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Carbon: Nitrogen (C:N) ratio level variation influences microbial community of the system and growth as well as immunity of shrimp (*Litopenaeus vannamei*) in biofloc based culture system

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ABSTRACT

Biofloc technology (BFT) is a novel modern aquaculture farming technique used to reduce toxic nitrogen concentration, act as *in situ* food source and eradicate pollutants using carbon and therefore to control C:N ratio in an aquaculture system. In this study, effect of different C:N ratios of a biofloc based system on water quality such as the level of Total ammonia nitrogen (TAN) nitrite-nitrogen (NO_2^-–N) and nitrate nitrogen (NO_3^-–N) were explored. Further, the growth and immunity status of shrimp *L. vannamei* under the influence of different C:N ratios were evaluated. Two of the C:N ratios (15 and 20) could significantly ($P < 0.05$) reduce TAN, NO_2-N and NO_3-N levels (0.456 ± 0.01, 0.145 ± 0.09, and 0.102 ± 0.02 ppm) compared to control (1.45 ± 0.1, 0.749 ± 0.14 and 0.675 ± 0.16 ppm). Large variations in the frequency distribution of operational taxonomic units (OTUs) for the bacterial community in water with different C:N ration (BFT) and control were observed. Vibrios often considered as opportunistic pathogens, where the most dominant bacterial flora of water in control (79%) and C:N5 (37%) group. In C:N10, *Thauera* (62%) was most represented genus. Similarly, *Attheyaceae* (56%), followed by *Peridiniaceae* (30%) were the most dominant groups in C:N15 treatment. The diversity of bacterial flora was more spread in C:N20 treatments with *Psychrobacter* (26%), *Proteobacteria* (25%) and *Peridiniaceae* (20%) as the major groups. The trend of Vibrio dominance decreased with the increase in C:N ratios and thus confirming the dominance of heterotrophic bacteria in high C:N ratio groups. Upon challenge with pathogens, shrimps from C:N10, C:N15 and C:N20 groups showed significantly higher survival ($P < 0.05$) compared to the C:N5 and control group. Similarly, better growth rate was also observed in BFT tanks compared to control both during the culture and at harvest. Comparatively higher expression of four immune-related genes (ras-related nuclear gene (RAN), serine proteinase gene (SP), prophenoloxidase activating enzyme (PPAE), and crustin were observed in different C:N ratio ponds than control and these were in increasing trend with the C:N ratio. Gene expression analysis showed that the transcripts of those immune genes were significantly increased among all C:N treatments than that of control. Overall, these findings demonstrated that with optimum C:N ratio, BFT can be used to optimize the bacterial community composition for both optimal water quality and optimal shrimp health. This study thus indicates the possibility of obtaining better performance of *L. vannamei* culture with proper adjustment of C:N ratio in a biofloc based system.

1. Introduction

Extensive use of inputs through intensification of shrimp aquaculture practice brings stress and makes the animals susceptible to different diseases. Generation of toxic metabolites such as ammonium and nitrite from the accumulated aquaculture wastes in the form of feces and unutilized feed is main culprit behind this stress. It results in severe adverse implication on the overall production bringing huge economic loss to farmers [1–6]. As intensification is inevitable to achieve high production, modification of existing culture practices has

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become quite necessary for the establishment of better environment; to maintain good health and growth of animals.

Biofloc based culture practice is one such technology (BFT) during recent time which has added several advantages including of biosecure system due to zero or minimal water exchange, improved water quality through the self-generated bioremediation process and improved growth and immune system of shrimps through diverse heterotrophic bacterial system. In addition to this, biofloc also serves as a high nutrients food source rich in amino acids, proteins, fatty acids and lipids in the form of different microorganisms, and thus substantially reduces external feed supply to make it more economical. The diverse microbial community of flocs not only provides supplemental nutrition, but also acts as consumers of dissolved oxygen, as nutrient recyclers, and as a food source for different microbial diversity from higher trophic levels in aquaculture [1,5,7–9]. Several studies reported that the composition, structure, and stability of bio-flocs could be affected by diverse types of organic carbon sources (molasses, corn, wheat, glucose acetate, glycerol, and tapioca) and their ratio. Different carbon-nitrogen ratio also makes the differences in the bioflocs nutrients composition such as carbohydrate, protein, lipid and fatty acid [9–14].

In most of the aquaculture feeds, the C: N ratio is about 7–10:1, whereas bacterial population present in ponds needs around 20 units of carbon to assimilate one unit of nitrogen [15]. The heterotrophic bacteria populations in aquaculture ponds will not get expanded to desired level with such a low C:N ratio. Therefore, addition of extra carbon sources becomes inevitable to increase the heterotrophic bacterial population to a dense mass in pond, and use of local resources can make it more economical [16,17].

Microbial studies in aquaculture are focused on the understanding of symbiotic and antagonist interrelationships of microbes with eukaryotes such as fish, crustacean, and molluscans. Microorganisms of aquatic system were used as biomarkers or sentinel, effluent bioremediators, probiotics and a direct food source for the cultured species [18–20]. Despite such vigorous and constant growth of the use of microorganisms, most of the bacterial species thriving within culture systems and their particular roles in such microsomes are not clear. Determination of metabolic process performed by microbes in aquaculture system is important to point to achieve better understanding. It increases the possibilities of manipulating the microcosms created by aquaculture to understand the biogeochemical cycles of nutrients within and outside of ponds, the modification of bacterial communities and disruption of key processes that lead to disease [21]. Studies on diversity, abundance, symbiotic and antagonist of bacteria in the aquatic system will pave the way to understand and optimize nutrient cycles, disease control, water quality, farming production, the environmental impact of effluents [18–20]. Metagenomics studies allowed researchers to study the diversity and quantity of particular microbes or genes along spatiotemporal patterns to make stronger associations among given microbial communities and host genotype or phenotype [22–24].

It is also well known that, diverse range of microorganisms of biofloc and their cell wall components have been used as probiotics and potent immunostimulants to develop innate immunity, antioxidant status and disease resistance of shrimps against invading pathogens. Microbial components of biofloc also contain potent bioactive compounds including carotenoids, chlorophylls, polysaccharides, phytosterols, taurine and fat-soluble vitamins and shown to be involved in improving the immunity of shrimp species [9,10,16].

