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Carbohydrate sources deferentially influence growth performances, microbial dynamics and immunomodulation in Pacific white shrimp (*Litopenaeus vannamei)* under biofloc system

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ABSTRACT

This study aims to evaluate the influence of different carbohydrate sources on water quality, growth performance and immunomodulation in pacific white shrimp and to find an alternate for molasses in biofloc system. The experiment consists of 8 biofloc treatments with different carbon sources, C1 (maida flour), C2 (wheat flour), C3 (gram flour), C4 (millet flour), C5 (rice flour), C6 (corn flour), C7 (molasses), C8 (multigrain flour) and un-supplemented control C0 was conducted in 200 L tank system for 120 days. Shrimp juveniles of average weight 1 g were stocked at the rate of 300 nos/m^3. Shrimp reared in C8, C7 and C4 treatments had similar growth, survival rate, and disease resistance and were significantly higher ($P < 0.05$) than other treatments including control. Immune parameters like total hemocyte count (THC) and prophenoloxidase (ProPO) activity showed significantly higher ($P < 0.05$) levels in biofloc treatment groups. The genes targeting the proPO cascade (PX, BGBP) and antioxidant defense systems (SOD, MnSOD, CAT) revealed significant upregulation in the transcript levels indicating an enhancement in the immune-regulatory functions in the BFT groups. The results suggest that millets and multigrain flour can effectively replace molasses as the carbohydrate source for biofloc system and the biofloc system offers higher growth, survival, and immunomodulation than control.

1. Introduction

Shrimp farming is one of the leading sectors in aquaculture accounting for 55% of the world's crustacean production (*Litopenaeus vannamei)* remarkably exceeding 3.66 MT (million tons) in 2014 [1]. The demands of shrimp in the international market led to an expansion of shrimp culture area and production which was supported by innovative scientific methods to increase the farm productivity. The biofloc technology (BFT) is considered as one such technique which gained momentum and positive reviews in shrimp farming recently. BFT is a pioneering notion which implements the rearing of aquatic animals intensively by manipulating the microbial communities under controlled conditions [2,3]. The environmentally benign system helps recycling the water in the culture system and cause lesser environmental impacts on water source [4]. This approach was shown to enhance the production of marine animals at high stocking densities in a bio-secure manner [5]. It is well established that the microbial populations in BFT maintain the basal water quality, assist in oxidation of organic matters [6] and enhance the biosecurity of the shrimp cultures by preventing the onset of various diseases [7] and the nursery phase with high-density culture can avoid disease outbreak such as EMS, RMS, EHP using minimal water exchange system [8].

The biofloc system critically depends on the effective manipulation of C: N ratio through the supply of carbon source and feed addition. Carbon source addition enhances the conversion to heterotrophic bacteria and also helps in balancing the C: N ratio [3,9,10]. These heterotrophs effectively absorb the inorganic nitrogen and facilitate faster reduction of TAN than the process of nitrification. Total ammonia nitrogen (TAN) is a major concern in shrimp farming. TAN consists of ionized (NH_4^+) and unionized ammonia (NH_3) with later being are highly toxic to the cell membranes of shrimps and other aquatic organisms [11]. To minimize the TAN levels, it is necessary to use carbon sources with lower dissolution rates to favor the carbon: nitrogen ratio

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(C: N ratio). On the other hand, different organic carbon sources can probably affect the composition of biofloc [12].

The use of carbon sources like molasses in biofloc based *L. vannamei* farming have shown to promote heterotrophic bacterial growth which effectively controlled the TAN levels in the culture system [7]. Molasses and dextrose are reported to efficiently control the ammonia levels in *L. vannamei* biofloc systems [13]. Apparently, among the carbon sources tested, molasses and dextrose were shown to possess faster dissolution rates [14]. Faster degradation provides higher levels of carbon as a substrate for heterotrophic bacteria to metabolize ammonia. Brewery residues were also shown as a promising source of organic carbon for *L. schmitti* farming in BFT system [15] evidencing the improvement on the zootechnical performance of the species and the low cost compared to the other sources reported.

Carbon sources in maintaining a balanced C: N ratios have divergent roles including the formation of biofloc, reducing the TAN levels, and consequently improving the water quality. It is also hypothesized that carbon sources may play a vital role in enhancing the shrimp's immunostimulant activities. Understanding the transcriptome profile of several immune genes involved with it is still a challenge, though recently few studies are addressing it [16–18]. The objective of the study was to evaluate growth performance and immunomodulation in pacific white shrimp *L. vannamei* reared under BFT supplemented with different carbon sources.

2. Materials and methods

2.1. Experimental site and tank preparation

The experiment was conducted for 120 days, in a 200 L fiber-reinforced plastic (FRP) tanks (dimensions: width 67 cm x Length 126.5 cm x Height 52.5 cm) at Muttukadu experimental station of ICAR-CIBA. *L. vannamei* juveniles (1.01 ± 0.03 g) were stocked at the rate of 300 nos m^{-3} into assigned experimental tanks. The experiment consists of 8 treatments of different carbon sources for the development of biofloc and an autotropic control in triplicates viz. C1 (maida flour) C2 (wheat flour), C3 (gram flour), C4 (millet flour), C5 (rice flour), C6 (corn flour), C7 (molasses), C8 (multigrain flour) and a control treatment C0 (without carbon/Biofloc). All the experimental units were filled with disinfected seawater (30 ppt) and were provided with adequate aeration by a 5 HP blower. For the generation of biofloc, all the items were added based on the recommendation of Avnimelech (1999) [9] and the respective carbon sources were added to the treatments as per the experimental design. The respective carbon sources and probiotic consortium *Bacillus subtilis* (MTCC 2756) & *Saccharomyces cerevisiae* (IAM 14383T) at the rate of 5.4×10^9 CFU/ml was fermented with 1 L of sterile seawater for 24 h. After fermentation, 200 ml of inoculum was added to generate biofloc in respective treatment tanks.

2.2. Feed formulation and management

The experimental diet was formulated to contain crude protein level of 35%. The list of ingredients and feed proximate composition are depicted in Table 1. Fish meal, dried acetes, soya cake, and gingelly oil cake are in the ratio of 4:2:3:1 was used as protein source. A Mixture of Wheat, Broken rice and Maida (4:2:4) used as the carbohydrate source and both the sunflower oil and cod liver oil (1:1) used as a lipid source. Vitamins and minerals were added in the formulation as per the recommendation of Hu et al. and Xu et al. [19,20], respectively. The dried ingredients were pulverized to powder forms and were mixed thoroughly.

