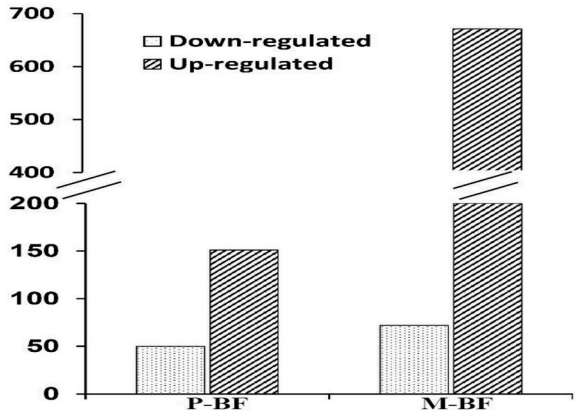

Fig. 2. Summary of significantly differentially expressed genes in P-BF and M-BF groups. P-BF and M-BF represented the two treatments of this study.

Table 4
The significantly over-represented GO terms resulted from GO enrichment analysis. The "Count ratio" column indicates study count ratio/population count ratio; the study count ratio is the ratio of genes associated with the GO term to all significantly differentially expressed genes in the M-BF treatment, and the population count ratio is the ratio of genes associated with the GO term to all genes in the reference set.

GO ID	GO term	P value	Count ratio (%)
GO:0009056	Catabolic process	0.006	3.7/1.7
GO:0016787	Hydrolase activity	0.005	6.4/3.5
GO:0051641	Cellular localization	0.004	3.7/1.6
GO:0071704	Organic substance metabolic process	0.020	16.5/12.2
GO:0044237	Cellular metabolic process	0.020	15.9/11.7
GO:0051234	Establishment of localization	0.043	6.4/4.2
GO:0006950	Response to stress	0.049	4.0/2.7

changes and to define the immune response mechanism of shrimp. Previous studies have analyzed the exposure of shrimp, fish and other aquaculture animals to stress in harmful aquaculture environments, including low salinity stress, nitrite stress, and TAN stress [20,21,23,39]. In those studies, the harmful environment could activate a physiological response in shrimp or fish and induce an anti-stress defense mechanism against the environment. However, most of the previous reports studied the response of shrimp or fish to stress caused by a single environmental factor in a short time [20,21,23,39]. The present study focused on the immune response of *L. vannamei* to multiple stresses after a 34-day experimental period.

There are several advantages of BFT systems such as reduced water exchange, availability of food sources, better FCR and increased bacterial diversity [5,8,11,14,15]. However, the addition of carbon sources may cause water quality problems, including a relatively high TSS, temporary low dissolved oxygen (DO) concentration and accumulation of certain toxic compounds such as nitrite [18,40]. In this study, the average nitrite, nitrate and TSS concentrations were highest in M-BFT. Therefore, the high concentration of nitrite, nitrate and TSS may have stressed the shrimp in M-BFT. Early studies showed that toxic compounds can accumulate in the hepatopancreas when shrimp are exposed to stress such as high concentrations of nitrite and nitrate [24,41]. Cheng and Chen reported that following 24 h of exposure to 0.360 mM nitrite, *Penaeus monodon* accumulated nitrite in muscle (0.404 μmoL/g), hepatopancreas (1.001 μmoL/g), gill (1.196 μmoL/g) and hemolymph (1.787 μmoL/ml), which were much higher than the ambient nitrite concentration [24]. Following long-term exposure, nitrate has been reported to cause damage in the peripheral blood and liver of *Oncorhynchus mykiss* and damage tissues of ganglionic neuropiles, muscle, foregut and midgut of *P. monodon* postlarvae [42].