There is no information on the role of carbon ratio in improving the microbial diversity in a biofloc based system, growth and immune response of shrimps reared in it. The knowledge of the microbial composition, structure, and stability of a biofloc and its nutritional value results in the improvement of cost-effective shrimp feed preparations. The goal of present study was to evaluate the effects of four different ratios of Carbon and Nitrogen (CN: 5; 10; 15; 20) on growth, and immune status of *L. vannamei* and variation in microbial community in the culture system with zero water exchange.

2. Materials and methods

2.1. Experimental site and tank preparation

The study was carried out for 120 days in the Institute facility. Experimental tank systems of 500 L capacity kept in an open-air structure in a semi-translucent roof. All the tanks were covered with nylon nets to prevent escape of the animals. Tanks were filled with prechlorinated and filtered 32 ppt seawater (sand filtered), 10% of which were regularly exchanged on a fortnight basis. Initially, the tanks were treated with agricultural lime ($CaCO_3$) @ 20 ppm, and inorganic fertilizers like urea (@ 15 ppm) and single superphosphate (@ 15 ppm) to develop the system autotrophically followed by carbohydrate addition for driving the system heterotrophically.

2.2. Experimental design

The experiment was conducted with four different level of C:N ratio (5, 10, 15 and 20) and designated as C:N 5:1, C:N10:1, C:N15:1 and C:N 20:1. All treatments had three replicates and allocation for each treatment was completely randomized to generate the biofloc in all the treatment tanks. Molasses as carbohydrate source (200 ml), probiotics consortium (*Bacillus strains* (5.4×10^9 CFU/ml), were mixed in autoclaved seawater (10 L) and dissolved thoroughly and brewed for 24 h for fermentation. The fermented inoculum was applied in all the treatment tanks @ 50 ml/tank every day for five days to generate the heterotrophic bio-floc. Then C:N ratio was maintained followed by the method of [25] for transition of the heterotrophic system.

2.3. Animal stocking and biofloc management

After the nursery period of 35 days, *L. vannamei* juvenile shrimp with graded weight, 1.0 ± 0.01 g were stocked at 150/m^3 with working volume of 500 L. Formulated pellet feed containing 35% of crude protein (CP) were used as feed in all the treatments. Daily feeding started at 8% of body weight and gradually reduced to 2.5% towards the end of the experiment. The feed was distributed equally to shrimps in all the experimental units, thrice daily at 6:00 a.m., 11:00 a.m. and 6:00 p.m. initially for two months followed by one additional feeding ratio at 10:00 p.m. up to the end of the experiment.

2.3.1. Carbohydrate addition

In this study, the molasses was selected as a carbon source (28% carbon w/w and specific gravity of 1.2). These four different levels of C:N ratios were calculated by C:N contents of the feed and the carbon content of the molasses. The C:N ratio of the applied feed was 7:1 which is adjusted at the base level. A tank without molasses supplementation was referred as a control. To achieve the C:N ratios to C:N 5:1, C:N 10:1, C:N 15:1 and C:N 20:1 above the base level of inherent CN in feed; 0.32 mL, 0.64 mL, 0.96 mL, and 1.28 mL of molasses were added daily for every 1 g of the feed offered, respectively. Control groups was maintained in autotrophic way by developing bloom using above mentioned fertilizers and fed with same feed without addition of any carbon sources. The composition of experimental diet [26] was tabulated in Table 1.

Continuous aeration and agitation were provided by one 5HP blower passing through sand stones aerator, fixed at 10 cm above the ground, with a capacity of injecting 7.5 m^3 air/tank/minute. In the biofloc treatment tanks, minimal water exchange that is up to 10% of water was exchanged in every 15 days interval, and sludge removal was done regularly on a daily basis, whereas for control on a weekly basis 50% of water was exchanged. This was followed throughout the experimental period.

Table 1
Feed formula for biofloc experiments.

Composition	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	2.2
Lecithin	1
Vitamin and Mineral Mix[a]	2
Binder[b]	1

[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.

2.4. Assessment of water quality parameters

Water quality was checked on a weekly basis at 09.00 a.m. Water parameters such as temperature (thermometer), pH (pH-Scan-Eutech instruments, Singapore), Salinity (hand refractometer), Total Ammonia Nitrogen (TAN) (Phenol hypochlorite method), NO_2-N, NO_3-N, phosphate-P (PO_4-P), total alkalinity, turbidity and dissolved oxygen were recorded during the experiments following the usual methods described in Ref. [27]. Total suspended solid was determined on fortnight interval [27]. Biofloc volume was quantified by measuring through the Imhoff cone on a daily basis to understand the dynamics of feed addition, quality of water and microorganisms.

2.5. Estimation of growth and production parameters

The growth parameters of shrimps in all the groups were recorded through biweekly sampling (n = 50). The growth parameters included length, weight gain (%), feed efficiency ratio (FER), feed conversion ratio (FCR), and specific growth rate (%) (SGR) as follows. Weight gain (%) = (FW-IW) x 100/IW, FCR = Feed given (DW)/bodyweight gain (WW), FER = 1/FCR, SGR (%) = [ln (FW) − ln (IW)/N] × 100. Where, FW = final weight, IW = initial weight, DW = dry weight, WW = wet weight, ln = natural log and N = number of culture days.

2.6. Determination of microbial biomass

Total heterotrophic bacterial count and Vibrio count of water samples were determined at initial and ten days interval up to 130 days of experiment. Water samples were collected in a sterile poly-propylene bottle from the center of the tanks. Samples were maintained at 4 °C and immediately brought to laboratory. Two hundred milliliter of the sample was homogenized and subsequently, tenfold serial dilution was made in normal saline solution (NSS), and 0.1 ml of appropriate dilutions were plated in duplicates on Zobell marine agar for the total count and thiosulfate citrate bile salts sucrose agar (TCBS agar) for *Vibrio* count. Plates were incubated at room temperature for 24 h and colony in the range of 30–300 were counted and expressed as bacterial colony forming unit (CFU/mL).

