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Effect of carbon and nitrogen ratio (C:N) manipulation on the production performance and immunity of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) in a biofloc-based rearing system

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Abstract

A 16-week indoor culture trial was conducted to evaluate the effect of varying C:N ratio on growth performance, physico-chemical parameters, microbial dynamics, feed utilization, and immunological parameters. The experiment comprised of five biofloc treatment groups (with varying C:N ratio 5:1, 10:1, 15:1, 20:1) and a control with three replicates each, having 100 nos/m^3 as stocking density in 500 L tanks with constant aeration. The C:N ratios of the treatments were manipulated using molasses as an organic carbon source whereas there was no carbon source added in control. The water quality parameters monitored throughout the experiment were found to be within permissible limits in shrimp culture. At the end of the experiment, it was observed that there were significant differences between the treatment groups and the control regarding absolute growth, SGR, FCR, PER, and FER. Furthermore, a considerable difference in immunological parameters, namely, THC, phagocytosis, and PO activity (17.5×10^6 cells per ml, 43.5%, 0.112 Units min^{-1} mg min^{-1}), was recorded among the treatments compared to that of the control groups (6.2×10^6 cells per ml, 31.5%, 0.051 Units min^{-1} mg min^{-1}) respectively. Enhanced growth and survival with substantial disease resistance were recorded in C15 treatment. The results indicate that the CN15 ratio coupled with minimal water exchange is optimal for improved survival, growth, and immune activity.

KEYWORDS
C:N ratio, molasses, water quality, zero-water exchange

1 | INTRODUCTION

Availability of specific pathogen-free shrimp has resulted in increased shrimp farming in terms of culture as well as production. The choice of *Litopenaeus vannamei* over *Penaeus monodon* is primarily due to enhanced production, SPF availability, higher yield after processing, and higher market demand. Of late, biofloc technology (BFT) has gained momentum and positive response in shrimp and tilapia farming. In India, farmed shrimp production increased from <1 lakh tonnes in 2009 to 3.5 lakh tonnes in 2014. In 2016–2017, the production was over 5 lakh tonnes, accounting for 38% in quantity and 64.5% in value (Rs. 24,426 crores) of the total Indian seafood export worth 5.78 billion dollars (Rs. 37,870 crores) (MPEDA, 2017). The expanding culture system influences water quality and environmental factors. Due to intensification with higher stocking densities, there is

more use of water, feed, and fertilizers which leads to increased production of waste and disease (Beveridge, Phillips, & Macintosh, 1997; Moss, Moss, Arce, Lightner, & Lotz, 2012; Otta et al., 2014; Robledo, Navarro-Angulo, Lozano, & Freile-Pelegrín, 2012). Several studies in the past have highlighted the low-cost eco-based technology and its improvement strategies (Avnimelech, 1999, 2007 ; Hari, Madhusoodan, Johny, Schrama, & Verdegem, 2004; Panigrahi et al., 2018; Panigrahi, Sundaram, Ravichandran, & Gopal, 2014), which seem to be a promising option for farmers adopting sustainable shrimp culture. The Pacific white shrimp, *Litopenaeus vannamei*, is one of the most preferred species worldwide due to its fast growth, high survival, and is the most compatible shrimp for the biofloc system (Ballester et al., 2010; Panigrahi et al., 2018; Xu & Pan, 2012). Sustainable shrimp production with greater emphasis on environment and high disease resistance is the need of the hour. However, intensive shrimp culture with improper management leads to eutrophication in the receiving water bodies, due to the overrichness of nutrients in the drained water which promotes diseases and accumulation of nitrogen metabolites (Robledo et al., 2012).

Biofloc is naturally dynamic, artificially cultivated, natural food agglomerate composed of bacteria, algae, protozoa, rotifers, nematodes, hypotrichs, dead organisms, uneaten feed, and shell moults. These microbial communities are formed by adding organic carbon to a water body, with the C:N ratio maintained at a stable level (Haslun, Correia, Strychar, Morris, & Samocha, 2012; Jorand et al., 1995; Schryver, Crab, Defoirdt, Boon, & Verstraete, 2008). One of the primary requisites of biofloc formation is to maintain the carbon and nitrogen ratio in the culture system. For the development of biofloc, the C:N ratio should be within the range from 10:1 to 20:1 (Abreu, 2007; Asaduzzaman et al., 2010; Avnimelech, 1999; Ballester et al., 2010; Emerenciano, Ballester, Cavalli, & Wasielesky, 2012). The C:N ratio manipulation can improve the water quality by utilizing the nitrogen and regenerate new bacterial cells thereby reducing the waste effluent from the culture system.

Aquaculture production often gets crippled due to deterioration of water quality, particularly due to nitrogen metabolites from unutilized feed and the waste metabolite of the animal. By adding a source of carbon, this problem is addressed. Inclusion of carbon supports the formation of biofloc that results in the improvement of water quality (Avnimelech, 2007; Crab, Avnimelech, Defoirdt, Bossier, & Verstraete, 2007; Hargreaves, 2006; Hari et al., 2004; McIntosh, 2000) and increase in dissolved oxygen in the system (Lananan et al., 2014; Lananan, Jusoh, Ali, Lam, & Endut, 2013). Biofloc is corsortium of heterotrophic microorganism which helps in maintaining water quality by reducing ammonia and other nitrogenous metabolites from the system. Earlier studies suggest that optimum C:N ratio can also help in increasing the nitrogen retention from feed by 7%–13% (Hari et al., 2004; Schneider, Sereti, Eding, & Verreth, 2005). In turn, the microbial floc so developed can also act as supplementary food to the culture species (Avnimelech, 1999; Browdy, Bratvold, Stokes, & McIntosh, 2001; Burford & Lorenzen, 2004; Moss, Pruder, & Samocha, 1999; Xu & Pan, 2013).