Later, oil and water were added to the mixture, which was passed through a ring die pellet extruder. For efficient storage, the extruded pellets were oven dried and stored in a plastic bag. The proximate composition of the diet was estimated by following the method of AOAC [21]. The experimental animals were fed four times daily at 06.00, 11.00, 18.00 and 22:00 h initially for 2 months at the rate of 8% of body weight which declined gradually to 2.5% towards the end of the experiment.

2.3. Assessment of water quality parameters

Water parameters such as temperature (mercury thermometer), pH (pH-Scan-Eutech instruments, Singapore), Salinity (hand refractometer), TAN (Phenol hypochlorite method), NO_2—N, NO_3—N, phosphate-P (PO_4—P), total alkalinity, turbidity, and dissolved oxygen were recorded based on the methodology described in APHA (1998) [22]. Total suspended solid was determined every fortnight following the methods of APHA (1998) [22]. Biofloc volume was quantified by measuring through the Imhoff cone on daily basis.

2.4. Assessment of growth performances

For estimation of the growth performance, 50 numbers of shrimp were sampled from each treatment and control. The growth indices like weight gain (AWG) (g week − 1), final biomass (g m − 3), survival (%), growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) were measured by following our earlier work [23].

2.5. Bacterial quantification

The total heterotrophic count and vibrio count were analyzed at every 10 days interval using the Zobell marine agar (ZMA) and thiosulfate citrate bile salts sucrose agar (TCBS agar) (Hi Media, Mumbai) spread plates respectively till the end of the experiment. For efficient proliferation of bacteria, the plates were incubated for 48 h at room temperature. The colonies on the plates were counted and represented as bacteria colony forming unit (CFU).

2.6. Analysis of amino acid composition

The biofloc samples from each replicate tank was collected on monthly intervals and immediately freeze dried. The dried biofloc samples were processed for hydrolysis using 6 N hydrochloric acid in a sealed tube filled with nitrogen for 22 h at 110 °C in an oven. The acid was dried using vacuum rotary evaporator (IKA, RE 10C S84) and the residue was brought into a diluent (0.1 N hydrochloric acid), and then filtered using a 0.2 μm membrane syringe filter. The amino acid profiles were analyzed using pre-column HPLC gradient system (Shimadzu Corp, LC 30 AD) [24]. The YMC-Triart C18, RRH (1.8 μm, 2.1 × 100 mm) column was used to separate the amino acids after derivatization with mercaptopropionic acid, O-pthaladehyde and fluorenylmethoxycarbonyl chloride under gradient elution using phosphate buffer (20 mmol as mobile phase A) and combination of acetonitrile: methanol: water (45:40:15 as mobile phase B) at the flow rate of 0.3 ml/min. The gradient was changed by increasing mobile phase B concentration at the rate of 11%–13% at 3 min, 31% at 5 min, 37% at 15 min, 70% at 20 min, 100% at 25 min. Amino acids were qualified and quantified by a fluorescent detector (RF-20AXS) using an amino acid mixer as an external standard (Sigma Aldrich, Cat. No: AAS18) and nor leucine as an internal standard. Tryptophan was measured after alkali hydrolysis by the spectrophotometric method at 500 nm which is responsible for acid hydrolysis as mentioned by Sastry and Tummuru (1985) [25]. The partial oxidation of sulfur-containing amino acids like cysteine and methionine was prevented by adding 0.1% of phenol during acid digestion as mentioned by Jajic et al. (2013) [26]. The Essential amino acid index was calculated based on the amino acid requirements for *L. vannamei*.

Table 1
Ingredients of experimental diet (as % fed basis), proximate composition of carbon sources (as 100 gm dry basis) and amino acid profile of biofloc samples.

Feed ingredients	35% CP
Fish meal	23.6
Acetes	11.8
Soyabean meal	17.7
Gingelly oil cake	5.9
Wheat	13.92
Broken rice	6.96
Maida	13.92
Fish oil + sunflower oil	2.2
Lecithin	1
Vitamin and Mineral Mix[a]	2
Binder[b]	1

Proximate composition of various carbon sources used in the experiment

Carbon sources (gm)	Maida (C1)	Wheat (C2)	Gram (C3)	Millet (C4)	Rice (C5)	Corn (C6)	Molasses (C7)	Multigrain (C8)
Crude protein	10.33	13.0	22.0	11	7.2	6.9	0	14.8
Ether extract	0.98	2.5	6.7	4.3	2.8	3.9	0.1	2.02
Crude fiber	2.7	11	11.0	3.5	4.6	7.3	0	7.9
Others (Vitamin & Minerals)	9.68	1.5	2.3	6.2	9.4	4.9	24.9	1.77
NFE[c]	76.31	72.0	58.0	75.0	76.0	77	75	73.51

[a] Vitamins (mg kg-1): Vitamin A 20.0, Vitamin D 4.0, Vitamin E 120.0, Vitamin K 60.0, Choline chloride 6000.0, Thiamine 180.0, Riboflavin 240.0, Pyridoxine 180.0, Niacin 1080.0, Pantothenic acid 720.0, Biotin 2.0, Folic acid 30.0, Vitamin B12 0.150 Inositol 1500.0, Vitamin C 9000.0. Minerals (g kg-1): $CaCO_3$ 28.0, K_2SO_4 10.0, $MgSO_4$ 12.5, $CuSO_4$ 0.2, $FeCl_3$ 0.5, $MnSO_4$0.5, KI 0.01; ZnSO4 1.0, CoSO4 0.01, Cr_2SO_4 0.05, Bread flour 7.14.
[b] Poly MethylolCarbamide.
[c] Nitrogen free extract calculated by difference = 100-(Crude protein% + Crude fibre% + Ether extract% + Total ash%).

2.7. Challenge study

At the end of the experimental period, 20 healthy shrimps at an inter-moult stage from each biofloc treatment and control were challenged with the pathogenic strain of *Vibrio parahaemolyticus* (MTCC 451, IMTCC, and Chandigarh India) in triplicates. The experimental shrimps (average body weight = 15.5 ± 1.55 g) were kept in a 100 L capacity plastic tank with 50 L of same culture water filled. The pathogen was inoculated into the culture tank and the final bacterial load of 1×10^4 CFU/ml was achieved. For negative control, the shrimps reared in water without vibrio inoculation. All the important water quality parameters and all the shrimps were carefully monitored after infection. No water was exchanged for the whole duration of the trial. During the challenge trail, water quality parameters, shrimp survival and cumulative numbers of dead shrimps were assessed every day. The present challenge trial was repeated twice and recorded.