2.7. Bacterial metagenome sequencing and bioinformatics analysis

At 12th week of experiment period, water samples were collected from triplicate of each group (control and BFT treatment tank) samples were pooled together in respective treatments and immediately processed for bacterial metagenome analysis. Total bacterial cells from each sample were separated and filtered through 8-μm qualitative filter paper to eliminate large suspended particles, and 1.0 L filtrate was later filtered by polycarbonate membranes of 0.8, and 0.22-μm pore size (47 mm diameter, Millipore, Corcaigh, Ireland), respectively. Total bacterial DNA was extracted from the samples using a QIAamp DNA stool mini kit according to the manufacturer's protocol. Then, V3-V4 region of the bacterial 16 S rDNA gene was amplified and sequenced to assess the microbial diversity of the samples. PCR was carried out in triplicate for each sample of a total reaction volume of 20 μL. Using the pair-end method of an Illumina MiSeq PE250 sequencer, the amplicons were sequenced. The raw 250 bps end sequence reads were pooled by FLASH v1.2.11. Using SEARCH GLOBAL, 16 S rDNA operational taxonomic units (OTUs) were selected from the pooled reads clustered with 97% identity. Then, using Ribosomal Database Project Classifier v.2.2 trained with the Green genes database, OTU representative sequences were taxonomically classified.

2.8. Gene expression analysis by qRT-PCR

Tissue samples (hepatopancreas) was collected randomly from the intermoult stages of shrimps (n = 6) in the CN ratio treatment and control groups and RNA was extracted, pooled and kept in −20 °C immediately for further use. The quantitative expression of different potential immune genes (Table 3) in pooled tissues was determined by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, USA). The cDNA synthesis was performed using Verso cDNA Synthesis Kit (Thermofisher, USA). The primer sequences for immune genes expression analysis are given in Table 3. The Real-Time PCR (Applied Biosystem's Real-Time PCR system Step One Plus®) was used for amplification, melting 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) and 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 PCR grade water. All the samples were analyzed in triplicate, and the relative expression was calculated by the comparative threshold value (CT) and ($2^{-\Delta\Delta CT}$ method) [28]. Beta-actin was used as a house keeping gene.

2.9. Statistical analysis

The survival, length-weight of the juvenile shrimps, water quality, and immunomodulatory parameters were compared by univariate ANOVA to find out any significant difference between the treatments by Duncan's multiple range test (DMRT). Similarly, keeping time as a group, univariate ANOVA was performed for above parameters using DMRT to know the significant difference between treatments. Statistical analysis was done using SPSS software package 17. The differences were considered to be significant for $P < 0.05$.

3. Results

3.1. Water quality parameters

No significant difference in salinity and temperature were found in C:N ratio treatments and control groups. pH ranges significantly decreased in C:N ratio treatments when compared to that of control. Similarly, TDS and TSS values were significantly increased with increasing C:N ratio groups such as CN ratio 20:1 followed by other CN treatments than control.(Table 2). However, TAN, NO_2-N and NO_3-N

Table 2
Mean values of physico-chemical parameters of water samples from varying C:N treatments.

Water quality parameters	Control	CN 5:1	CN 10:1	CN 15:1	CN 20:1	P Value
Salinity (ppt)	$30.15^{a} \pm 4.74$	$30.10^{a} \pm 3.25$	$30.15^{a} \pm 3.32$	$30.32^{a} \pm 5.48$	$30.77^{a} \pm 4.57$	NS
Temperature (°C)	$28.45^{a} \pm 5.58$	$28.90^{a} \pm 2.12$	$28.80^{a} \pm 1.41$	$28.45^{a} \pm 2.76$	$28.54^{a} \pm 2.83$	NS
pH	$8.58^{a} \pm 1.64$	$8.15^{ab} \pm 1.65$	$8.15^{b} \pm 1.61$	$8.07^{b} \pm 1.65$	$7.87^{c} \pm 1.13$	0.001*
EC (mS)	$26.90^{a} \pm 2.40$	$31.01^{b} \pm 1.97$	$33.03^{b} \pm 2.08$	$38.45^{c} \pm 2.90$	$41.65^{c} \pm 5.87$	0.001**
Turbidity (NTU)	$10.44^{a} \pm 1.15$	$12.27^{a} \pm 3.24$	$18.56^{b} \pm 2.80$	$23.21^{c} \pm 3.75$	$26.47^{d} \pm 3.13$	0.001**
TDS (ppm)	$8.41^{a} \pm 1.20$	$12.85^{b} \pm 2.90$	$15.25^{b} \pm 2.33$	$20.60^{c} \pm 3.11$	$22.38^{c} \pm 2.57$	0.001**
TSS (ppm)	$60.58^{a} \pm 16.94$	$123.53^{b} \pm 35.51$	$152.15^{b} \pm 37.26$	$317.05^{c} \pm 54.52$	$328.55^{c} \pm 36.84$	0.001**
TAN (ppm)	$1.45^{d} \pm 0.11$	$0.86^{c} \pm 0.06$	$0.60^{b} \pm 0.07$	$0.55^{ab} \pm 0.01$	$0.46^{a} \pm 0.01$	0.001**
NO_2 (ppm)	$0.75^{d} \pm 0.14$	$0.39^{c} \pm 0.13$	$0.24^{b} \pm 0.12$	$0.21^{ab} \pm 0.08$	$0.14^{a} \pm 0.09$	0.001**
NO_3 (ppm)	$0.67^{c} \pm 0.15$	$0.22^{b} \pm 0.04$	$0.21^{b} \pm 0.04$	$0.11^{a} \pm 0.02$	$0.10^{a} \pm 0.02$	0.001**
PO_4 (ppm)	$0.43^{a} \pm 0.12$	$0.52^{a} \pm 0.10$	$0.69^{b} \pm 0.09$	$0.80^{b} \pm 0.03$	$0.98^{c} \pm 0.32$	0.001**
DO (ppm)	$6.94^{a} \pm 1.05$	$5.95^{ab} \pm 2.19$	$5.00^{b} \pm 1.13$	$4.85^{bc} \pm 1.42$	$4.67^{d} \pm 0.81$	0.002*
COD (ppm)	$32.03^{a} \pm 13.39$	$54.65^{b} \pm 8.70$	$59.65^{b} \pm 7.85$	$58.25^{b} \pm 10.15$	$58.55^{b} \pm 6.24$	0.001**
Total Alkalinity (ppm)	$150.25^{a} \pm 25.10$	$140.25^{a} \pm 39.24$	$135.80^{a} \pm 28.57$	$130.22^{a} \pm 22.55$	$124.10^{a} \pm 10.35$	NS
Chlorophyll^{a} (mg/m^3)	$32.25^{a} \pm 6.01$	$62.08^{b} \pm 5.26$	$85.35^{c} \pm 7.28$	$121.50^{d} \pm 12.36$	$137.13^{e} \pm 12.10$	0.001**
Biofloc volume (ml/L)	$4.53^{a} \pm 1.82$	$16.03^{b} \pm 5.95$	$18.05^{b} \pm 3.45$	$23.58^{b} \pm 6.54$	$24.84^{b} \pm 4.84$	0.003*
THB (CFU/ml)	$4.55^{a} \pm 1.81$	$8.68^{b} \pm 1.14$	$10.27^{bc} \pm 1.13$	$12.14^{cd} \pm 1.78$	$13.31^{d} \pm 1.23$	0.001**
TVC (CFU/ml)	$16.66^{a} \pm 1.21$	$7.84^{b} \pm 2.12$	$6.08^{bc} \pm 1.08$	$3.04^{c} \pm 1.80$	$3.80^{c} \pm 1.28$	0.001**
V/T ratio	$3.69^{a} \pm 0.59$	$0.90^{b} \pm 0.11$	$0.599^{b} \pm 0.12$	$0.26^{c} \pm 0.05$	$0.29^{c} \pm 0.002$	0.001**