Highlights

- Growth, physico-chemical, and microbiological parameters were substantially higher in carbon and nitrogen (CN) ratio treatments compared to control.
- Optimization of C:N ratio in *L. vannamei* culture revealed an optimum ratio of 15 to be ideal for a biofloc-based system.
- Challenge study revealed higher mortality in control compared to CN-treated groups when challenged with the pathogen *Vibrio parahaemolyticus* (MTCC 451).
- Carbon supplementation appears to influence heterotrophic bacteria and provides immunity and protective response under BFT-based rearing.
- Immune responses like THC, phagocytic activity, and proPhenoloxidase activity were higher in treatments compared to control. The elevated immune response in CN15 indicates enhanced immune regulatory function.

Manipulation of C:N ratio helps in improving the immune activity as well as the antioxidant capacity of shrimp, both of which enhance the resistance against pathogens (Panigrahi et al., 2017; Vazquez et al., 2009). Biofloc contains rich bioactive compounds which increase the tolerance to stress and helps in activating the antioxidant activity in shrimp (Babin, Biard, & Moret, 2010). Furthermore, earlier studies have shown that biofloc-reared shrimps exhibit a higher proPhenoloxidase activity, phagocytic activity, and total haemocyte count (THC) compared to nonbiofloc control group (Ekasari et al., 2014; Kumar et al., 2015; Xu & Pan, 2013). The total haemocyte count in shrimp and natural immunostimulant in the biofloc system enhances protection against the pathogen (Smith, Brown, & Hauton, 2003). Recent studies have revealed that biofloc reduces the occurrence of acute hepatopancreatic necrosis disease (AHPND), also called as EMS (NACA, 2012). Experiments carried out at ICAR-Central Institute of Brackishwater Aquaculture (CIBA) revealed that constituents of bacterial cell walls in biofloc contain components that activate a cascade of reactions leading to the production of proPhenoloxidase and several other biochemical pathways (Panigrahi et al., 2017, 2018). Biofloc comprises beneficial bacteria that alter the biological and immunological status of shrimp by colonizing microbiota in the gut (Zhao et al., 2012).

The biofloc system can be a more cost-effective by fixing effective C:N ratio for sustainable shrimp production. As this system diminishes, the toxic nitrogenous metabolites through in situ bioremediation, it is a key tool for eco-friendly culture practices with zero-water exchange approach. This study evaluated different ratios of carbon and nitrogen levels on the physico-chemical parameters, growth, floc volume, and microbial dynamics along with growth and immune responses of the shrimp in the grow-out rearing of *L. vannamei* in a biofloc environment.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The experiment was carried out at the Muttukkadu Experimental Station (MES) of CIBA, about 35 km away from Chennai. *L. vannamei* was cultured in FRP tanks with minimal water exchange @ 10% of total volume, on a weekly basis. The protocol involved a conventional system without biofloc and a biofloc system with different C:N ratios. The combinations were control (C), C:N ratio 5:1 (CN5), C:N ratio 10:1 (CN10), C:N ratio 15:1 (CN15), and C:N ratio 20:1(CN20). The control tank was maintained autotrophically without the addition of any carbon source. Feed containing 35% crude protein with no additional carbon source was provided to the control. In the treatment groups, molasses was added as a carbon source to raise the C:N ratio to 5, 10, 15, and 20 to promote biofloc development. The experiment was run in triplicate in 500 L FRP circular tanks located in the indoor facility with diffused sunlight for a culture period of 120 days. Sea water was used during the entire experimental period. Initially, sea water was filled in FRP tanks and treated with chlorine @ 30 ppm. The next day, agricultural lime was applied to all the tanks @ 10 g/m^{-3}, after which the remaining ingredients such as urea, dolomite, and triple superphosphate (TSP) @ 10 g, 10 g, and 10 g/m^{-3}, respectively, were added to aid fertilization and generation of autotrophs. The biofloc inoculum was prepared by fermenting molasses (80 ml) and *Bacillus subtilis* (MTCC 2756) and *Saccharomyces cerevisiae* (IAM 14383 T) (40 ml: 10^8 cells per ml) in 4 litres of autoclaved seawater. The fermentation lasted 24 hrs. The biofloc inoculum was prepared and added to all the treatment tanks @ 1,000 ml/m^{-3}, whereas the control was maintained in an autotrophic manner, devoid of biofloc inoculum. Continuous aeration was provided to all the tanks. After generation of biofloc, 50 numbers of *L. vannamei* juveniles (avg. wt. 1.0 g) @ 100 per m^3 were stocked in all the treatment and control tanks.

An experimental diet prepared at the feed mill of CIBA was used; the details of which are listed in Table 1. The ingredients were powdered using a two-stage hammer mill, multipulverized, and thereafter sieved through a 300-micron mesh screen. All the ingredients including liquids were thoroughly mixed in a batch mixer. The mixture was then pelleted in a Ring-Die pellet mill with 16% moisture, at 95°C under steam as described by Panigrahi et al., 2017. Pellet feed of varying sizes as per shrimp's body size, namely, juveniles: 800–1,000 micron (crumble 2), subadults: 1.2–1.5 mm, and adults: 2.2 mm was prepared. The feed was provided manually till apparent satiation three times daily at 06:00, 14:00, and 22:00 hrs. The daily feeding rate was gradually reduced from approximately 5% of total body weight and biomass to 1.5% by the end of the experiment, and it was moderated daily according to feed intake, by hand net, to make sure that the diets were entirely consumed. Daily diet inputs were recorded in all the treatments (Hargreaves, 2006; McIntosh, 2001; Xu & Pan, 2012) and molasses was applied on a daily basis at 09.00 hrs in the morning.