2.8. Immunological parameters

2.8.1. Hemolymph collection and determination of immune parameters

Hemolymph samples were collected from the experimental animals of both the treatment and the control group from the ventral sinus using a 2 ml sterile syringe with a 21G needle. The syringe consists of 1 ml of ice cold cysteine anticoagulant saline solution containing 3 mg cysteine to 5 ml of physiological saline (KCl (13 mM), NaCl (340 mM), $MgCl_2$ (10 mM), NaH_2PO_4 (0.3 mM),$MgSO_4$ (11 mM), and glucose (1.6 mM) in 100 ml distilled water, pH 7.8) [27].

2.8.2. THC and phenoloxidase activity

THC was measured as per the modified methods of Söderhäll and Smith (1983) [27] where 10 µl of hemolymph from each sampled individual was introduced into the Neubauer hemocytometer. The concentration per cubic centimeter (milliliter) is calculated by counting the total number of cells in four 1-mm^2 area using the following formula:

THC = Total cell counted x dilution factor (10) x 10^4/number of 1-mm^2 areas counted

Phenoloxidase activity was determined by following methods of Söderhäll and Smith (1983) [27] in which the hemolymph was collected without using the anticoagulant from the animal and was allowed to clot for 30 min at room temperature. After which the clot was disturbed using glass rod and is subsequently centrifuged at the rate of 1500 rpm for 7 min. Then 10 µl serum was incubated with 20 µl of trypsin (2.1 mg/ml^{-1}) for 15 min at 25 °C. In control, trypsin was substituted with tris-HCl buffer (50 mM; pH 7.5). The mixtures were made to 200 µl with 5 mM L-DOPA and further incubated for 20 min at 25 °C. The optical density of both control and treatment samples was measured spectrophotometrically at 490 nm.

2.9. Quantitative PCR assays

Total RNA was isolated from the hepatopancreas of five shrimps from each treatment using Qiagen RNA isolation kit (Qiagen, USA). The presence of RNA bands was confirmed by agarose gel electrophoresis. Reverse transcriptase PCR reaction was performed to convert mRNA into complementary DNA. iScript 1st Strand cDNA Synthesis Kit (Bio-Rad, USA) was used. The cDNA thus obtained was serially diluted and used for relative quantification of the target genes. The Real-Time PCR (Applied Biosystem's Real-Time PCR system StepOnePlus®) was used for amplification, melt curve analysis and calculation of gene expression. The temperature cycling parameters for the two-step PCR reaction were as follows: Holding stage of 10 min at 95 °C (Initial denaturation), 45 cycles of 00.15 s at 95 °C (denaturation), 1 min at 60 °C (annealing and extension). The total reaction volume (20 µL) in each PCR tubes were as follows; 10 µL of 2X SYBR®Green qPCR master mix (Bio-Rad, USA), 1 µL each of forward and reverse primers (10 pmol), 1 µL of template DNA (30–60 ng) and 7 µL of nuclease-free water. Transcript levels of immune genes and the reference gene (β-actin) were quantified by comparative delta-delta C_t (DDCt) method using a Real-Time PCR. The designed oligonucleotides used for qPCR are listed in Table 2.

2.10. Statistical analysis

The data obtained from the experiment was analyzed by SPSS (Version-16). The homogeneity of the data was verified using normality "chi square" test. The comparison between all the treatment groups and

Table 2
List of primer pair sequences and amplicon size of the genes used for real-time PCR (qPCR).

Gene	Primer sequence (5'—3')	Accession no	Amplicon Size
SOD	F-GCTGAATTGGGTGAGGAACG R-CCTCCGCTTCAACCAACTTC	AY486424	172
cMnSOD	F– GGCACAGTCAGTCCTCAGAT R- GAGAGGTGGCAAAGCATGAG	DQ298207.1	346
BgBp	F– TTATACCCGAGACTCCACGC R- ACGTCCGTATCTGAAAGCGA	AY723297.1	235
Catalase	F– GCCCGTACAAGGAACTACCA R- CTGACGTTCTGCCTCATTCA	AY518322.1	231
Peroxinectin	F – GAGTCTGAACATCCATCGCG R – TATGCCACCCACGAAGAAGT	KC708021.1	187
β-actin	F-CAACCGCGAGAAGATGACAC R-TCGGTCAGGATCTTCATCAGG	GU732815.1	243

between the treatments ($P < 0.05$) was made using one way ANOVA and Duncan's multiple range test (DMRT) respectively. The probability level was kept at 5% for the statistical analysis.

3. Results

3.1. Water quality parameters

All the water quality parameters recorded in the experimental tanks were within favorable range for *L. vannamei* rearing throughout the experimental period. No significant difference ($P > 0.05$) among the treatment groups in terms of salinity, temperature, pH were noted. The temperature during the experiment was 28.8 ± 1.8 °C. All other parameters showed significant variations among the treatments (Table 2). The treatment C7 and C8 have significantly ($P > 0.05$) lower DO than that of control and other treatments. TAN, Nitrite-N, nitrate-N, PO_4 and total alkalinity in control was recorded higher than the biofloc treatment groups. The total alkalinity was significantly reduced in the C7 followed by other carbon sources when compared to control (Table 2). Significantly ($P < 0.01$) higher chlorophyll[a], TSS, floc volume (Fig. 1a), turbidity was recorded on C8, C7, and C4 than other treatments and control (Table 3).

3.2. Effect of carbon sources on FCR, survival rate and ABW

Table 3 indicates that a significant difference was recorded ($P < 0.05$) between the control and the biofloc treatments in terms of zootechnical performance. The growth performance i.e., SGR, ABW, ADG and FCR along with survival percentage were similar in C4, C7, and C8 biofloc treatment groups. However, higher growth and survival with minimum FCR was recorded in C8, C7, C4 followed by C6, C2, C1, C5, and C3 when compared to control (Table 4 and Fig. 1b).

3.3. Microbial dynamics

Supplementation of carbohydrates in the biofloc treatment significantly ($P < 0.01$) increased the total heterotrophic bacterial count compared to control. Increased THB load was reported in C8 and C7 treatment among the biofloc treatments. However, in case of total vibrio count, control had a significantly higher vibrio load compared to the biofloc treatments and C8 had the lowest which was statistically similar to C7 and C4 (Table 3).

3.4. Biofloc amino acid composition

The amino acid profiles of the biofloc are represented in Table 5 and are categorized as essential and nonessential amino acids based on the nutritional requirements of the shrimp as recommended by NRC (2011) [28]. The result revealed higher level of essential amino acids in C4, C7, and C8 compared to other treatments. The utilization of protein by the shrimp depends on the availability of essential amino acids in the diet. Among them certain amino acids like arginine, methionine and lysine are limiting amino acids as they are generally deficient in the diet. The biofloc samples from C4, C7, and C8 are having higher arginine, methionine and lysine levels of 1.35, 1.12, 2.13; 1.32, 1.17, 1.66 and 1.25, 1.093, 1.663 per 100 g dry floc, respectively compared to other treatments and control.