EC = Electrical conductivity, TDS = total dissolved solids, TAN = total ammonical nitrogen, DO = dissolved oxygen, COD = chemical oxygen demand, THB = total heterotrophic bacteria, TVC = total Presumptive vibrio count.The values are means (± SD, N = 15) of three replications and five sampling date for the treatment and control. * significant at 0.05, ** significant at 0.001, ^{a,b,c,d} significant at $P < 0.05$ based on DMR test & NS- Non-significant.

levels were significantly decreased in CN ratio 20:1 (whereas in control (Table 2) higher range was observed. Phosphate level was significantly ($p < 0.05$) increased in C:N ratio 20:1 whereas found lower in control (Table 2). DO level was significantly ($P < 0.05$) different between C:N ratio and control groups. Total alkalinity levels were significantly ($P < 0.05$) decreased in C:N ratio groups whereas control was stable but Chlorophyll^{a} was increased in C:N ratio 20:1 (137.1 ± 12.1 mg/m^3), 15:1 (121.5 ± 12.3 mg/m^3), 10:1 (85.35 ± 7.3 mg/m^3) and 5:1 (62.08 ± 5.3 mg/m^3) compared with control (32.2 ± 6.01 mg/m^3) shrimp rearing water (Table 2). Floc volume was gradually increased by CN ratio levels where as in control group, it was recorded low (Fig. 1).

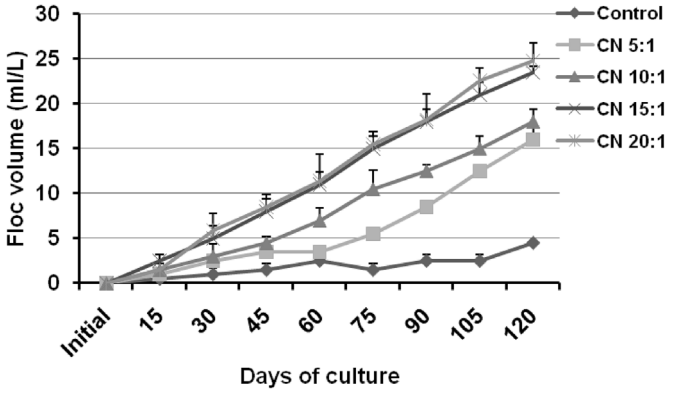

Fig. 1. Bio-floc volume ranges of carbon: nitrogen levels (C:N 5, C:N 10, C:N 15, C:N 20) and control tanks determined by Imhoff cone. Error bar indicates ± standard error.

3.2. Microbial biomass and community structure

Carbohydrate supplementation significantly increased the total heterotrophic bacterial (THB) count in increasing order from C:N5 to C:N20. As the culture proceeds, increasing biomass had a significant effect ($P < 0.01$) over total microbial load with higher level recorded in C:N15 and C:N20. Similarly, carbohydrate supplementation had a significant effect on total *Vibrio* count (TVC) in water ($P < 0.01$). TVC levels were greatly reduced in C:N10, C:N15 and CN20 reared water (91.80 ± 0.3%) whereas in control it was highest followed by C:N5. Carbohydrate supplementation resulted in 71.7 ± 3.3% decrease in TVC in water of BFT groups (Table 2). The proportion of Vibrio count to total heterotrophic bacterial count (V/T) was lower in the biofloc groups in decreasing order as we proceed from C:N5 to C:N20 compared to that of control group. The bacterial diversity and frequency distribution of bacterial phyla differed between treatments. Both in control and C:N5 treated water, the *Vibrio* of 79% and 37% were most dominant operational taxonomic unit respectively. In C:N10, *Thauera* (62%) was most represented taxa. In C:N15, *Attheyaceae* (56%) and *Peridiniaceae* (30%) were the most dominant phyla. In C:N20, the *Psychrobacter* (26%), *Proteobacteria* (25%) and *Peridiniaceae* (20%) phyla were found to be dominated phylum (Fig. 2 A, B, C, D & E).

Table 3
Primers of four selected immune related genes for qRT-PCR in this study.

Gene	Primer sequence (5′-3′)	Accession no	Amplicon size (bps)
RAN	F- CCAAGAGAAATTGGGAGGTCTTC R- GGGAACATTCTTGTACGTGACTCTAG	JX644455.1	93
Serine protease	F- CGTCGTTAGGTTAAGTGCGTTCT R- TTTCAGCGCATTAAGACGTGTT	AY368151	61
PPAE	F- CTGCAAGATCACTCAAGGCC R- TTATTGGGGACGACAGGGAG	JX644454.1	325
Crustin	F-ACGAGGCAACCATGAAGG R-AACCACCACCAACACCTAC	AF430076	141
β-actin	F-CAACCGCGAGAAGATGACAC R-TCGGTCAGGATCTTCATCAGG	GU732815	243