TABLE 1 Ingredients and proximate composition (g/100 g) of experimental diet formulated with 35% crude protein for culture of *Litopenaeus vannamei*

Composition	35% CP
Fish meal[a]	23.6
Acetes[a]	11.8
Soybean meal[a]	17.7
Gingelly oil cake[a]	5.9
Wheat[b]	13.92
Broken rice[b]	6.96
Maida[b]	13.92
Fish oil	2.2
Lecithin	1
Vitamin and Mineral Mix[c]	2
Binder[d]	1

[a]Protein base: Fish meal: *Acetes* sp.: Soya cake: Gingelly oil cake in the ratio of 4:2:3:1.
[b]Carbohydrate base: Wheat: Broken rice: Maida in the ratio of 4:2:3.
[c]Vitamins (mg/kg): 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): $CaCO_3$ 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.
[d]Poly MethylolCarbamide.

2.2 | Manipulation and maintenance of C:N ratio

The C:N ratio was calculated considering the feed protein/nitrogen and input carbon used in shrimp culture (Avnimelech, 1999; Avnimelech, Diab, & Kochva, 1992; Avnimelech, Kochva, & Diab, 1994; Avnimelech, Mokady, & Schroeder, 1989; De Schryver et al., 2008; Kochva, Diab, & Avnimelech, 1994). The carbon and nitrogen contents were calculated by the assumption of feed protein (35% protein feed) and the addition of various levels of carbon. Addition of molasses to one gram of feed depends on the assimilation and assumption of nitrogen in the system. In this study, molasses was used @ 0.32, 0.64, 0.96, and 1.28 g for CN 5, CN 10, CN 15, and CN 20, respectively, for one gram of feed (Anand et al., 2013; Avnimelech, 1999; Panjaitan, 2010). Water exchange (@10%) and removal of faecal matter were carried out in the CN treatment tanks once a week considering the evaporation loss. In the control tank, water was exchanged @ 50% twice a week, without the addition of carbon source.

2.3 | Growth performance

The average body weight (ABW), survival, SGR, and nutritional parameters (FCR, PER, FER) of the shrimps were determined fortnightly until the completion of culture. The total body weight (W) was recorded from each experimental container along with the number of live shrimps (N). The amount of feed used in each tank (W_f)

was recorded. The ABW was computed from *W* and *N*. The overall average values of survival (%), the growth rate of shrimp (gm/day), percentage weight gain, and feed conversion ratios (FCR) were computed as follows (Panigrahi et al., 2017).

$$SR\,\% = N_t/N_0 \times 100\%$$

where SR = the survival (%), N_t = the number of shrimp that survived until the end of the experiment, N_0 = the number of animals that were available at the beginning of the experiment.

$$SGR = (\ln \text{final weight} - \ln \text{initial weight})/\text{day of culture} \times 100$$

$$FCR = \text{Total feed used}/(\text{Dry weight})/\text{Total weight of the harvested shrimps (wet weight)}$$

$$PER = \text{Net weight gain (g)}/\text{Protein applied in feed (g)}$$

$$FER = \text{Weight gain}/\text{feed intake}$$

2.4 | Assessment of water quality parameters

Physico-chemical parameters, namely, temperature (using a thermometer), salinity (hand refractometer, Otago, Japan), pH (Eutech, Singapore), and electrical conductivity (Eutech Singapore) were recorded daily. Total dissolved solids (TDS), total suspended solids (TSS) dissolved oxygen, ammonia (TAN), nitrite (NO_2-N), nitrate (NO_3-N), turbidity, chemical oxygen demand, phosphate, total alkalinity, and chlorophyll a were measured following APHA (1998). The biofloc volume was determined from a litre of water in each experimental tank using Imhoff cones (Avnimelech & Kochva, 2009).

2.5 | Assessment of microbial load

The total heterotrophic bacteria were determined by counting the colonies which grew on Zobell Marine Agar (ZMA) plates with 1.0% NaCl (Jorgensen, Mørk, Høgåsen, & Rørvik, 2005). Before plating each sample onto agar medium, serial dilutions were made in a physiological saline solution composed of 0.9% NaCl (Sohier & Bianchi, 1985). The total *Vibrio* in water samples was counted using TCBS media (Hi-Media grade) by the spread plate technique after Harris, Owens, and Smith (1996). Levels of bacteria were expressed in colony-forming units per ml of water (CFU ml^{-1}).

2.6 | Challenge study

The bacterial pathogen *Vibrio parahaemolyticus* (MTCC 451) from Institute of Microbial Type Culture Collection (IMTCC), Chandigarh, India, used for challenge study was spread on TCBS agar and incubated for 24 hr at 30°C to form colonies. After that, a single colony was transferred to a 10-ml tryptic soy broth supplemented with 1% NaCl and incubated overnight at 30°C. The broth culture was then centrifuged at 7500x*g* for 10 min at 4°C. The precipitate was rinsed with sterile 0.9% saline, resuspended in sterile normal saline, and used as bacterial suspensions. A total of 10 healthy intermoult animals were used for the experiment, conducted in triplicate. Shrimps in the weight range of 15–18 g were injected intramuscularly in the third abdominal segment with 20 μl of bacterial suspension and transferred to 100 L FRP tanks. Control shrimp were also maintained which were injected with 20 μl of 0.9% saline. The shrimps were closely monitored for mortality.