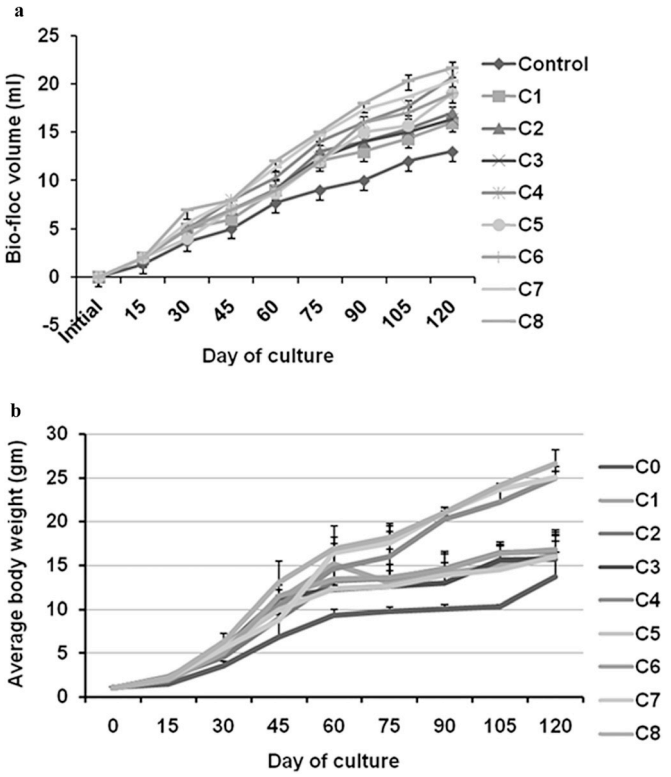

Fig. 1. a: Bio-floc volume of various carbon sources treatment for bio-floc based shrimp culture water. Significant difference ($p < 0.05$) between the groups is indicated by different letters on top of the bar. Fig. 1b: Effect of different carbon sources on ABW of *L. vannamei* measured at different in stages of growth. Data shown as mean with standard deviation as error bars (n = 50).

3.5. Challenge study

Cumulative mortalities in different carbon source treated groups were represented in (Fig. 2a). The challenge study shows that 90 ± 3.6% of mortality was observed in the control group whereas, in the treatment group, a maximum mortality (C1) of (up to 70 ± 3.4%) was recorded. The C4 and C8 groups were observed with the minimal percentage of mortality of 55 ± 1.8% after challenging with *V. parahemolyticus*. Similarly, C2, C5 and C7 treatments were observed with 60 ± 2.1% and in treatments C3, C6, 65 ± 2.6% mortality was recorded. There was a significant difference ($P < 0.05$) observed between control and treatments.

3.6. Immunological parameters

After 120 days of experimental period, Total Hemocyte Count of various CHO treated shrimp was significantly ($P < 0.05$) higher than that of control groups when challenged against *V. parahaemolyticus*. Similarly, Phenoloxidase activity was significantly higher ($P < 0.05$) in the various carbon source treated groups especially C8, C7 & C1, and others when compared to that of the control group (Fig. 2b and c).

Table 3
a) Mean values of physico-chemical parameters and b) biofloc measures of different carbon sources and control water samples during the experimental period. Significant difference ($p < 0.05$) between the groups is indicated by different letters on top of the data. C1 = Maida; C2 = Wheat flour; C3 = Gram flour; C4 = Millet flour; C5 = Rice; C6 = Corn flour; C7 = Molasses; C8 = Multigrain flour & C0 (without biofloc/carbon).

Parameters	C0	C1	C2	C3	C4	C5	C6	C7	C8
pH	8.4 ± 0.2^{b}	8.2 ± 0.1^{ab}	8.1 ± 0.3^{ab}	8.2 ± 0.8^{ab}	8.2 ± 0.9^{b}	8.3 ± 0.2^{b}	8.2 ± 0.2^{ab}	7.9 ± 0.2^{a}	8.3 ± 0.1^{ab}
Salinity (ppt)	31.8 ± 0.0^{a}	31.6 ± 0.7^{a}	31.6 ± 0.7^{a}	31.8 ± 1.4^{a}	31.3 ± 0.8^{a}	31.1 ± 1.5^{a}	31.3 ± 2.5^{a}	31.8 ± 1.7^{a}	31.8 ± 1.2^{a}
TAN (ppm)	1.865 ± 0.9^{d}	0.830 ± 0.2^{c}	0.167 ± 0.0^{a}	0.189 ± 0.08^{ab}	0.151 ± 0.07^{a}	0.199 ± 0.08^{ab}	0.675 ± 0.04^{bc}	0.096 ± 0.02^{a}	0.073 ± 0.04^{a}
NO2–N (ppm)	1.262 ± 0.9^{c}	0.410 ± 0.32^{b}	0.083 ± 0.01^{ab}	0.106 ± 0.07^{ab}	0.075 ± 0.01^{ab}	0.088 ± 0.08^{ab}	0.174 ± 0.17^{ab}	0.026 ± 0.03^{c}	0.016 ± 0.02^{c}
NO3–N (ppm)	0.732 ± 0.23^{d}	0.214 ± 0.12^{c}	0.122 ± 0.04^{abc}	0.155 ± 0.09^{bc}	0.047 ± 0.01^{ab}	0.082 ± 0.06^{dc}	0.089 ± 0.07^{ab}	0.018 ± 0.02^{a}	0.005 ± 0.01^{a}
PO4–P (ppm)	0.104 ± 0.05^{a}	0.142 ± 0.07^{a}	0.163 ± 0.01^{a}	0.126 ± 0.08^{a}	0.135 ± 0.08^{a}	0.124 ± 0.05^{a}	0.149 ± 0.14^{a}	0.172 ± 0.10^{a}	0.162 ± 0.12^{a}
TA (ppm)	161.1 ± 22.9^{b}	125.7 ± 24.9^{a}	107.3 ± 17.4^{a}	115.4 ± 25.8^{a}	118.0 ± 24.3^{a}	118.7 ± 24.5^{a}	124.2 ± 36.4^{a}	126.8 ± 27.9^{a}	125.2 ± 26.1^{a}
DO (ppm)	6.4 ± 1.02^{d}	5.2 ± 0.86^{bc}	5.1 ± 0.70^{bc}	5.3 ± 0.83^{bc}	4.7 ± 0.38^{ab}	5.1 ± 0.58^{bc}	5.7 ± 0.85^{c}	4.2 ± 0.48^{a}	4.3 ± 0.73^{a}
Chlorophyll^{a} (mg/m^{3})	18.7 ± 3.8^{a}	81.3 ± 8.2^{b}	139.8 ± 12.69^{c}	88.5 ± 15.5^{b}	191.2 ± 44.9^{d}	120.5 ± 10.6^{bc}	97.4 ± 18.5^{bc}	220.0 ± 43.3^{d}	232.4 ± 20.6^{d}
TSS (ppm)	118.1 ± 25.7^{a}	161.0 ± 34.8^{ab}	179.0 ± 61.1^{ab}	168.0 ± 29.5^{ab}	244.0 ± 33.7^{c}	193.3 ± 14.2^{bc}	157.3 ± 25.2^{ab}	305.6 ± 21.5^{d}	327.0 ± 39.2^{d}
FV (ml)	9.4 ± 2.9^{a}	13.4 ± 2.6^{abc}	15.2 ± 3.8^{bc}	15.6 ± 2.2^{bc}	25.1 ± 2.7^{d}	17.3 ± 3.9^{c}	12.9 ± 2.5^{ab}	23.9 ± 3.8^{d}	24.9 ± 3.4^{d}
Turbidity (NTU)	11.07 ± 1.6^{a}	14.5 ± 1.8^{b}	18.4 ± 1.6^{c}	13.7 ± 0.9^{ab}	25.1 ± 2.7^{de}	17.3 ± 3.9^{cd}	12.9 ± 2.5^{b}	23.9 ± 3.8^{ef}	24.9 ± 3.4^{f}
THB (X 10^{6} CFU/ml)	3.4 ± 0.47^{a}	8.22 ± 1.37^{b}	8.21 ± 1.41^{b}	8.78 ± 1.47^{b}	9.19 ± 1.56^{bc}	9.61 ± 1.53^{bc}	8.77 ± 1.42^{b}	10.1 ± 1.58^{bc}	11.54 ± 1.55^{c}
TVC (X 10^{3} CFU/ml)	3.2 ± 0.31^{d}	2.2 ± 0.22^{c}	2.1 ± 0.21^{bc}	1.9 ± 0.17^{ab}	1.4 ± 0.16^{d}	1.8 ± 0.21^{b}	1.8 ± 0.12^{b}	1.3 ± 0.13^{a}	1.2 ± 0.12^{a}

