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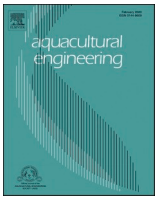

Effects of feed, carbohydrate addition and stocking density on Pacific white shrimp (*Litopenaeus vannamei*) production

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ABSTRACT

Shrimp culture rearing systems are dynamic with numerous processes influencing system performance. This research investigated the effects of stocking density, feeding level and C:N ratio on shrimp production, water quality and microbial community composition in a biofloc shrimp rearing system, using a 3 × 3 factorial design. Pacific white shrimp (*Litopenaeus vannamei*) were stocked at 27, 120, or 300 individual m^{-3} and fed 100, 80 or 60% of the recommended daily feed ration. For each combination of stocking density and feeding level, C:N ratios of 7.4, 12 or 16 were created by feeding a mixture of casava and rice bran besides the daily pelleted feed input to culture tanks. After 12 weeks of culture, the harvested shrimp biomass was the highest in the rearing tanks with the highest stocking density, feeding level and C:N ratio ($P < 0.05$), while the individual shrimp size at harvest decreased with increasing stocking density ($P < 0.05$). Biofloc biomass (e.g. total suspended solids TSS, volatile suspended solids VSS) and, to a lesser degree, water quality (total ammonia nitrogen TAN, NO_2-N) were affected by $_2$ and 3-way interactions of the main tested factors. The ash, protein, fat and nitrogen free extract (NFE) content in biofloc were affected by stocking density ($P < 0.05$), and ash and NFE content by C:N ratio ($P < 0.05$). Regarding the microbial community composition in the biofloc at the highest stocking density, increasing feeding level and C:N ratio led to an increasing microbial richness and diversity ($P < 0.05$) as a result of nutrient input. Overall, the shrimp production and biofloc biomass were influenced in a similar way by the stocking density, feeding level and C:N ratio applied in the biofloc system. Future research should focus on how the type of carbohydrates and methods of administration affect shrimp growth and biofloc formation, and how this may promote health benefits through the microbial community manipulation.

1. Introduction

In Pacific white shrimp (*Litopenaeus vannamei*) culture, stocking density, feeding level, and C:N ratio have been described as major factors affecting growth and survival; in fact, stocking density has been reported to often exhibit an inverse relationship with those parameters (Araneda et al., 2008; Sandifer et al., 1987; Sookying et al., 2011; Williams et al., 1996). Reduced growth and survival at high densities have been attributed partly to crowding, leading to a competition for space and feed, and to poor water quality. Such conditions can contribute to increased stress and potentially to immunosuppression (Araneda et al., 2008; Liu et al., 2017; Williams et al., 1996). On the other hand, increasing feeding level and C:N ratio in biofloc systems through carbohydrate addition can improve shrimp growth. Specifically, a higher C:N ratio has been reported to improve water quality and biofloc growth (Avnimelech, 1999; Crab et al., 2012; Lara et al., 2017). Biofloc can contribute up to 29% of the daily feed intake of Pacific white shrimp (Burford et al., 2004) and thus by carbohydrate addition to increase the C:N ratio, a better shrimp production with less additional feed can be achieved. Actually, in systems with high primary production or supplemented with bioflocs, feeding 25% less than the recommended feed ration did not to affect shrimp growth (Lara et al., 2017; Roy et al., 2012). Moreover, as bioflocs are formed by aggregation of diverse microbial groups (heterotrophs and autotrophs), nutrient input through C:N manipulation or different feeding levels can largely affect the microbial community composition (Bossier and Ekasari, 2017); specifically, C:N ratio is known to affect the heterotrophic microbial populations (Deng et al., 2018; Zhu et al., 2022). Whether the shifts in diversity are beneficial or not for the shrimp it is largely unexplored. Consequently, questions still remain regarding the optimal feeding level at different C:

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N ratios and the effects on biofloc microbial diversity, without negative impacts on shrimp growth and possible interactions.

Therefore, the current research examined the main effects of three factors: stocking density, feeding level and C:N ratio, as well as their interactions, on Pacific white shrimp production reared in a biofloc systems. We assessed the impacts both at the production level by evaluating shrimp growth, and at the system level by evaluating water quality, biofloc biomass and the prokaryotic microbial community composition in the biofloc.