2.7 | Immunological parameters

2.7.1 | Total haemocyte count

Healthy intermoult stage shrimp were collected, and about 100 μl of haemolymph from control and CN-treated shrimps was withdrawn using a syringe containing 900 μl of ice-cold anticoagulant saline (ACS). The syringe was shaken gently for rapid mixing of haemolymph and ACS. A drop of haemolymph suspension was introduced into an improved Neubauer haemocytometer, and the number of haemocytes was determined microscopically. The THC was computed after Söderhäll, (1982):

$$\text{Total hemocyte count} = \frac{\text{Total number of cells counted} \times \text{dilution factor} \times 10^4}{\text{Number of fields counted}}$$

2.7.2 | Phenoloxidase activity

The haemolymph collected from the animal was allowed to clot for 30 min at room temperature (28°C). After that, the clot was disturbed and centrifuged at 1,500 *g* for 7 min. The clear supernatant (serum) was collected. The serum collected from each experimental group animal was used for measuring the phenoloxidase (PO) activity spectrophotometrically (Shimadzu, Japan) from the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) as described by Asokan, Arumugam, and Mullainadhan (1998). Briefly, 0.01 mol/L solution of L-DOPA was prepared in 0.1 mol/L phosphate buffer (0.2 mol/L Na_2HPO_4, 0.2 mol/L NaH_2PO_4, pH 6.0). An equal volume of serum and L-DOPA (100 μl each) was added to a spectrophotometer cuvette (10 mm) containing 3 ml of phosphate buffer. The mixture was agitated at 2-min intervals for 60 min. The optical density (OD) was measured at 490 nm. Phenoloxidase activity was determined from the increase in OD per min. Total haemolymph protein was determined spectrophotometrically as per Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

2.7.3 | Phagocytosis

Haemolymph (100 μl) was collected in 2 ml of trisodium citrate buffer (30 mmol/L trisodium citrate, 340 mmol/L NaCl, 10 mmol/L EDTA, 120 mmol/L dextrose; pH 7.55). It was spread on an alcohol-washed, clean, dry glass slide over an area of 2 cm^2 and kept in a moist chamber for 30 min at 23°C to obtain haemocyte monolayer

(50 μl). For calculating the viability of haemocytes in monolayers, the procedure by Garvey, Cremer, and Sussdorf (1979) was followed using the trypan blue dye exclusion technique. Human A blood collected in Alsever's solution was fixed in glutaraldehyde following the method of Nowak, Haywood, and Barondes (1976).

Phagocytosis of human A erythrocyte in five haemocyte monolayers was prepared using haemolymph samples obtained from *L. vannamei*. The first and second pair of monolayers were overlaid with 200 μl human A erythrocyte (0.5%) and observed at 5 min interval for 1 hr. The mean of five determinations was recorded and values computed as below:

$$\text{Phagocytosis}(\%) = (\textit{No.}\ \text{of phagocytotic hemocytes}/\text{Total no. of hemocytes}) \times 100$$

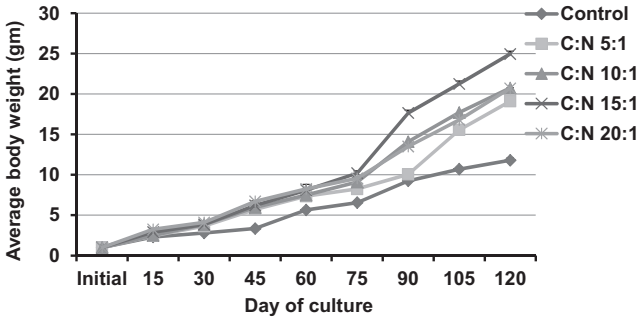

FIGURE 1 Mean values of body weight of *L. vannamei* in fifteen days interval of four CN ratio groups and control group. Values are means (±*SD*) of three replicate tanks per sampling time in each group

2.8 | Statistical analysis

Water quality parameters, growth, and immunological assessment were subjected to ANOVA following Duncan Multiple range test. The correlation between water quality and growth as well as related parameters was computed. The analysis was carried out using SPSS package 22.

3 | RESULTS

3.1 | Survival and growth

Survival of C:N-treated shrimp was higher than that of control. At the end of the experiment, the survival was highest in CN15 (99%) followed by CN20 (96%), CN10 (89%), and CN5 (81%) whereas it was lowest in control (77%) (Table 2). Average body weight was significantly ($p < 0.001$) higher in C:N treatments compared to control (Figure 1). Highest ABW (24.71 ± 2.61 g) was achieved in CN15 compared to control (11.8 ± 2.93 g). Specific growth rate (SGR), protein efficiency ratio (PER), feed conversion ratio (FCR), and feed efficiency ratio (FER) showed significantly higher values among the treatments ($p < 0.01$) compared to those of control (Table 2). A higher FCR was observed in CN15 and CN20 compared to other treatments and control (Table 2). The FER values revealed no significant difference between CN15 and 20 treatments whereas significant differences were observed in CN5 and CN10 treatments compared to control (Table 2). However, there was no significant difference among other CN treatment groups. The PER was significantly ($p < 0.001$) higher in CN15, CN20, CN10, and CN5 compared to control (Table 2).

3.2 | Water quality parameters

The water quality parameters like salinity, pH, and temperature did not differ between the treatments and control. The EC and turbidity levels increased proportionately with the progress of culture (Table 3). Similarly, TDS and TSS values significantly ($p < 0.001$) increased in CN treatments compared to control. However, TAN, NO_2-N, and NO_3-N levels significantly ($p < 0.001$) decreased in CN20 followed by other CN treatments whereas in control, higher values were recorded (Table 3). Phosphate levels were significantly ($p < 0.001$) higher in the water samples of C:N treatments compared to control where the values were low. The DO levels varied significantly ($p < 0.05$) between the treatments and control (Table 3). Total alkalinity levels were not significantly different among the treatments and control, as the alkalinity was maintained by adding lime. However, chlorophyll a increased in the treatments

TABLE 2 Average body weight, survival, specific growth rate, protein efficiency ratio, feed conversion ratio, and feed efficiency ratio of *Litopenaeus vannamei* in a 16-week trial comparing effect of different CN ratio in a biofloc-based, zero-exchange tank system stocked with juveniles at a density of 100 shrimp m^{-3}