3.7. Effect of carbon sources on immune gene expression

The transcript levels of SOD, MnSOD and BGBP were significantly up-regulated in shrimps reared in C4, C7 and C8 indicating that increase in the ABW and ABL has proportionally enhanced the immune regulatory functions. Superoxide dismutase (SOD) had a considerable higher expression pattern in sources C4 to C8. Shrimps reared with C4 showed approximately 11-fold up-regulation in the SOD transcript levels, while C7 and C8 showed 12-fold and 11-fold respectively (Fig. 3a). Similarly, up-regulation was measured in MnSOD transcripts (C4 – 16 fold, C7 -7 fold and C8 -5 fold) (Fig. 3b).

Further, the transcripts of β-1, 3-glucan-binding protein (BGBPβ) of white shrimp was significantly increased in C6 (4 fold) and C7 (9 fold) respectively while C4 (1 fold) remains unaffected (Fig. 3c). Enhanced BGBP mRNA transcripts suggest that the activation of these systems which indirectly control the immunomodulation in the shrimps. Additionally, peroxinectin (PX) transcripts were upregulated in C4 treated samples (Fig. 3d) and catalase (CAT) transcripts were upregulated in C7 treated samples (Fig. 3e).

4. Discussion

The water quality parameters are maintained in the optimal range for *L.* vannamei culture [29]. The low concentration of TAN and nitrite in the biofloc treatment than the control suggest total oxidation of ammonia to nitrate in the biofloc treatment [30]. Further lowering of nitrate level in the biofloc treated group indicates that the microbial communities in the biofloc removed these obnoxious gases from the system, which is very essential for a zero-water exchange system [31]. The chlorophyll^{a} concentration was higher in the biofloc system compared to the control group (autotrophic) which can be attributed to the high rate of nutrient recycling within the biofloc system. In the present study, there is no difference between the biofloc treated groups in terms of chlorophyll^{a} concentration.

The ability of Pacific white shrimp to utilize natural productivity and its effect on enhancing shrimp growth is well documented [7,32,33]. Ju et al. (2009) [34] suggests that microalgae in the microbial floc may play a key role in improving shrimp growth. Biofloc enhances the growth performance of *Penaeus monodon* [35,36] *Litopenaeus vannamei* [7,37], and *Farfantepenaeus paulensis* [38]. Apart from being a source of quality proteins, biofloc constituents are a rich source of growth promoters, bioactive compounds [34] and improves health status of the cultured shrimps [39]. The high survival and improved growth of the shrimp in two biofloc treatments [37] can also support the view that the shrimp can grow in a healthy condition (biofloc) with carbohydrate addition. The present study revealed better shrimp performance in terms of survival, growth in carbon sources such as C4, C7 and C8 treatments than control. Better essential amino acid composition in the millet, molasses and multigrain atta would have resulted in enhanced growth performance in the reared animals [40].

Similarly, other carbon source used in the study is showing promising results in the biofloc system [41]. A comparison of wheat flour and corn flour as a carbon sources for biofloc production in freshwater tilapia showed that there were no significant effects on water quality between the biofloc groups and the control. However, in terms of weight gain and feed conversion ratio (FCR), wheat flour performed better than corn flour [42]. In our present study, a similar trend was observed with wheat and corn flour.

The utilization of microbial protein depends on the ability of the target animal to harvest the bacteria and its ability to digest and utilize the microbial protein [9]. The higher yield in the carbohydrate added treatments of the present study showed that *L. vannamei* can well utilize the additional protein derived from the increased bacterial biomass. Burford et al. (2004) [32] suggested that flocculated particles rich in bacteria and phytoplankton could contribute substantially to the nutrition of the *L. vannamei* in intensive shrimp ponds. Ju et al. (2009)

Table 4
Growth parameters of *Litopenaeus vannamei* culture based on bio-floc system using various carbon sources and control groups. Significant difference ($p < 0.05$) between the groups is indicated by different letters on top of the data. ABW = average body weight; ABL = average body length; ADG = average daily growth; SGR = specific growth rate; FCR = feed conversion ratio.