Under environment stress, aquatic animals usually show an enhanced antioxidant response, which is represented by high antioxidant enzyme levels (e.g., SOD), to prevent reactive oxygen species (ROS) induced oxidative stress (e.g., lipid peroxidation) [25]. Meanwhile, phagocytes also mediate their innate immunological response by releasing products, such as lysozyme and elastase, to eliminate pathogens using an oxygen-independent mechanism [43]. In the present study, shrimp in the M-BF system showed apparent cellular stress, which was indicated by the up-regulation of superoxide dismutase [Fe] (Fe SOD). Fe SOD was involved in the response to stress (GO:0006950), and it showed significantly up-regulated expression in shrimp cultured in M-BF. Generally, higher SOD activity indicates that there are more radicals that need to be reacted. Therefore, significantly higher SOD activity in shrimp in M-BF might have indicated that the stress in the molasses biofloc system induced the oxygen-dependent reactivity in phagocytes of *L. vannamei* [20]. Similar changes in enzymatic activity levels in response to biofloc stress have been reported in *Fennerpenaeus chinensis*, *L. vannamei* and *Oreochromis niloticus* [25,44,45], indicating that the M-BF culture environment may stimulate the oxidase system and cause oxidative damage.

In this study, several significantly enriched GO terms induced various metabolic processes such as the organic substance metabolic process (GO:0071704) and cellular metabolic process (GO:0044237) in shrimp cultured in M-BF. It has been reported that a high nitrite concentration in the environment can induce TAN excretion in shrimp, and the degradation of nitrogenous compounds was accomplished through organic substance metabolic, amino metabolic and nucleic metabolism [39,46,47]. Therefore, we hypothesize that the alterations in the

Table 5
Detailed information about the differentially expressed genes involved in each of the pathways.

Gene name	Contig ID	M-BF
Cellular stress response		
Heat shock protein 7A	TRINITY_DN22392_c0_g1_i1	2.90
Heat shock protein 7B	TRINITY_DN29533_c3_g1_i1	3.84
Heat shock 70 kDa protein cognate 4	TRINITY_DN29767_c5_g5_i1	−6.83
Heat shock protein homolog SSE1	TRINITY_DN29533_c1_g1_i1	3.78
Heat shock protein 90	TRINITY_DN22864_c0_g1_i1	3.63
Heat shock protein 83	TRINITY_DN29840_c0_g2_i1	−6.69
Solute carrier family 35 member C2	TRINITY_DN17651_c0_g1_i1	3.32
Superoxide dismutase [Fe]	TRINITY_DN23025_c0_g1_i1	3.16
Immune response		
Aquaporin	TRINITY_DN75000_c0_g1_i1	3.52
Integrin beta-8	TRINITY_DN33799_c1_g3_i2	−3.28
C-type lectin domain family 12 member B	TRINITY_DN26833_c0_g2_i3	4.00
C-type lectin domain family 17, member A	TRINITY_DN27668_c1_g1_i3	8.39
C-type lectin domain family 4 member F	TRINITY_DN37422_c3_g3_i3	5.02
C-type lectin domain family 6 member A	TRINITY_DN37527_c1_g1_i2	2.81
Ferritin	TRINITY_DN28841_c0_g1_i12	3.76
Galactose-specific lectin nattectin	TRINITY_DN29733_c1_g3_i1	2.33
Hepatic lectin	TRINITY_DN26833_c0_g1_i1	2.97
Lysozyme	TRINITY_DN33717_c2_g1_i2	2.69
Pathogenesis-related protein 2	TRINITY_DN26783_c0_g1_i1	−7.68
Techylectin-5B	TRINITY_DN28641_c0_g2_i14	7.11
TNF receptor-associated factor 3	TRINITY_DN34019_c2_g3_i1	2.86
TLR4 interactor with leucine rich repeats	TRINITY_DN34514_c2_g2_i2	5.34
L-type lectin-like domain-containing protein C4F6.05c	TRINITY_DN1291_c0_g1_i1	3.41
Pathogen recognition		
CD209 antigen	TRINITY_DN29959_c0_g1_i4	2.61
CD44 antigen	TRINITY_DN36818_c2_g3_i1	3.35
Protein slit	TRINITY_DN33960_c4_g4_i1	2.89
Slit homolog 3 protein	TRINITY_DN36145_c1_g1_i1	3.31
Macrophage mannose receptor 1	TRINITY_DN32127_c3_g2_i2	5.94
C-type mannose receptor 2	TRINITY_DN33408_c1_g3_i1	4.31
NLRC3	TRINITY_DN30384_c0_g1_i2	2.53

metabolism processes that were related to nitrogen excretion may represent a strategy to handle endogenous nitrogenous compounds in order to avoid nitrite toxicity in the environment.