Treatments	ABW (gm)	Survival rate (%)	SGR	PER	FCR	FER
Control	11.85^{a} ± 2.93	76.50^{a} ± 2.12	2.01^{a} ± 0.04	1.35^{a} ± 0.04	2.32^{c} ± 0.08	0.43^{a} ± 0.02
CN 5:1	19.01^{b} ± 2.74	81.05^{b} ± 1.41	2.40^{b} ± 0.05	2.99^{b}±0.09	1.03^{b} ± 0.09	0.86^{b}±0.17
CN 10:1	20.77^{b} ± 3.19	88.55^{c} ± 0.78	2.49^{b} ± 0.07	3.47^{c} ± 0.03	0.90^{a} ± 0.03	1.11^{c} ± 0.05
CN 15:1	24.71^{c} ± 2.61	99.00^{d} ± 1.45	2.68^{c} ± 0.05	3.84^{d} ± 0.02	0.81^{a} ± 0.04	1.23^{d} ± 0.04
CN 20:1	20.26^{b} ± 4.52	96.00^{d} ± 1.48	2.49^{b} ± 0.06	3.83^{d} ± 0.01	0.82^{a} ± 0.02	1.23^{d} ± 0.07
p value	0.003^{*}	0.001^{**}	0.001^{**}	0.001^{**}	0.001^{**}	0.001^{**}

Note. The value indicates mean ±standard deviation of biofloc measured in treatments and control groups.
Different superscripts like ^{a,b,c,d} are depicted for significant difference at *p* value mentioned based on DMR test.

CN20, CN15, CN10, and CN5, and it was significantly higher in all the treatments compared to control ($p < 0.001$). The biofloc volume gradually increased over the period in the treatments, and low values were recorded in control, significant difference ($p < 0.03$) (Table 3).

3.3 | Assessment of microbial load

The microbial load in the culture system, as revealed by total heterotrophic count, was significantly higher ($p < 0.001$) in the water samples of the treated groups compared to control. Mean values of total heterotrophic bacterial counts were comparatively higher in CN20 (Figure 2). The total presumptive *Vibrio* count significantly increased ($p < 0.001$) in water samples of control proportionate to the culture period but gradually decreased in samples of various CN treatments. Vibrio load was higher in the water samples of than control the CN treatments (Figure 3).

3.4 | Challenge study

Survival of shrimp challenged with *V. parahaemolyticus* was significantly ($p < 0.05$) higher in CN treatment groups compared to control. The cumulative mortality was 100% in control group followed by 80% (CN5 and CN10) and 70% (CN15 and CN20). Mortality increased after 5 days postchallenge in control whereas, in the biofloc treatments, mortality decreased after fifth day (Figure 4).

3.5 | Immunological parameters

The total haemocyte count was significantly ($p < 0.05$) higher in CN15 compared to control (Figure 5). Similarly, THC was significantly ($p < 0.01$) different in CN20, CN10, and CN5 treatments, (12.2 ± 0.24, 11.1 ± 0.43, and 10.8 ± 0.35) X 10^6 cells ml^{-1}, respectively. Phenoloxidase activity of serum C:N-treated shrimps was significantly higher ($p < 0.05$) in CN15 followed by CN20, CN10, and CN5 compared to control (Figure 6). Phagocytosis percentage showed significantly ($p < 0.05$) higher values for CN15 (43.5 ± 2.8) and CN20 (41.0 ± 3.1) compared to that of CN10 and CN5 whereas the control group (31.5 ± 2.9) exhibited the least value (Figure 7).

4 | DISCUSSION

4.1 | Growth performance

Biofloc technology enhances water quality through microbial manipulation, thereby facilitating healthy growth of cultured shrimp. BFT being a zero-water exchange system, the addition of carbohydrate promotes the development of diverse and balanced microbial communities originating from the rearing water (Haslun et al., 2012). These active and dense microorganisms together with suspended organic particles tend to form the biofloc, which can continuously be consumed by the shrimp as a natural food source (Burford, Thompson, McIntosh, Bauman, & Pearson, 2004; Kent, Browdy, & Leffler,

TABLE 3 Mean values of physico-chemical parameters of water samples from control and four C:N treatments. The values are means (±*SD*, *N* = 15) of three replications and five sampling date for the treatment and control

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

Note. Mean values within a row with the same superscripts are not significantly different ($p < 0.05$).
Different superscripts like ^{a,b,c,d} are depicted for significant difference at $p < 0.05$ based on DMR test and NS—Non-significant.

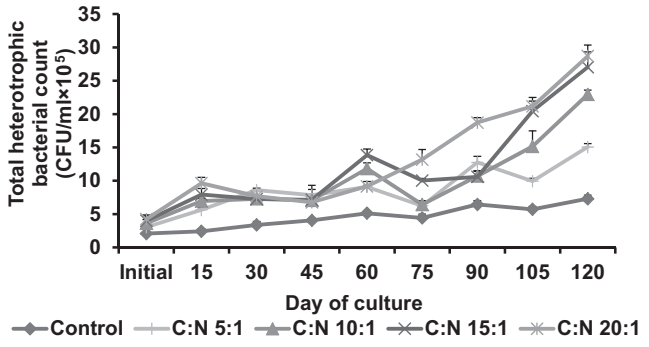

FIGURE 2 Mean values of total heterotrophic bacterial count in water samples *L. vannamei* culture in fifteen days interval of four CN ratio groups and control group. Values are means (±*SD*) of three replicate tanks per sampling time in each group