2. Materials and methods

2.1. Experimental design

This experiment was done in the aquatic experimental facilities at College of Aquaculture and Fisheries, Can Tho University, Vietnam, using a three-way factorial design. The feeding level variable had three levels, including 100% (FL100), 80% (FL80), and 60% (FL60) of the recommended feeding level by the feed provider (3–22% body mass per day depending on shrimp size). The carbon:nitrogen (C:N) ratio (on mass basis) had three levels: 7.4 (C:N7), 12 (C:N12) and 16 (C:N16). Each combination of the feeding level and C:N ratio was tested in three stocking densities, including 27 ind/m^3 (SD27), 120 ind/m^3 (SD120) and 300 ind/m^3 (SD300). The three stocking densities were tested in separate 12-week grow-out trials. In total, this experiment comprised twenty seven treatments executed in triplicate. During each grow-out trial, the treatment SD120-FL100-C:N7 was executed as inter-phase control, with 3 replicates per trial. The acronyms FL100, FL80, and FL60 refer to tanks where shrimp were fed 100%, 80%, and 60% of the feed level recommended by the feed company, respectively. The same holds for the acronyms C:N7, C:N12, and C:N16 or SD27, SD120, and SD300.

2.2. Experimental site preparation

The experiment was conducted outdoor to simulate farming conditions. Nevertheless, a transparent plastic film was used to cover the experimental site to prevent interference of rain water, while still allowing ambient light to the experimental tanks. Thirty circular plastic tanks (0.5 m^3 total volume) were cleaned prior to the addition of saline water, and employed during each 12-week trial period.

Sea water (80 ppt salinity) was diluted to 15 ppt salinity, disinfected with chlorine and adjusted to 140 mg/L alkalinity using sodium bicarbonate. Three hundred liters treated water were pumped into each experimental tank. All tanks were continuously aerated with one airstone ca. 10 cm above the bottom in the middle of the tank to maintain the dissolved oxygen level above 6 mg/L. Air was provided to all tanks from one air blower (RB-022, APP Co. Ltd, Taiwan) serving all experimental tanks.

2.3. Experimental animals

For every experimental trial, Pacific white shrimp postlarvae (PL15) were obtained from the same nearby hatchery, and nursed in a 2-m^3 tank at 15 ppt salinity for one month. During this period, shrimp were fed three times daily at 08:00, 12:00, and 16:00 h. Shrimp of 0.13 g were stocked in the experimental units. Per tank, 8, 36, or 90 shrimp were stocked to obtain the stocking densities of SD27, SD120, and SD300, respectively.

2.4. Feeding and carbohydrate addition

Shrimps were fed a complete feed (42% protein, C:N ratio 7.4) (Skretting, Vietnam) three times per day at 08:00, 12:00, and 16:00 h, applied evenly over the whole tank surface area. The shrimp in FL100 tanks were fed 3–20% BW/day according to shrimp size, following the recommendations of the feed company. The shrimp in treatment tanks FL80 and FL60 were fed each day 20% and 40% less feed compared to shrimp in treatment tanks FL100, respectively. The feeding level was adjusted every two weeks, based on the average shrimp individual body mass in the inter-trial control treatment SD120-FL100-C:N7.

A mixture of rice bran and cassava (3:7 ratio) was used as carbohydrate (CHO) source to adjust the C:N ratio. Treatment tanks with C:N7 (which received feed with a C:N ratio 7.4) did not receive CHO besides the pelleted feed. To increase the C:N ratio to 12 and 16, 681 and 1278 g of CHO, respectively, was added per kilogram of feed applied. The CHO was weighed, and incubated in 60 °C water for 24 h prior to addition to the treatment tanks. The proximate composition of the feed and carbohydrate applied is shown in Table 1.

Table 1
The proximate composition of shrimp feed and carbohydrate (CHO) in this research.

Samples	Moisture (%)	Ash (% DM)	Crude Fat (% DM)	Crude Protein (%DM)	NFE (%DM)
Feed	10.9	11.4	8.3	47.3	33.0
CHO	11.3	3.6	3.1	7.0	86.3

2.5. Water quality monitoring

The ammonia, nitrite, and nitrate concentrations were checked using commercial test kits (sera GmbH, Germany), while the water pH and temperature were monitored weekly two hours after the mid-day feeding period using an electric probe. Every two weeks, filtered and disinfected tap water was added to each tank to compensate water loss due to evaporation.