Treat-ments	Initial weight (gm)	Initial length (mm)	Survival (%)	ABW (gm)	ABL (mm)	ADG (gm)	SGR (%)	FCR	Improvement (%)
C0	1.10 ± 0.07^{a}	46.3 ± 3.2^{a}	70.5 ± 2.1^{a}	13.7 ± 2.8^{a}	99.3 ± 1.3^{a}	0.082 ± 0.0^{a}	2.55 ± 0.01^{a}	2.25 ± 0.3^{c}	-
C1	1.04 ± 0.03^{a}	44.5 ± 1.7^{a}	86.5 ± 2.1^{c}	16.2 ± 2.5^{b}	120.7 ± 2.6^{d}	0.119 ± 0.01^{b}	2.73 ± 0.01^{b}	1.5 ± 0.1^{b}	18.25 ± 1.03^{a}
C2	1.10 ± 0.15^{a}	44.0 ± 0.1^{a}	84.0 ± 1.4^{bc}	16.6 ± 1.1^{b}	121.3 ± 1.8^{d}	0.138 ± 0.01^{b}	2.76 ± 0.07^{b}	1.5 ± 0.1^{b}	21.17 ± 8.26^{a}
C3	1.05 ± 0.03^{a}	43.5 ± 0.5^{a}	81.0 ± 1.4^{b}	15.7 ± 2.8^{b}	116.3 ± 5.2^{c}	0.125 ± 0.01^{b}	2.76 ± 0.09^{b}	1.6 ± 0.2^{b}	14.6 ± 1.03^{a}
C4	1.12 ± 0.11^{a}	46.0 ± 1.1^{a}	96.5 ± 2.1^{d}	24.95 ± 0.8^{c}	129.8 ± 1.3^{b}	0.208 ± 0.01^{c}	3.18 ± 0.03^{d}	0.93 ± 0.1^{a}	82.12 ± 5.68^{ab}
C5	1.02 ± 0.14^{a}	45.2 ± 3.5^{a}	83.0 ± 4.2^{b}	16.0 ± 2.4^{b}	118.2 ± 3.1^{cd}	0.131 ± 0.01^{b}	2.78 ± 0.06^{bc}	1.5 ± 0.1^{b}	16.79 ± 1.55^{a}
C6	1.03 ± 0.14^{a}	43.9 ± 2.4^{a}	82.5 ± 3.5^{b}	16.85 ± 2.2^{b}	110.6 ± 0.9^{b}	0.114 ± 0.01^{b}	2.83 ± 0.05^{c}	1.5 ± 0.2^{b}	22.99 ± 1.66^{a}
C7	1.08 ± 0.04^{a}	43.5 ± 1.2^{a}	99.0 ± 1.1^{de}	25.05 ± 1.2^{c}	128.9 ± 1.8^{e}	0.209 ± 0.00^{c}	3.19 ± 0.01^{d}	0.91 ± 0.1^{a}	82.85 ± 1.55^{ab}
C8	1.02 ± 0.02^{a}	44.6 ± 1.0^{a}	100.0 ± 0.0^{e}	26.55 ± 1.6^{c}	131.8 ± 0.5^{e}	0.221 ± 0.00^{c}	3.25 ± 0.01^{e}	0.87 ± 0.1^{a}	93.80 ± 1.55^{b}

Table 5
Amino acid composition (g/100 g dry floc) of biofloc produced with different carbon sources.

	C1	C2	C3	C4	C5	C6	C7	C8
Essential amino acids								
Arg	1.143	1.187	1.113	1.353	1.160	1.273	1.320	1.253
His	2.803	2.663	2.740	2.920	2.567	2.440	3.030	2.907
Ile	1.313	1.307	1.193	1.317	1.240	1.173	1.360	1.293
Leu	2.150	2.057	2.050	2.137	1.493	1.727	2.153	1.900
Lys	1.393	1.343	1.463	1.560	1.270	1.257	1.660	1.663
Met	1.007	0.967	1.077	1.127	0.903	0.863	1.170	1.093
Phe	1.547	1.860	1.717	1.803	1.647	1.507	1.773	1.790
Thr	1.653	1.750	1.730	1.870	1.850	1.750	1.810	1.850
Trp	0.290	0.290	0.290	0.330	0.280	0.323	0.367	0.350
Val	1.383	1.373	1.310	1.490	1.373	1.277	1.367	1.313
Non-essential amino acids								
Ala	2.677	2.560	2.227	2.267	2.450	2.430	2.287	2.277
Asp	2.863	2.493	2.433	2.553	2.673	2.327	2.367	2.373
Cys	0.417	0.393	0.437	0.453	0.370	0.517	0.457	0.477
Glu	3.277	3.617	3.663	3.407	3.060	3.367	3.433	3.600
Gly	2.183	2.443	2.303	2.150	2.820	3.383	3.463	3.427
Pro	1.290	1.293	1.270	1.287	1.367	1.437	1.290	1.313
Ser	1.350	1.550	1.253	1.383	1.483	1.383	1.440	1.360
Tyr	1.223	1.053	1.133	0.963	1.000	0.987	1.147	1.163

[34] reported that increase in organic carbon promotes the dominance of heterotrophic bacterial community. Several studies have reported that an increased resistance of shrimps to pathogenic Vibrio through probiotic administration [43,44]. Our data pointed out a lower concentration of vibrio recorded in carbon sources treated groups than control. Our findings are in harmony with Kumar et al. (2014) [45]. This indicates that the biofloc have beneficial microorganism that competes and reduces the pathogens especially vibrios.

The challenge test helps us to deduce the health of animals reared in the system. *Vibrio parahemolyticus* is one of the common disease-causing bacteria in the aquatic system, which causes a huge loss to the farmers. The animals were reared in the biofloc system with different carbon sources. The carbon source used which results in the growth of abundant microflora and microbiota in the system. in turn, improves shrimp's health [9,36,46]. The biofloc naturally act as anti-bacterial compound and the same as the positive influence on the immunity of cultured species [47–50]. These shrimps were infected with opportunistic pathogenic bacteria to understand the performance of carbon source on shrimp's health and BFT reared animals. BFT reared animals exhibited better resistance against the *V. parahaemolyticus* compared to control irrespective of the CHO source. Our results are in agreement with other findings on *L. vannamei* [16] and *P. monodon* [51]. The study supports the use of different carbon source in reducing the stress of toxic metabolites thereby reducing the chances of disease occurrence. Avnimelech (2009) [2] stated that molasses are the most widely used carbon source for biofloc production. Hence the present study also used molasses as one of the carbon sources thus by increasing the availability carbohydrate in the system and showed better survivability after challenging *V. parahaemolyticus*. The results were comparable to Samocha et al. (2007) [46] and Krummenauer et al. (2014) [52]. There was a difference in the performances of different treatments especially Millet (finger millet), wheat, multigrain flour, and molasses.