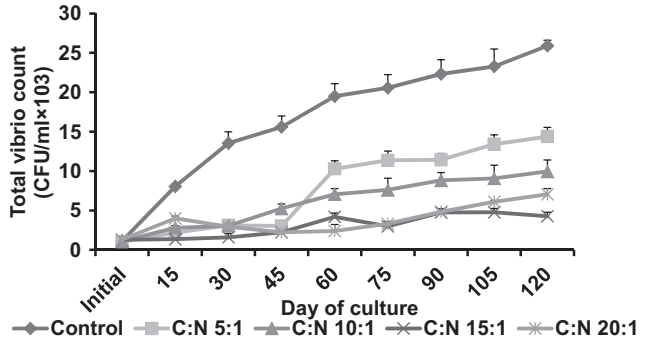

FIGURE 3 Mean values of total presumptive vibrio count in water samples of *L. vannamei* culture in fifteen days interval of four CN ratios groups and control groups. Values are means (±*SD*) of three replicate tanks per sampling time in each group

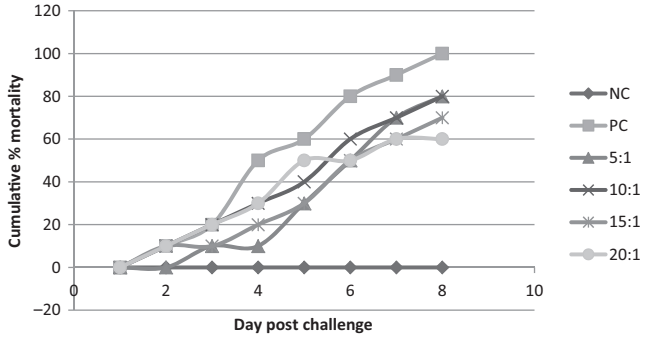

FIGURE 4 Cumulative percent mortality of control and biofloc with different C: N ratio (CN5, CN10, CN15, & CN20) group of animals challenged with *V. parahemolyticus* (MTCC, 451 from IMTCC, Chandigarh, India). Cumulative mortality was recorded at 24 hr intervals. Experiments were repeated two times with similar results obtained. The differences in cumulative mortality between treatments were analysed by DMR test (**$p < 0.01$)

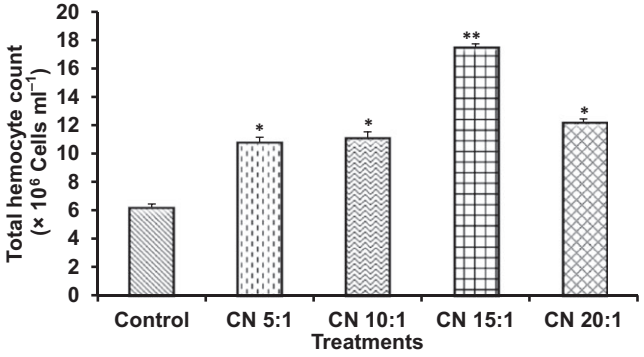

FIGURE 5 Mean values total hemocyte count in shrimp reared in four C:N ratio (CN5, CN10, CN15, & CN20) and control shrimps. Data shown as mean with standard deviation as error bars ($n = 10$). Significant difference ($p < 0.05$) between the groups is indicated by asterisk mark on top of the bar

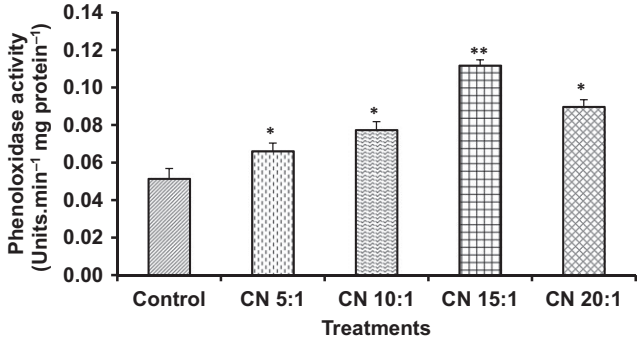

FIGURE 6 Mean phenoloxidase activity of shrimp reared in four C:N ratio (CN5, CN10, CN15, & CN20) and control. Data shown as mean with standard deviation as error bars ($n = 10$). Significant difference ($p < 0.05$) between the groups is indicated by asterisk marks on top of the bar

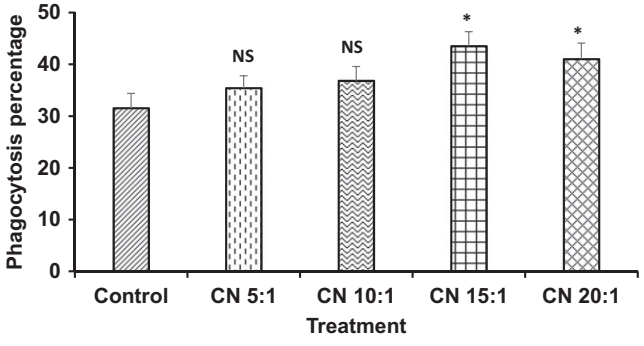

FIGURE 7 Mean value of phagocytosis activity of shrimp reared in four C:N ratio (CN5, CN10, CN15, & CN20) and control. Data shown as mean with standard deviation as error bars ($n = 10$). Significant difference ($p < 0.05$) between the groups is indicated by asterisk marks on top of the bar

2011; Wasielesky, Atwood, Stokes, & Browdy, 2006). It also improves water quality, microbial dynamics, and immunological parameters of the cultured *L. vannamei* and *P. monodon* (Burford et al., 2004; Epp, Ziemann, & Schell, 2002; Moss & Pruder, 1995; Tacon et al., 2002; Kumar et al., 2004). Although the underlying mechanisms of BFT in promoting shrimp growth are mostly

unknown, it is expected that the beneficial effect of BFT has several interrelated causes. Furthermore, many studies have demonstrated the beneficial effects of biofloc on shrimp culture (Ballester et al., 2010; Haslun et al., 2012; Ray, Dillon, & Lotz, 2011; Wasielesky et al., 2006; Xu & Pan, 2012; Zhao et al., 2012). Several researchers suggested that apart from maintaining clean and stable water quality, the established biofloc in the culture system can improve feed utilization, thereby increasing growth performance. Similar findings were reported on different shrimp species, namely, *Penaeus monodon* (Arnold, Coman, Jackson, & Groves, 2009), *P. semisulcatus* (Megahed, 2010), *Farfantepenaeus paulensis* (Ballester et al., 2010), *L. vannamei* (Xu & Pan, 2012), and *Marsupenaeus japonicus* (Zhao et al., 2012).