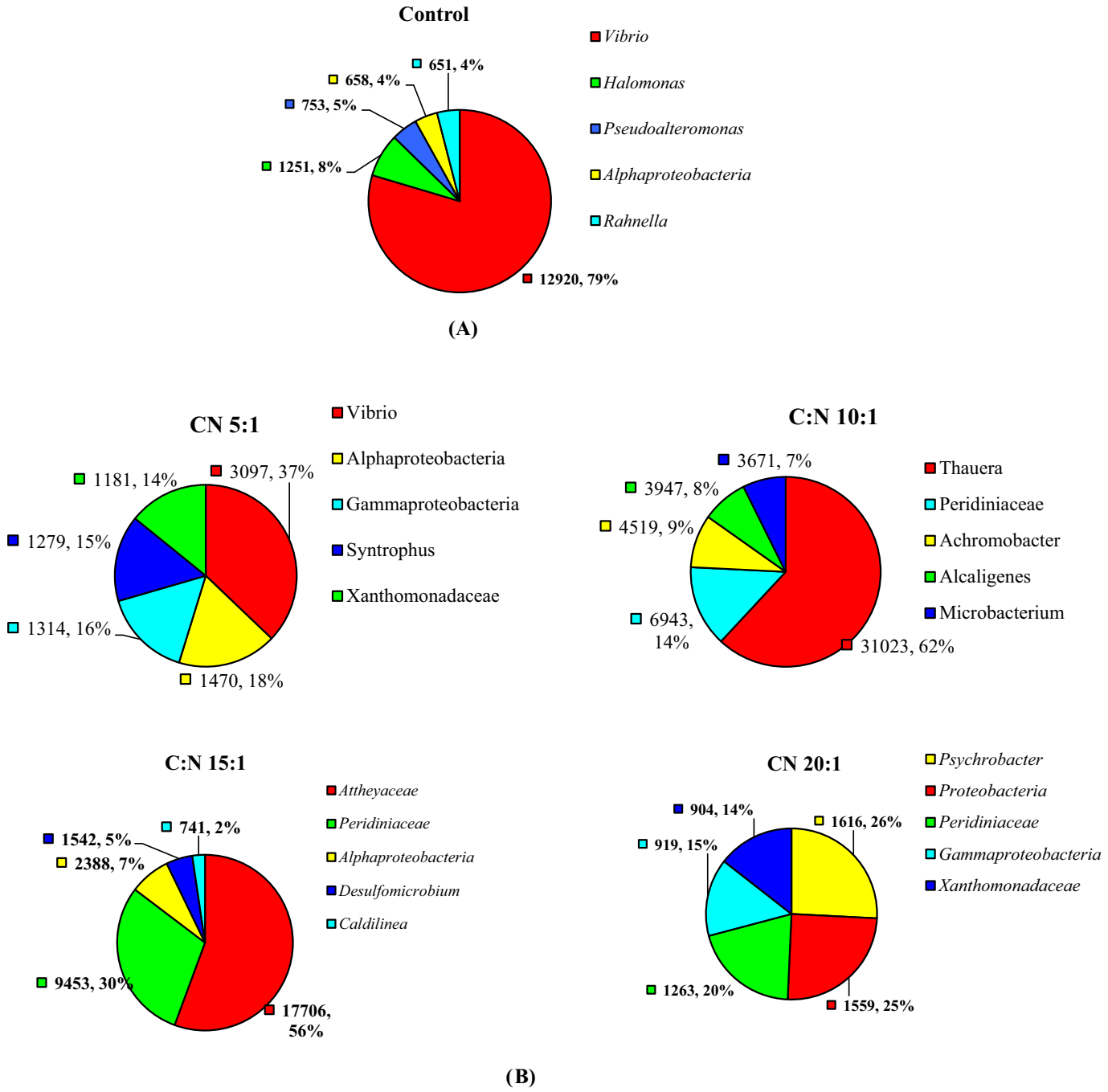

Fig. 2. Relative abundance of the most frequently identified microbial phyla in water from different rearing conditions: clear water and biofloc (BFT) with different CN ratio. 1 A - Control; 1 B - C:N 5; 1C - C:N 10; 1D- C:N 15; 1 E − C:N 20.

3.3. Performance of the shrimp in differentially intense biofloc system

The survival rate of C:N treated tanks were higher than that of control tanks. CHO supplementation showed significantly ($P < 0.05$) higher survival against that of the control group. At the end of culture period survival rate of control and C:N 5:1, C:N 10:1, C:N 15:1 and C:N 20:1 treated tanks were 76.5 ± 2.1, 81.0 ± 1.4, 88.5 ± 0.7, 99.0 ± 1.4 and 96.0 ± 1.4 respectively (Table 4). Survival rate of the experimental tanks showed significant interaction ($P < 0.05$) with that of C:N ratio. Survival rate of the groups with C:N10, C:N15 and C:N20 showed significantly higher survival compared to the C:N5 group. Average daily growth (ADG) (Fig. 3) and average body weight (ABW) (Table 4) was found to be higher in C:N 15 than that of C:N 5, 10, 20 and control.

3.4. Immune-related gene expression analysis by real-time PCR

The mRNA expression of RAN (Ras-related nuclear protein), SP (Serine protease), PPAE (proPhenoloxidase activating enzyme) and crustin is shown in Fig. 4 (a), Fig. 4 (b), Fig. 4 (c) and Fig. 4 (d). The transcript levels of Ran gene in shrimps were upregulated by 2.6 fold in C:N15 treatment and in treatment C:N5, C:N10,C:N20 and it was 1.0, 2.4, 1.8 fold expression, respectively. The enhanced immune regulation in biofloc with C:N15 was higher than other C:N ratio groups. The transcript level of serine proteinase in C:N15 was found to have 1.8 fold up-regulations whereas same in C:N5 was downregulated, C:N10 was

Table 4
Mean values of growth parameter of the experimental groups.