2.6. Sampling and sample analysis

The number of shrimp stocked and the average shrimp body mass and length were determined at the start (Day 0), and from then onwards at bi-weekly intervals (Days 14, 28, 42, 56, and 70), until harvest (Day 84). In the SD27 grow-out trial, 4 shrimp were randomly sampled from each tank for the determination of average body mass and length every two weeks. In the SD120 and SD300 grow-out trials, 10 shrimp per tank were collected for the average body mass and length determination. At harvest, the number of shrimp harvested from each tank were counted to determine the survival rate.

Water samples were collected at the end of culture weeks 6 and 12 in each grow-out trial. These samples were analyzed for total suspended solids (TSS), volatile suspended solids (VSS), chlorophyll a (Chla), total ammonia nitrogen (TAN) and nitrite nitrogen (NO_2-N) concentration following Standard Methods for the Examination of Water and Wastewater (APHA, 1995).

Biofloc volume was measured every two weeks using Imhoff cones. One liter of water was taken from each tank and let to settle in an Imhoff cone for 20 min. The biofloc volume was then recorded as the amount of settled material in mL L^{-1}. At the end of the experiment, biofloc samples were collected and analyzed for proximate composition, following the Official Methods of Analysis (AOAC, 2000). During the SD300 grow-out trial, biofloc samples were collected from every tank by filtering 1 L of culture water through 1.5 μm-pore size filters. Biofloc samples were then mixed with DNA-later at the 1:1 ratio, and stored at − 40°C for the analysis of microbial community composition.

2.7. Microbial community composition analysis

For microbiota analysis, DNA was extracted from biofloc samples coming from the highest stocking density group (SD300). The biofloc samples, were collected at the end of the experiment by filtering through a 0.22 μm (seston part) pore size sterile filter. The samples were

Table 2
Shrimp production parameters in three repeats of the inter-phase control (treatment SD120-FL100-C:N7). Values are mean ± standard deviation of 3 replicate tanks per repeat. No difference was observed between 'repeats' ($P > 0.05$) for each of the production parameters.

	Final BW	Final BL	Total biomass	Survival*	FCR
	(g/ind)	(cm/ind)	(g)	(%)	
Repeat 1	4.7 ± 0.5	8.2 ± 0.2	161 ± 15	95 ± 2	1.06 ± 0.10
Repeat 2	4.3 ± 0.2	8.3 ± 0.1	149 ± 10	96 ± 4	1.10 ± 0.08
Repeat 3	4.6 ± 0.1	8.4 ± 0.1	145 ± 8	92 ± 5	1.19 ± 0.07
P values	0.292	0.501	0.281	0.355	0.212

subjected to lysis by lysozyme buffer and proteinase K before DNA extraction using DNeasy PowerSoil kit (Qiagen, Valencia, CA). The harvested DNA was quantified using the Nanodrop spectrophotometer. Sequencing of the PCR-amplified V4 region of the 16 S rRNA (prokaryotic microbial communities), using primers 515 F (5′-CTAGTGCCAGCMGCCGCGGTAA −3′) and 806 R (5′-CTAGGACTACHVGGGTWTCTAAT-3′) was performed using a MiSeq PE300 Next Generation system (Illumina) by Genome Quebec, following the company's protocol. Sequencing data can be found at the NCBI (SRA) database under the study accession code PRJNA728072, accession numbers SRX10835974 - SRX10836033.