Early studies suggested that stress factors (e.g. nitrite and nitrate) can induce the immune resistance of crustaceans, as shown by measurements of antioxidant enzyme activities, total hemocyte count (THC), differential hemocyte count (DHC), phenoloxidase activity and bacteriolytic activity [24,48–50]. In the present study, our results also reflected the influence of multiple stresses in M-BF on the immune resistance of *L. vannamei*. Lysozyme, an important hydrolytic enzyme, kills bacteria by catalyzing the hydrolysis of bacterial cell walls, and it has strong bacteriolytic activities against several *Vibrio* species [51]. Lectins work in concert with other immune factors in a series of reactions that involve the recognition, defense, agglutination, engulfment and encapsulation of foreign invaders as well as the subsequent wound repair [52]. The main role of lectins may be in the agglutination of foreign molecules, which can cause pathogens to lose their ability to further invade the body and spread in the tissue for the purpose of immune defense. C-type lectin is a type of lectin that was discovered earlier. Existing studies have shown that C-type lectin has a great relationship with the enhancement of blood cell phagocytic activity of shrimp. In this study, the up-regulation of lysozyme, hepatic lectin and C-type lectin suggests that molasses biofloc exposure affected the immune response.

As a solid carbon source, insoluble biodegradable polymers, such as 3-hydroxybutyric acid-co-3-hydroxyvaleric acid (PHBV), poly-β-hydroxybutyric (PHB), poly L-lactic acid (PLA), poly-caprolactone (PCL), and poly butanediol succinate (PBS), are new alternative to traditional carbon sources [17]. Compared with traditional carbon sources, insoluble biodegradable polymers needed to be added only once at the beginning of the study, which greatly reduced difficulties associating with system management. Furthermore, there was no significant difference in most of the GO enrichment and KEGG pathway analyses between P-BF and CW systems. Therefore, PHBV may be a better carbon source in shrimp aquaculture systems, as it was easy to use and better to the shrimp. The usefulness of the other biodegradable solid carbon sources was also reported by other studies [53,54].

5. Conclusion

Molasses and PHBV were used as the carbon sources in biofloc systems. In the present study, the immune response in *L. vannamei* reared in biofloc systems was investigated using the next generation sequencing technique for the first time. The results revealed that molasses-based BFT systems may cause various immune responses. Thus, PHBV may be a better alternative of carbon sources in biofloc systems. This study will be of great value in understanding the molecular mechanisms of *L. vannamei* in response to stress in biofloc systems.