Parameters	Control	CN5	CN10	CN15	CN20	P value
ABW (g)	$11.85^{a} \pm 2.93$	$19.01^{b} \pm 2.74$	$20.77^{b} \pm 3.19$	$24.71^{b} \pm 2.61$	$20.26^{b} \pm 4.52$	0.003**
ADG (g)	$0.098^{a} \pm 0.003$	$0.159^{b} \pm 0.005$	$0.173^{c} \pm 0.007$	$0.208^{d} \pm 0.005$	$0.173^{c} \pm 0.006$	0.001**
SGR	$2.01^{a} \pm 0.04$	$2.40^{bc} \pm 0.05$	$2.49^{c} \pm 0.07$	$2.68^{d} \pm 0.05$	$2.49^{c} \pm 0.06$	0.001**
Survival rate (%)	$76.50^{a} \pm 2.12$	$81.05^{b} \pm 1.41$	$88.55^{c} \pm 0.78$	$99.00^{d} \pm 1.45$	$96.00^{d} \pm 1.48$	0.001**
FCR	$2.32^{a} \pm 0.08$	$1.03^{b} \pm 0.09$	$0.90^{c} \pm 0.03$	$0.81^{d} \pm 0.04$	$0.82^{d} \pm 0.02$	0.001**

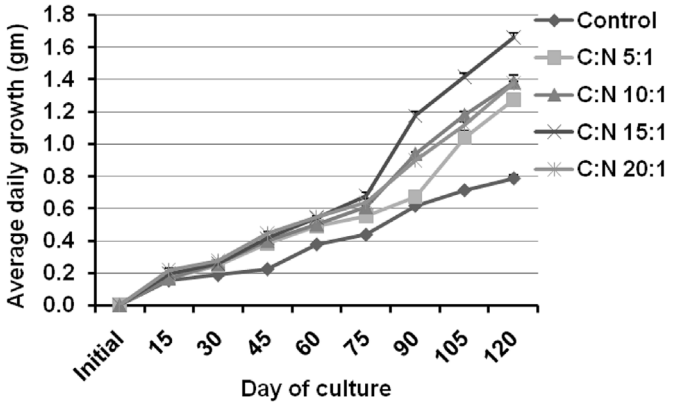

Fig. 3. Average daily growth of shrimp in varying C:N treatments (C:N 5, C:N 10, C:N 15, C:N 20) and control. Data shown as mean with standard deviation as error bars (n = 50).

showing one fold change and C:N20 was showing 2.2 fold upregulation. A similar trend was observed in prophenoloxidase activating an enzyme which was 2.5 fold higher in C:N15 but 7.6 fold in C:N20. Similarly, C:N10 was showing 3.4 fold upregulation compared to 1.6 fold in C:N5. Enhanced immune gene expression suggests the activation of these systems which indirectly control the immunomodulation in shrimps.

4. Discussion

BFT is considered as a modern aquaculture farming system for many of the recently cultured species in benefitting environment, effluent discharges, biosecurity, feed management, production intensification and thus the overall economy. The biofloc serves as high nutrients rich food source throughout the day and thus reduces external feed supply and thereby reducing substantial cost. Biofloc is rich in amino acids, native proteins, fatty acids and lipids in the form of different micro-organisms.

The diverse microbial community of bioflocs not only provides supplemental nutrition, but also acts as nutrient recyclers, and as a food source for different microbial diversity from higher trophic levels in

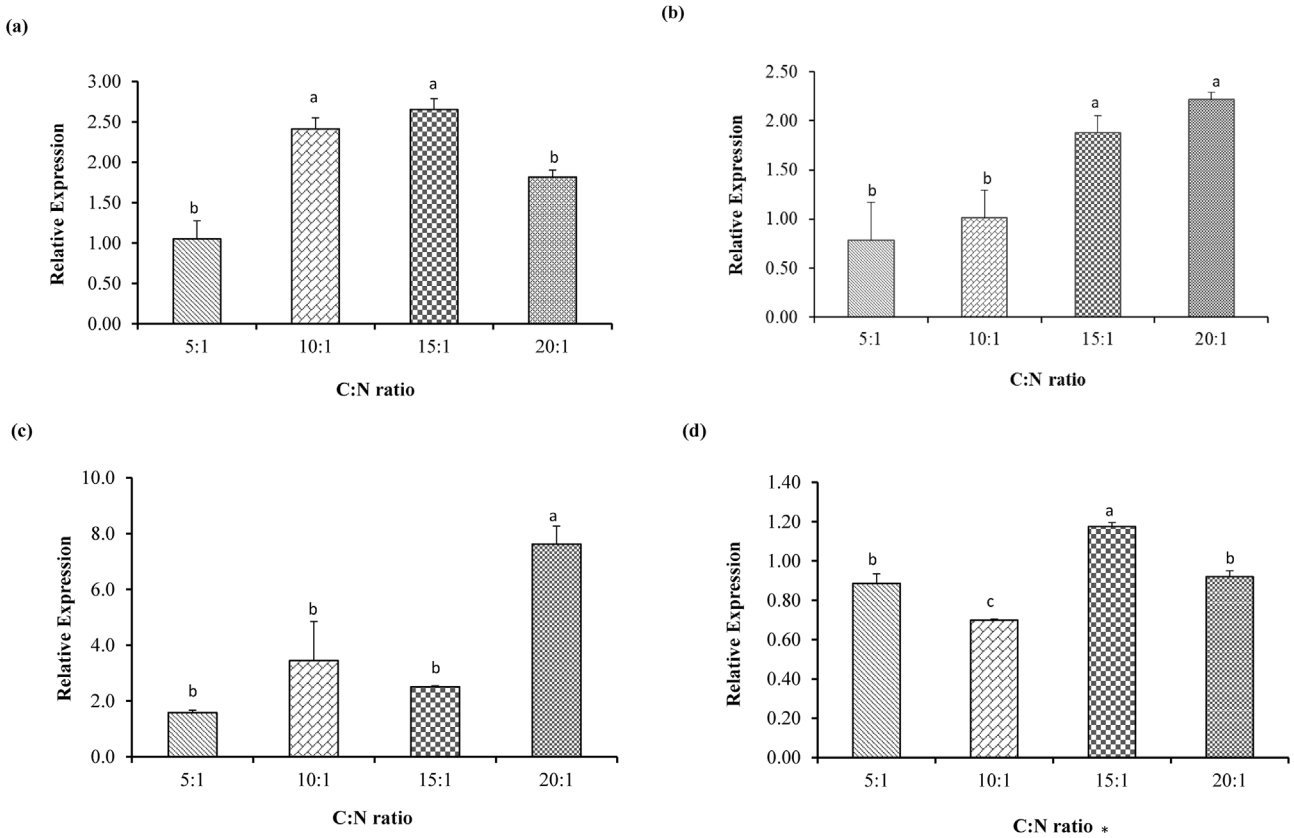

Fig. 4. Comparative mRNA expression levels of (a)ras-related nuclear gene (Ran) gene, (b) serine proteinase gene (SP) gene, (c) prophenoloxidase activating enzyme (PPAE) gene and (d) Crustin gene in *L. vannamei* reared in biofloc system supplemented with varying concentrations of C:N ratios in comparison to that of the control as determined by real time PCR. Five individual shrimps were analyzed from the control and each of the C:N treatment groups. Data are means ± SD of gene expression in the different C:N treatments. Significant differences between different C:N groups are marked with letters (a, b, and c) ($P < 0.05$).