The present study confirmed the significant role of C:N ratio levels in the biofloc-based shrimp culture system in promoting growth. Our results revealed that survival was significantly higher in CN treatments (82%–99%) compared to that of the control (77%). Moreover, higher growth was observed in CN15 and other treatments compared to control. Similarly, related studies have shown that survival of shrimp in CN treatments ranged from 80% to 100% compared to control (Anand et al., 2013; Panjaitan, 2011; Xu, Morris, & Samocha, 2016). Ju, Forster, and Dominy & W. G. (2009) suggested that microalgae in the microbial floc may play a key role in improving shrimp growth. Our results indicate that growth, SGR, FER, and PER significantly ($p < 0.05$) increased in all the CN treatment shrimps compared to that of control. The FCR varied significantly in CN treatments (0.81–1.03) compared to control (2.32). The FCR was higher in CN5 treatment although no significant differences between different biofloc groups could be observed. Wasielesky et al. (2013) reported an FCR of 0.95–1.61 in the BFT system. Improved FCR values were observed with biofloc treatments suggesting that biofloc improved feed utilization (Megahed, 2010; Ray et al., 2011; Xu &Pan, 2013).

4.2 | Water quality parameters

Our results revealed no significant differences between the treatments and control with regard to salinity and temperature. The pH levels recorded were significantly low compared to that in control because of the inclusion of carbon sources. We maintained the optimum level by adding lime. Organic/nitrogenous metabolites are converted into bacterial biomass by addition/balancing with carbon and nitrogen to reduced ammonium concentration in the biofloc-based shrimp culture system (Schneider et al., 2005). By adding carbohydrates to the pond, bacterial growth is stimulated, and nitrogen uptake through the production of microbial proteins takes place (Avnimelech, 1999). This enhanced nitrogen uptake by bacterial growth decreases the ammonium concentration more rapidly than nitrification (Hargreaves, 2006). Our results revealed that TAN levels reduced gradually when the addition of carbon sources increased. The CHO addition to CN20 reduced TAN concentration significantly. Similarly, NO_2-N and NO_3-N were reduced in the water of all the CN treatments, whereas in control, the values recorded were significantly ($p < 0.05$) higher. The TAN level was negatively correlated ($r = -0.868$; $p < 0.01$), indicating that the presence of ammonia in the system strongly influences the survival and also affects the body weight and hence the growth in shrimp ($r = -0.789$; $p < 0.01$), ($r = -0.899$; $p < 0.01$ respectively). These studies revealed that phosphate concentration was higher in the water of the CN treatments whereas in control, the value was low. The PO_4-P concentrations remained below the 40 mg/L obtained by Ray, Lewis, Browdy, and Leffler (2010) but were higher than the values in other BFT systems (Krummenauer, 2008; McIntosh et al., 2000). The accumulation of phosphorus is due to noningested feed and decomposition of excreta which favours eutrophication (Peñaflorida, 1999). This accumulation does not directly affect the development of shrimp but may cause favourable conditions for the proliferation of filamentous cyanobacteria, which could obstruct the shrimp gills and produce harmful toxins (Wasielesky et al., 2006). Silva (2009) reported that the nitrogen and phosphorus dynamics in *L. vannamei* and *Farfantepenaeus paulensis* culture in BFT system affirms that the accumulation of phosphorus and the microbial floc inability to retain great quantities of the same make excess phosphorus removal necessary. Wurts (2002) has reported that the phosphorus level in the system will enhance the growth of the phytoplankton. In the present study, the PO_4 was positively and significantly correlated with the chlorophyll and growth rate ($r = 0.861$ and 0.652 respectively). Electrical conductivity was recorded to be higher in CN treatments whereas in control, the value was low. Similarly, turbidity levels were significantly ($p < 0.05$) higher in the CN15 and 20 compared to other treatments and control because of biofloc abundance. Total dissolved solids were significantly ($p < 0.05$) higher in the water of CN treatments whereas in control, the values were low. The TSS levels increased in the CN15, CN20, and other treatments compared to control because of higher density of microbial abundance in the biofloc system. According to Schveitzer et al. (2013), when *L. vannamei* were cultured in zero-exchange superintensive tank systems with TSS concentration higher than 800 mg/L, the final shrimp yield was lower than those cultured in the concentration of 200–600 mg/L. These results indicate that the addition of carbon source promotes higher natural bacterial protein biomass and increases the TSS and improves shrimp growth in the biofloc systems. Similarly, biofloc volume was significantly ($p < 0.05$) higher when carbon sources were increased whereas, in control, lower volume was recorded. Bioflocs are a good source of vitamins and minerals, especially phosphorus, they may also have probiotic effects. Maintaining settleable solids concentration of 25–50 ml/L provides good functionality in biofloc systems for tilapia (Avnimelech, 1999). In lined biofloc shrimp ponds, 10–15 ml/L is the typical target range (Hargreaves, 2013). Allan, Moriarty, and Maguire (1995) recorded faster growth of prawns in well-prepared ponds with an abundant meiofauna. The present study showed significantly higher chlorophyll a concentration in the CN treatment water samples. Utilization of microbial protein depends on the ability of the target animal to harvest the bacteria and to digest and utilize the microbial protein (Avnimelech, 1999; Burford et al., 2004; Burford, Thompson, McIntosh, Bauman, & Pearson, 2003; Hari et al., 2004). Reported lethal levels of dissolved oxygen for *L.*

vannamei are far below 1.0 mg/L (Hopkins, Hamilton, Sandifer, Browdy, & Stokes, 1993) and 0.5 mg/L (Zhang, Zhang, Li, & Huang, 2006). The COD concentrations were significantly higher in all the CN treatments compared to control (Table 3). Total alkalinity levels were significantly reduced in the water of the CN treatments whereas in control, they were higher (Ebeling, Timmons, & Bisogni, 2006).