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References

[1] J. Rodriguez, M.G. Le, State of the art of immunological tools and health control of penaeid shrimp, Aquaculture 191 (1) (2000) 109–119.
[2] C.E. Boyd, E.L. Torrans, C.S. Tucker, Dissolved oxygen and aeration in ictalurid catfish aquaculture, J. World Aquacult. Soc. 49 (1) (2018).
[3] J. Haslun, E. Correia, K. Strychar, T. Morris, T. Samocha, Characterization of bioflocs in a no water exchange super-intensive system for the production of food size pacific white shrimp *Litopenaeus vannamei*, Int. J. Aquac. (2012).
[4] Y.L. Li, C.E. Boyd, Laboratory tests of bacterial amendments for accelerating oxidation rates of ammonia, nitrite and organic matter in aquaculture pond water, Aquaculture 460 (2016) 45–58.
[5] N.S. Abu Bakar, N. Mohd Nasir, F. Lananan, S.H. Abdul Hamid, S.S. Lam, A. Jusoh, Optimization of C/N ratios for nutrient removal in aquaculture system culturing African catfish, (*Clarias gariepinus*) utilizing Bioflocs Technology, Int. Biodeterior. Biodegrad. 102 (2015) 100–106.
[6] M.E. Azim, D.C. Little, The biofloc technology (BFT) in indoor tanks: water quality, biofloc composition, and growth and welfare of Nile tilapia (*Oreochromis niloticus*), Aquaculture 283 (1–4) (2008) 29–35.
[7] A.J. Ray, J.M. Lotz, Comparing a chemoautotrophic-based biofloc system and three heterotrophic-based systems receiving different carbohydrate sources, Aquacult. Eng. 63 (2014) 54–61.
[8] W.J. Xu, T.C. Morris, T.M. Samocha, Effects of C/N ratio on biofloc development, water quality, and performance of *Litopenaeus vannamei* juveniles in a biofloc-based, high-density, zero-exchange, outdoor tank system, Aquaculture 453 (2016) 169–175.
[9] C.L. Browdy, D. Bratford, A.D. Stokes, R.P. Mcintosh, Perspectives on the Application of Closed Shrimp Culture Systems, Aquaculture, The World Aquaculture Society, Baton Rouge, USA, 2001.
[10] T.M. Samocha, S. Patnaik, M. Speed, A.M. Ali, J.M. Burger, R.V. Almeida, et al., Use of molasses as carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei*, Aquacult. Eng. 36 (2) (2007) 184–191.
[11] Y. Avnimelech, Biofloc Technology: a Practical Guide Book, (2012).
[12] M. Deng, J. Chen, J. Hou, D. Li, X. He, The effect of different carbon sources on water quality, microbial community and structure of biofloc systems, Aquaculture 482 (2017).
[13] J.A. Hargreaves, Photosynthetic suspended-growth systems in aquaculture, Aquacult. Eng. 34 (3) (2006) 344–363.
[14] M.G.C. Emerenciano, L.R. Martínez-Córdova, M. Martínez-Porchas, A. Miranda-Baeza, Biofloc Biofloc Technology (BFT): a Tool for Water Quality Management in Aquaculture, (2017).
[15] A. Boley, W.R. Müller, G. Haider, Biodegradable polymers as solid substrate and biofilm carrier for denitrification in recirculated aquaculture systems, Aquacult. Eng. 22 (1–2) (2000) 75–85.
[16] W. Wu, L. Yang, J. Wang, Denitrification performance and microbial diversity in a packed-bed bioreactor using PCL as carbon source and biofilm carrier, Appl. Microbiol. Biotechnol. 97 (6) (2013) 2725–2733.
[17] T.L. Qiu, Y. Xu, M. Gao, M.L. Han, X.M. Wang, Bacterial community dynamics in a biodenitrification reactor packed with polylactic acid/poly (3-hydroxybutyrate-co-3-hydroxyvalerate) blend as the carbon source and biofilm carrier, J. Biosci. Bioeng. 123 (5) (2017) 606–612.