aquaculture [5,29]. Several studies reported that composition, structure, and stability of bio-flocs could be influenced by diverse types of organic carbon sources (molasses, corn, wheat, glucose acetate, glycerol, and tapioca) and their ratio. Different carbon ratio also makes the differences in the bioflocs nutrients composition such as carbohydrate, protein, lipid and fatty acid [9,12,17].

Sound knowledge of the microbial composition, structure, and stability of a biofloc and its nutritional value will give sound information to understand environment-microbiota-host relationship, optimize the bacterial community composition for optimal water quality, growth performance, and immune modulatory potential in rearing system. The aim of the present investigation was to evaluate the effects of four different ratios of molasses (C:N5; 10; 15; 20) as a carbon source on microbial community, growth and immune status in *L. vannamei* culture with minimal water exchange.

4.1. Effect on water quality parameters

Water quality management is playing a crucial role in any aquaculture endeavor. It is strongly influenced by stocking density of the cultured animal, environmental parameters, species combination, quality and quantity of nutritional input added to the system. In the current study, there was an increase in TDS and TSS values among the treatments. This may be attributed to continuing input of feed and organic carbon supplementation [7]. Further, a reduction of TAN, NO_2-N, and NO_3-N was noted in all different CN BFT treatments than those of the control. Nutrient cycling or microbial assimilation by certain microbes present in the bio-floc which has potential to assimilate TAN into microbial biomass might have been reason for this. PO_4^- level was also reported to be at a higher level in all the CN treatments than that of the control. This increase in phosphate level could be due to dominant heterotrophic bacteria because of BFT system and fewer microalgae and in addition to algal die-offs. This study indicates that biofloc culture system to have good microbial environment through floc formation, where diatom, nitrogen and sulfur cycle bacteria work together in a culture environment instead of a particular group of bacteria, these can control water quality very effectively.

4.2. Microbial community characterization and dynamics

Microbial communities inhabiting on soils and water are some of most complex known science. The microbes influencing aquaculture productivity are also poorly understood despite their economic importance. Microbial consortia perform a wide variety of ecosystem services necessary for aquatic plant and animal growth, including bioremediation, nutrient cycling, disease suppression, and sequester iron and other metals. Functional metagenomics strategies are being used to explore interactions between plants and microbes through the cultivation-independent study of these microbial communities [30].

Bacterial diversity and frequency distribution of bacterial phyla differed between the treatments. In both control and C:N 5 treated water, Vibrio sp., (79% and 37% respectively) were most dominant operational taxonomic unit. In C:N5 treated water, *Alphaproteobacteria* (18%), *Gammaproteobacteria* (16%), *Syntrophus* (15%) were other operational taxonomic units apart from *Vibrio* species. In C:N10, *Thauera* (62%) was most represented bacteria. In C:N15, *Attheyaceae* (56%) and *Peridiniaceae* (30%) were most dominant ones. In C:N20, *Psychrobacter* (26%), *Proteobacteria* (25%) and *Peridiniaceae* (20%) phyla were found to be dominated phylum.

Xu et al. [7] reported that addition of molasses in the bio-floc treatment was aimed at increasing C:N ratio and promoting the development of heterotrophic bacteria in culture tank water. In heterotrophic biofloc based shrimp culture systems, the driving force is dense populations of active heterotrophic bacteria which can be promoted by increasing the C:N ratio of feed input and assimilate waste nitrogen from culture water resulting in the production of new microbial biomass (cellular proteins) [29,31,32]. Bacterial communities particularly use organic matter and nitrogen compounds for growth and require support to grow on. These two conditions are very much prevalent in a BFT system, particularly are rich in organic matter and suspended particles in water column. This ability to attach the surfaces and to use organic matter may be a significant physiological characteristic of bacteria in biofloc [33].

Proteobacteria such as *Alphaproteobacteria, Gammaproteobacteria, Syntrophus* were observed to be most abundant phylum in C:N5 treatment and its relative abundance ranged between 15 and 18%. About 25% of microbial diversity in C:N20 treatment was also found to be dominated by *proteobacteria.* This phylum is widely dispersed in marine environment and plays an important role in the process of nutrient cycling and mineralization of organic compounds [33]. Vibrio species were represented 79% and 37% both in control and C:N5 groups respectively than other C:N treatments. This could be due to less abundance of total heterotrophic bacteria in control and C:N5 either due to the complete absence or partial presence of biofloc system. Vibriosis belongs to *Vibrionaceae* family, has been recognized as pathogens for aquatic organisms such as crustacean larvae and juveniles [33,34]. Because of comparatively low level or relative absence of *Vibrio* in C:N10, 15 and 20 which was possibly due to direct biocontrol activity of biofloc through mechanisms such as quorum quenching, it is predicted that shrimps could remain healthy throughout the culture period. Stressed shrimp can be more susceptible to facultative pathogenic microorganisms that are part of their natural microbial flora and aquatic environment [35], [36]. Mohney et al. [37] reported that *Vibrio* sp. detected in control group was higher in penaeid shrimp culture than that of probiotic treated groups. However, some of *Vibrio* species were potential causative agents for diseases in aquaculture systems [38]. Balcázar [39] demonstrated that addition of a mixture of bacterial strains (*Bacillus* and *Vibrio* sp.) positively influenced the growth and survival of juveniles of white shrimp and presented a protective effect against pathogen *Vibrio harveyi*. Our studies confirmed that significantly reduced Vibrio levels in C:N ratio maintained water than control.