4.3 | Microbial dynamics

Xu et al. (2016) reported that the addition of molasses to the biofloc treatment increased the CN ratio and promoted the development of heterotrophic bacteria in the culture tank water. The TAN was assimilated by utilization of heterotrophic bacteria to promote/convert microbial biomass in the biofloc-based system (Ebeling et al., 2006; Hargreaves, 2006). In heterotrophic biofloc-based shrimp culture systems, the driving force is dense populations of active heterotrophic bacteria which can be promoted by increasing the CN ratio of feed input. Assimilating the waste nitrogen from culture water resulted in the production of new microbial biomass (cellular proteins) (Avnimelech, 2006; Crab et al., 2007; Ebeling et al., 2006). The present study revealed that CN treatments exhibited significantly ($p < 0.05$) higher ranges of THB compared to control (Table 3). Moreover, *Vibrio* load as significantly ($p < 0.05$) reduced in the water samples of CN treatments whereas in control, it was high. It is observed that stressed shrimp can be more susceptible to facultative pathogenic microorganisms, part of their natural microbial flora and aquatic environment (Lightner, 2005). Moriarty (1998) reported that *Vibrio* sp. detected in the control group was higher than the probiotic-treated group. However, some *Vibrio* species are the potential causative agents for diseases in aquaculture systems (Immanuel, Vincybai, Sivaram, Palavesam, & Marian, 2004; Mohney, Lightner, & Bell, 1994). Balcazar (2003) demonstrated that the administration 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 the pathogen *Vibrio harveyi*. The present study confirmed these findings based on the fact that *Vibrio* levels were significantly ($p < 0.001$) lower in the water samples for CN treatments compared to control.

4.4 | Challenge study

The C:N ratio plays a critical role in the development of the microbial community (Panigrahi et al., 2018), which enhances the immunity of the cultured organisms. (Crab, Chielens, Wille, Bossier, & Verstraete, 2010; Haslun et al., 2012; Xu & Pan, 2013; Xu et al., 2016; Zhao et al., 2012). In the biofloc system, carbohydrates are added to promote diverse microbial growth (Haslun et al., 2012). As the biofloc is manipulated to an appropriate C:N ratio, the health of the animal is maintained. However, nitrogen is required to produce the protein-rich microbial cells. Inorganic nitrogen is immobilized into bacterial cells when the metabolized organic substrates have high C:N ratio (McCarty & Rittmann, 2001). Carbon is used for the utilization of microorganisms, especially heterotrophic bacteria to assimilate the nitrogen metabolites in the culture system under aerobic conditions. The C:N ratio in an aquaculture system can be increased by adding different locally available cheap carbon sources and a reduction in protein content in the feed (Avnimelech, 1999; Hargreaves, 2006). Our study suggests that the defensive action of biofloc-reared shrimps against *V. parahaemolyticus* resulted in lower mortality in CN15 and CN20 compared to other treatments. Our results are in agreement with those reported by Xu and Pan et al., (2012) who worked in the biofloc system and challenged the biofloc-based shrimps against pathogenic organisms.

4.5 | Immunological assessment

Biofloc is a mechanism that provides shrimp with pattern recognition and other molecules that lead to stimulation of the nonspecific immune system. There is an energy cost associated with constant immunostimulation although it is difficult to conclude whether this effect is deleterious or not. Biofloc enhances the immune system, but it is not fully activated until a pathogen is encountered. Avnimelech (2007) reported that biofloc could reduce pathogenic bacteria compared to clear water. Antagonistic activity between the pathogen and other bacteria might limit the pathogens. A similar effect may occur between dense heterotrophic bacteria and *V. parahaemolyticus* as the causative agent. Although biofloc has been confirmed as being rich in natural microorganisms and bioactive compounds, not much effort has been made to study the effect of biofloc on the physiological health of cultured shrimp, particularly concerning immune and antioxidant defence systems (Panigrahi et al., 2018; Xu et al., 2013). Jang et al. (2011) found that the expression of a proPhenoloxidase activating enzyme (lvPPAE1) in haemocytes of *L. vannamei* was enhanced significantly when shrimps were reared in biofloc water over the long term. The present study revealed that total haemocytes count significantly ($p < 0.05$) increased in CN15 and other CN treatments compared to control. Phagocytosis percentage was found to be highly elevated in the CN15 group followed by CN20 compared to control, but no significant differences were discernible in other/lower CN treatments.

5 | CONCLUSION

The relationship of carbon and nitrogen is an essential phenomenon of biofloc-based shrimp culture systems. The ability to degrade the nitrogen metabolites and maintain the water quality through manipulation of C:N ratio influences the environment and productivity immensely in the biofloc-based system. Also, recycling the waste product excreted/additives can be regenerated/converted to useful microbial product for shrimp as a natural diet, thereby maintaining a cleaner environment. Addition of carbohydrates for reduction in ammonia promotes biofloc concentration and heterotrophic bacterial generation/production of microbial proteins, improves shrimp immunity, and reduces pathogenic strains. The present study revealed that CN treatments had a significant effect on growth, water quality,

microbial dynamics, and immunological parameters compared to control, and it could, therefore, be inferred that the treatment CN15 ratio exhibited an optimal range for maintaining a biofloc system in *L. vannamei* culture.

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