Bioflocs are deliberated to be in one of the richest sources of many bioactive compounds such as chlorophylls, carotenoids, phytosterols, bromophenols, polysaccharides, fat soluble vitamins and amino sugars [34,53]. These bio-active compounds produced in the biofloc improve the health of cultured species and this is due to the amount of essential amino acids, fatty acids and other nutritional elements [54]. The heterotrophic bacteria quickly decompose the simple carbohydrates, which are added continuously to maintain C: N level in the tank. However complex carbohydrates are decomposed slowly. As millets are highly nutritive, it is one of the conventional foods in south India. 72.6 g of carbohydrates are obtained from 100 g of millet it provides cheap protein, minerals, and vitamins to the cultured species. Having starch as its major component it also have amylopectin, amylase and 69.73% of soluble carbohydrate in which 16.2% and 1.2% are composed of starch and reducing sugars respectively [55]. Since no study had reported on millet as a carbon source, we used finger millet powder and the results were found to be positive on growth, survival as well as immunity when compared to other carbon sources.

Multigrain flour is a mixture of six natural grains providing wholesome nutrition.100 g of multigrain atta is made of 72.48 g of carbohydrates in which 6.18 g are sugar and 14.43 g of proteins. After challenging *V. Parahemolyticus*, the result was in harmony with the result obtained from millet. The survival rate was up to 45%. Since multigrain powder, millet and wheat are complex carbohydrates they dissolve slowly and releases carbon in the culture water.

The biofloc clearly elucidate the shrimp innate immune response. For instance, the activity of phenoloxidase and total hemocyte count was upregulating in the shrimp reared in a biofloc system than control without any induced infection. This stimulation effect seems to be a general feature of the biofloc system [23], although the extent of the stimulation implies to be a carbon source dependent. Improvement in immunomodulatory functions of *L. vannamei* in the biofloc reared shrimps suggest that these carbon sources can modulate and trigger the immune genes when challenged with pathogens. The circulating hemocytes of crustaceans are essential in performing functions such as phagocytosis, encapsulation, and storage and release of the prophenoloxidase system [56]. Phenoloxidase is an enzyme activating melanization of foreign cells and prevents them from further infection. This enzyme is highly stimulated by microbial cell wall components such as lipopolysaccharides (LPS) and ß −1, 3-glucans [57–59]. As the shrimps were cultured in BFT-based systems they evidently consumed the microbial floc *in situ,* [3,16] so that the increase in total hemocyte number and PO activity point in the direction of a stimulatory effect of the

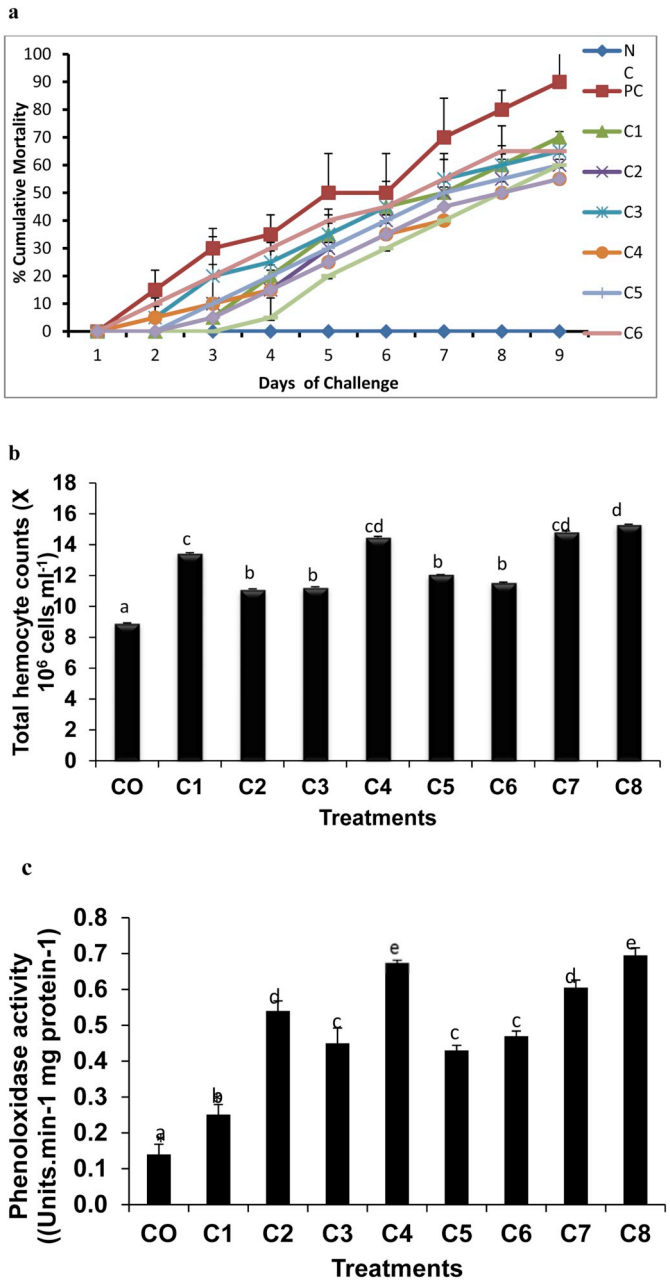

Fig. 2. a): Cumulative mortality of *Litopenaeus vannamei* different carbon sources of biofloc and control experimental groups post challenged against *Vibrio parahaemolyticus* MTCC 451. b) THC and c) Phenoloxidase activity of *L. vannamei* after 120 days treated with different CHO and control group (n = 10). Data shown as mean with standard deviation as error bars (n = 50). Significant difference ($p < 0.05$) between the groups is indicated by different letters on top of the bar. THC = total hemocyte count; PO = phenoloxidase.

(digested) biofloc on shrimp immunity. Similarly, our data showed C8, C7, C4 and other carbon sources had significantly increased in the level of THC and Phenoloxidase activity than control shrimp. It is also interesting to note that the application of biofloc in shrimp culture results in similar effects in terms of growth, feeding efficiency, pathogenic bacteria inhibition and immune responses as the application of probiotics [60].