4.3. Growth performance

Result of the current study revealed that CN15 had considerable influence on growth performance in comparison with other CN treatments and control. Similar advantage in bio-floc based grow-out systems has also been reported by many studies. As 20–30% of shrimp feeding is taken care by floc particles, there is a potential gain in FCR. Growth might be enhanced by continuous consumption of “native protein”, including growth factor [2,14]. Selective breeding program for Pacific white shrimp, *L. vannamei*, requiring a grow-out evaluation of selected families and involving super-intensive shrimp culture with bio-floc has been conducted at Oceanic Institute in Waimanalo, Hawaii, USA, since 1997. These trials are conducted in a 75-m^3 super-intensive BFT raceway stocked at 300–400 shrimp/m^3 in Oceanic Institute's Nucleus Breeding Center. In one of our experimental microbial floc system, shrimp fed with a feed with less than 24% crude protein performed similarly to shrimp raised under regular intensive culture with a 38% protein diet. Bio-floc system also delivered more consistent survival rates, especially at higher density [2,14,40,41].

Growth trail of *P. monodon* was conducted under bacterial and periphytic algae floc's to investigate the effect of integration of substrate in carbon-nitrogen ratio manipulated systems. A 42% increase in final body weight was recorded in substrate integrated floc system through provision of natural food in the form of bacterial floc and periphytic algae [2,42].

4.4. Immune-related gene expression by RT-PCR

CN ratio manipulation has been done to maintain an optimal growth environment for microbial community in biofloc system. It is assumed

that microbes in the system naturally enhance the immunity of cultured shrimps. The shrimps respond to pathogen in minimal level in the biofloc system as the beneficial microbes population seems to be high. Microbial cell wall components have substances that have potential to activate shrimp immune system [10,11,41,43–46]. Microbial community of the host intestine proved to be playing a very important role in developing host immunityeither through forming a physical barrier defense mechanism against pathogens invasion or by inhabiting an ecological niche [47–49]. Ferreira et al. [50] reported that probiotic's such as *Bacillus cereus* and *Bacillus licheniformis* were isolated from the bioflocs in an intensive system and they increased Total hemocyte count THC while *L. vannamei* were fed with a feed containing *Bacillus* sp. The abundant level of THC can give more protection for crustacean species against pathogenic infection which could be because of hemocytes play a major role in various immune cell reaction and activation [51]. The diet containing beneficial bacteria like lactic acid bacteria has also been showed to reduce adherence and colonization of pathogenic bacteria and to improve fish health [52,53] and found to have an immunomodulatory role [54,55]. The expression of potential immune genes including prophenoloxidase (proPO1 and proPO2), serine protease (SP1), prophenoloxidase activating enzyme (PPAE1), masquerade as serine protease (MAS) and Rat-sarcoma-related nuclear protein necessitate directly or indirectly in the activation of shrimp immune response and reported to have significant upregulation in biofloc-grown shrimp [56]. Immune stimulation could be playing a major role in biofloc-grown shrimp to control diseases. Previous report said that lower prevalence of acute hepatopancreatic necrosis disease (AHPND) found on farms that maintained under BFT [57]. AHPND is currently causing very large problems in the culture of shrimp postlarvae in Asia [58].

In our present study, some of the potential immune genes were taken into consideration to study their expression pattern. RAN (RAs-related Nuclear protein) gene is also involved in antiviral activity. It will interact with light chain of myosin to form a protein complex to eliminate pathogen by phagocytosis activity [59]. There was an enhanced activity of RAN gene as RAN transcript was significantly upregulated in C:N15 and similar results were observed by Ref. [60]. Wu et al. [61], reported that mas and serine proteinase homologs (SPHs) are entailed in proPO cascade pathway activation in invertebrates, whereas, the RAN gene was said to be involved in the antiviral defense of *Marsupenaeus japonicus* [59]. Previously [62,63], identified a cDNA fragment which is highly homologous to Ran proteins from WSSV resistant shrimp. Exposure to bio-flocs stimulates non-specific immune system in shrimp. Constituents of bacterial cell walls in biofloc components activate a cascade of reactions leading to the production of prophenoloxidase system and other biochemical pathways.

Activation of proPO cascade requires proteolytic activity that can be activated by serine proteinase. PPAEs transcript expression level was up-regulated after *Vibrio harveyi* infection [64]. Similar results were observed in the present study. Ekasari et al. [10] reported that C:N15 was optimal regarding biofloc generation and its impact in the study conducted by the group.

Pattern recognition receptors (PRRs) binds to pathogen cell wall components including LPS, PG and β-1, 3-glucans to trigger a series of responses activating the host defense system [65]. This continuous immune response is known as the prophenoloxidase (proPO) activating system, which is one of the most important innate immune responses in invertebrates [66]. In case of injury or infection, non-self-molecules, such as LPS, PG and β-1, 3-glucan, recognized by PRRs, leads to activation of the proPO cascade [67]. The proPO cascade pathway entails several proteolytic processes that are catalyzed by multiple clip domain-SPs. Serine proteinase (SP) activate the inactive proPO into its active form is known as prophenoloxidase activating enzyme (PPAE). This reaction has been characterized in most of the insects and crustacean [68–70]. Shrimp β-glucan binding protein (BGBP) seems as a constitutive plasma protein that after binding to β-glucan reacts with hemocytes surface and stimulates the release of hemocytic granules. The contents exist in the granules get activated in the presence of plasma Ca^{2+} to activate the proPO1 and proPO2 [71,72]. PPAE can activate proPO system directly and is also a key member of the proPO activating system [66]. Crustin is one of the most important antimicrobial peptides found in crustaceans. The transcript s of crustin gene was substantially elevated in C:N15 when compared to other treatments which are consistent with the study of [73]. Finding of this experiment goes very well with studies mentioned about growth performance as well as immunomodulation.

5. Conclusion

BFT is a recent aquaculture farming technique in aquaculture regarding as a direct food source, immunity booster, consequent detoxification of the system for cultured organisms and others. The results of the present study demonstrated that manipulating C:N ratio had a significant impact on development and characteristics of biofloc system, water quality parameters, growth performance, immune system and environment-microbiota-host relationship. Present investigation provides the first information relating the complex biofloc microbial community structure, growth performance, and immune functions in Pacific whit shrimp *L. vannamei* in response to different BFT C:N ratio. It could give strong insights to understand the environment-microbiota-host relationship, optimize the bacterial community composition for both optimal water quality, growth performance, and immune modulatory potential in the rearing system.

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