[18] G. Luo, Z. Liu, L. Shao, H. Tan, Using poly-β-hydroxybutyric as an additional carbohydrate for biofloc in a shrimp *Litopenaeus vannamei* bioflocs nursery system with brackish water, Aquaculture 506 (2019) 181–187.
[19] Z. Jun, W. WeiNa, W. AnLi, H. WenYin, Z. QiTing, L. Yuan, et al., Glutathione S-transferase in the white shrimp *Litopenaeus vannamei*: characterization and regulation under pH stress, Comp. Biochem. Physiol., C 150 (2) (2009) 224–230.
[20] Q. Zhao, L. Pan, Q. Ren, D. Hu, Digital gene expression analysis in hemocytes of the white shrimp *Litopenaeus vannamei* in response to low salinity stress, Fish Shellfish Immunol. 42 (2) (2015) 400–407.
[21] H. Guo, J.A. Xian, A.L. Wang, Analysis of digital gene expression profiling in hemocytes of white shrimp *Litopenaeus vannamei* under nitrite stress, Fish Shellfish Immunol. 56 (2016) 1–11.
[22] D.V. Lightner, Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance, J. World Aquacult. Soc. 36 (3) (2010) 229–248.
[23] H. Guo, J.A. Xian, B. Li, C.X. Ye, A.L. Wang, Y.T. Miao, et al., Gene expression of apoptosis-related genes, stress protein and antioxidant enzymes in hemocytes of white shrimp *Litopenaeus vannamei* under nitrite stress, Comp. Biochem. Physiol., C 157 (4) (2013) 366–371.
[24] S.Y. Cheng, J.C. Chen, Accumulations of nitrite and nitrate in the tissues of *Penaeus monodon* exposed to a combined environment of elevated nitrite and nitrate, Arch. Environ. Contam. Toxicol. 43 (1) (2002) 64–74.
[25] W. Ren, L. Li, S. Dong, X. Tian, Y. Xue, Effects of C/N ratio and light on ammonia nitrogen uptake in *Litopenaeus vannamei* culture tanks, Aquaculture 498 (2019) 123–131.
[26] G. Liu, S. Zhu, D. Liu, X. Guo, Z. Ye, Effects of stocking density of the white shrimp *Litopenaeus vannamei* (Boone) on immunities, antioxidant status, and resistance against Vibrio harveyi in a biofloc system, Fish Shellfish Immunol. 67 (2017) 19–26.
[27] R. Morin, M. Bainbridge, A. Fejes, M. Hirst, M. Krzywinski, T. Pugh, et al., Profiling the HeLa S3 transcriptome using randomly primed cDNA and massively parallel short-read sequencing, Biotechniques 45 (1) (2008) 81–94.
[28] Y. Chu, D.R. Corey, RNA sequencing: platform selection, experimental design, and data interpretation, Nucleic Acid Therapeut. 22 (4) (2015) 271.
[29] X. Qian, B. Yi, Q. Zhuang, G. Zhong, RNA-Seq technology and its application in fish transcriptomics, Omics-A Journal of Integrative Biology 18 (2) (2014) 98.
[30] S. Smith, L. Bernatchez, L.B. Beheregaray, RNA-seq analysis reveals extensive transcriptional plasticity to temperature stress in a freshwater fish species, BMC Genomics 14 (1) (2013) 375-375.
[31] J.H. Xia, P. Liu, F. Liu, G. Lin, F. Sun, R. Tu, et al., Analysis of stress-responsive transcriptome in the intestine of Asian seabass (*Lates calcarifer*) using RNA-Seq, DNA Res. 20 (5) (2013) 449–460 20,5(2013-6-10).
[32] X. Jian, P. Ji, B. Wang, Z. Lan, W. Jian, Z. Zhao, et al., Transcriptome sequencing and analysis of wild amur Ide (*Leuciscus waleckii*) inhabiting an extreme Alkaline-Saline lake reveals insights into stress adaptation, PLoS One 8 (4) (2013) e59703.
[33] Y. Avnimelech, Carbon nitrogen ratio as a control element in aquaculture systems, Aquaculture 176 (3–4) (1999) 227–235.
[34] D.L. Eaton, L. Clesceri, A.S. Greenberg, A. Grenberg, R. Trussel, Standard Methods for Examination of Water and Wastewater, (1995).