The biofloc system consists of beneficial microbes in high density which is assumed to enhance the immune system. The present finding of biofloc induced immunomodulation are in agreement with the earlier studies [16,17,23,48]. The immune system of shrimp is mainly activated by the microbial cell wall material comprise of peptidoglycan, lipopolysaccharides and β-1,3-glucans [17,23,61,62]. Superoxide dismutase (SOD) is frequently used as a biomarker for measuring immune parameters in *L. vannamei* [63]. The expression level of SOD increases after infection with Vibrio sp. as bacterial infection induces the activity of SOD significantly. Anchalee et al. (2013) [64] observed similar findings in their study. β-1, 3-glucan-binding protein (BGBP) gene plays an essential role in shrimp-pathogen interactions and reflexes. They respond to the pathogens by activating a series of downstream cascade which triggers the proPO system, coagulation cascade and the expression for antibacterial effectors [65]. Pattern recognition protein of LPS and β-1, 3-glucan in the shrimp proPO-activating system, enhance the phenoloxidase activity [64]. The protein further acts as an opsonin to increase phagocytosis [66]. Enhanced BGBP mRNA transcripts suggest the activation of these systems which indirectly control the immunomodulation in the shrimps. CAT enzymes are also known to play a vital role in the antioxidant defense system, hence protecting the shrimps from free radicals [67,68]. The catalase expression level was up-regulated in this study. Similar results were reported by Anchalee et al. (2013) [64] where PX is involved in the proPO cascade system [57]. Beneficial bacteria (probiotics) also show a promising result in activating the immune response in shrimp and the mechanisms by which the bacteria influence shrimp performance have been reviewed by several authors [59,69,70]. For instance, Zokaeifar et al., (2012, 2014) [60,71] reported that adding *Bacillus subtilis* into the water or into the feed of white shrimp resulted in better growth, survival, inhibition of Vibrio growth in the intestine, as well as the up-regulation of immune related genes such as LGBP, proPO, peroxinectin, and serine protease. Biofloc act as a natural probiotic which acts against pathogenic stimulants and the carbon source used in the biofloc increase the beneficial bacteria naturally [72]. The bio active compound of biofloc may have an effect on the health of cultured shrimps, particularly on immune and antioxidant defense system by the addition of different carbon source [50]. In our present study better immune responses were exhibited as a result of having a better nutritive composition in different carbon source.

5. Conclusion

Biofloc science is gaining importance in aquaculture alongside the commercial success of its adoption for high-density aquaculture to achieve high productivity through a sustainable approach. This study highlights the importance of different carbon sources in improvising the biofloc system and shrimp performance and warrants the use of millet, multigrain as an alternate to molasses without compromising generation of biofloc and enhanced growth performance with improved diseases resistance in *L. vannamei.* The gene expression study revealed that carbon sources, in specific millet, multigrain and molasses showed immunomodulatory action in *L. vannamei* by triggering the proPO activating and antioxidant defense systems. Considering the fact that gene expression does not inevitably refer to functional proteins, further investigation in this regard is justified for prolific documentation. It is important to define the advantages of available CHO sources in improving the zootechnical performance of the species and reducing the cost of production compared to other sources reported.

Conflicts of interest

The authors declare that they have no conflict of interest.

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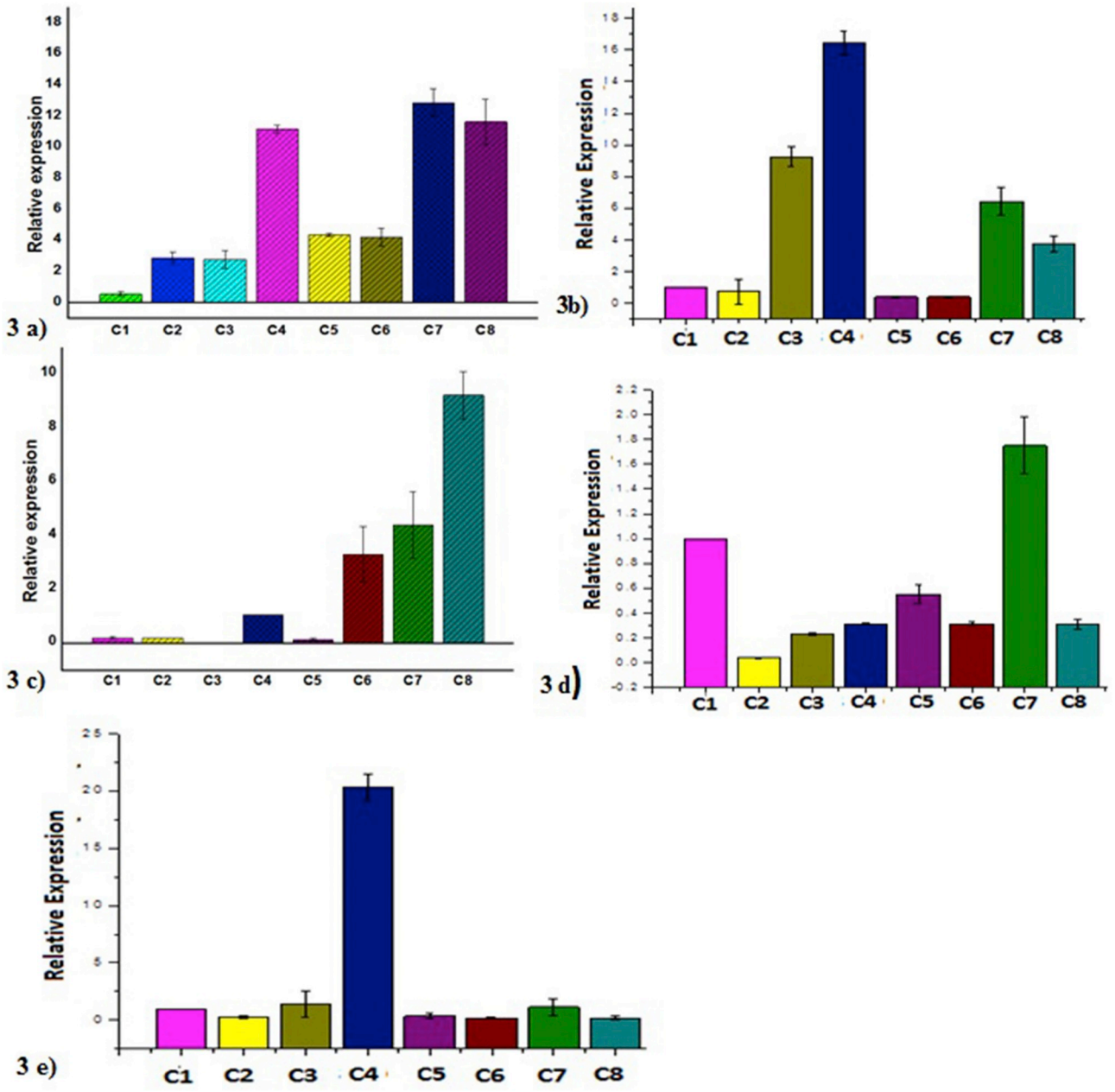

Fig. 3. Expression of a) SOD b) MnSOD c) BGBP d) PX and e) CAT mRNA transcripts in *L. vannamei* reared in a BFT system supplemented with various carbon sources in comparison to that of the control as determined by real time PCR. Five individual shrimps were analyzed from the control and each of the CHO treatment groups. Data are means ± SD of gene expression in the different C:N treatments. SOD = superoxide dismutase; cMnSOD = cytoplasmic manganese superoxide dismutase; BGBP = beta-glucan binding protein; PX = peroxinectin; CAT = catalase.

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