[35] B. Langmead, S.L. Salzberg, Fast gapped-read alignment with Bowtie 2, Nat. Methods 9 (4) (2012) 357–359.
[36] M.D. Robinson, D.J. Mccarthy, G.K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data, Bioinformatics 26 (1) (2010) 139.
[37] Y. Jia, WEGO 2.0: a Web Tool for Analyzing and Plotting GO Annotations, 2018 Update, (2018).
[38] Y.B. Liu, M.L. Liu, X.R. Li, B. Cao, X.F. Ma, Identification of differentially expressed genes in leaf of reaumuria soongorica under PEG-induced drought stress by digital gene expression profiling (SCI), PLoS One 9 (4) (2014) e94277.
[39] Q. Ren, L. Pan, Digital gene expression analysis in the gills of the swimming crab (*Portunus trituberculatus*) exposed to elevated ambient ammonia-N, Aquaculture 434 (434) (2014) 108–114.
[40] J. Li, G. Liu, C. Li, Y. Deng, M.A. Tadda, L. Lan, et al., Effects of different solid carbon sources on water quality, biofloc quality and gut microbiota of Nile tilapia (*Oreochromis niloticus*) larvae, Aquaculture 495 (2018).
[41] S.Y. Cheng, J.C. Chen, The time-course change of nitrogenous excretion in the Kuruma shrimp *Penaeus japonicus* following nitrite exposure, Aquat. Toxicol. 51 (4) (2001) 443–454.
[42] P.R. Muir, D.C. Sutton, L. Owens, Nitrate toxicity to *Penaeus monodon* protozoea, Mar. Biol. 108 (1) (1991) 67–71.
[43] R. Thiagarajan, A. Munusamy, M. Periasamy, Agglutinin-mediated phagocytosis-associated generation of superoxide anion and nitric oxide by the hemocytes of the giant freshwater prawn *Macrobrachium rosenbergii*, Fish Shellfish Immunol. 24 (3) (2008) 337–345.
[44] A.T. Mansour, M.A. Esteban, Effects of carbon sources and plant protein levels in a biofloc system on growth performance, and the immune and antioxidant status of Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol. 64 (2017) 202–209.
[45] M.S. Kim, E. Min, J.H. Kim, J.K. Koo, J.C. Kang, Growth performance and immunological and antioxidant status of Chinese shrimp, *Fennerpenaeus chinensis* reared in bio-floc culture system using probiotics, Fish Shellfish Immunol. 47 (1) (2015) 141–146.
[46] J.C. Chen, S.Y. Cheng, Hemolymph oxygen content, oxyhemocyanin, protein levels and ammonia excretion in the shrimp *Penaeus monodon* exposed to ambient nitrite, J. Comp. Physiol. B 164 (7) (1995) 530–535.
[47] M. Regnault, Nitrogen excretion in marine and fresh-water cruster, Biol. Rev. 62 (1) (2010) 1–24.
[48] X.L. Liu, Q.Y. Xi, L. Yang, H.Y. Li, Q.Y. Jiang, G. Shu, et al., The effect of dietary Panax ginseng polysaccharide extract on the immune responses in white shrimp, *Litopenaeus vannamei*, Fish Shellfish Immunol. 30 (2) (2011) 495–500.
[49] M. Hong, L. Chen, X. Sun, S. Gu, L. Zhang, Y. Chen, Metabolic and immune responses in Chinese mitten-handed crab (*Eriocheir sinensis*) juveniles exposed to elevated ambient ammonia, Comp. Biochem. Physiol. C. 145 (3) (2007) 363–369.
[50] L. CH, C. JC, Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to Vibrio alginolyticus, Fish Shellfish Immunol. 16 (3) (2004) 321–334.
[51] S. Hikima, H. Ji, J. Rojtinnakorn, I. Hirono, T. Aoki, Characterization and function of kuruma shrimp lysozyme possessing lytic activity against *Vibrio species*, Gene 316 (1) (2003) 187–195.
[52] G.J. Arason, Lectins as defence molecules in vertebrates and invertebrates, Fish Shellfish Immunol. 6 (4) (1996) 277–289.
[53] F.G. Vilani, R. Schveitzer, R.D.F. Arantes, F.D.N. Vieira, C.M.D.E. Santo, W.Q. Seiffert, Strategies for water preparation in a biofloc system: effects of carbon source and fertilization dose on water quality and shrimp performance, Aquacult. Eng. 74 (2016) 70–75.
[54] A.T. Mansour, M.Á. Esteban, Effects of carbon sources and plant protein levels in a biofloc system on growth performance, and the immune and antioxidant status of Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol. 64 (2017) 202–209.