An open-source software package, DADA2 (Callahan et al., 2016), was applied to model and correct Illumina-sequenced amplicon errors. Data were demultiplexed into forward and reverse reads according the barcode sequence into sample identity, and trimming was performed, according to Kokou et al. (2020). For the forward reads and based on the quality profiles, the first 250 nucleotides were kept, and the rest were trimmed, while for the reverse reads, the last 220 nucleotides were kept. DADA2 resolves differences at the single-nucleotide level and the end product is an amplicon sequence variant table, recording the number of times each exact sequence variant (ESV) was observed in each sample (100% sequence identity). Taxonomy was assigned using the Ribosomal Database Project Classifier (Wang et al., 2007) against the 16 S gene

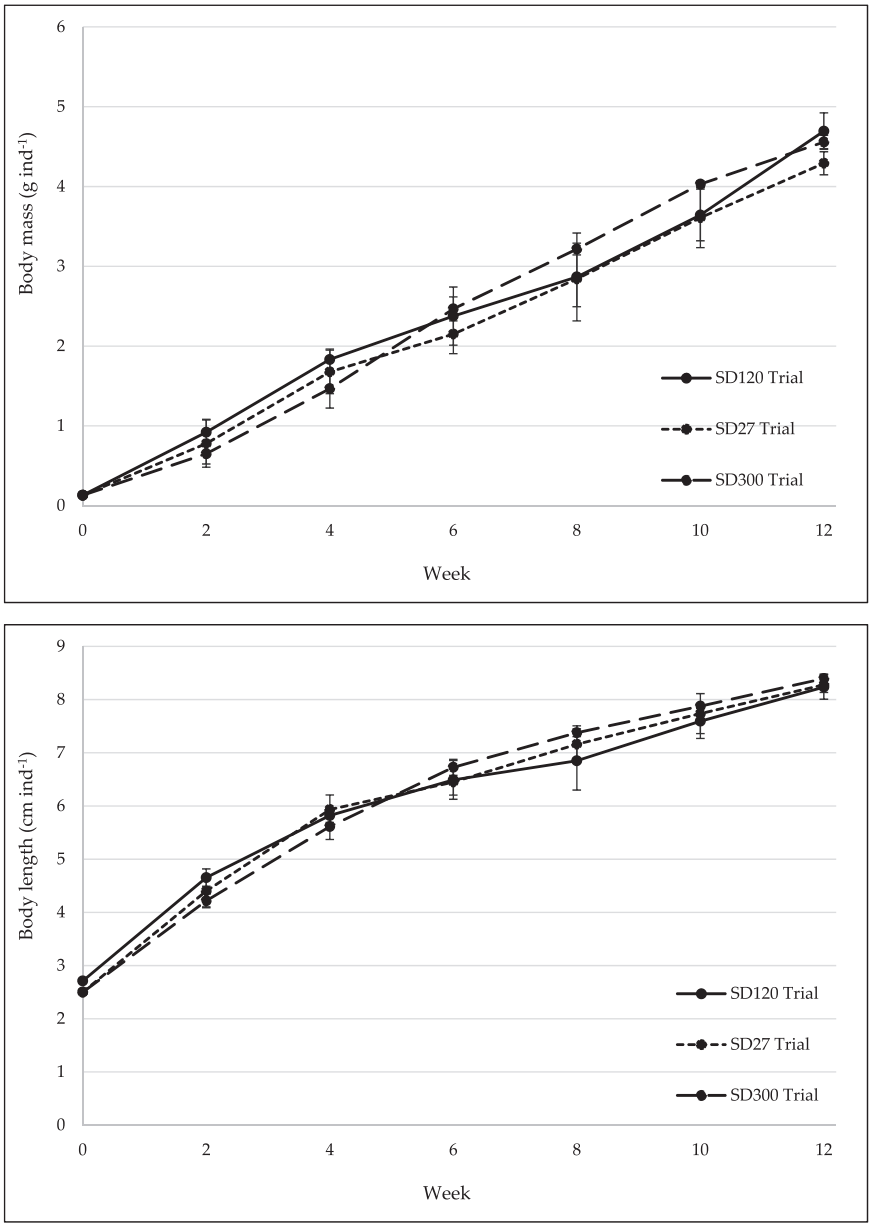

Fig. 1. Average shrimp mass (top) and length (bottom) among repeats of the inter-phase control (treatment SD120-FL100-C:N7). Data points are mean ± standard deviation of 3 replicate tanks per trial.

Table 3
Probability (P) values for the main and interaction (*) effects of the three independent factors on shrimp production parameters. P values in bold indicate significant effects ($P < 0.05$). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio, FCR = feed conversion ratio, AGR = absolute growth rate, SGR = specific growth rate.

Parameters	P values						
	SD	FL	C:N	SD*FL	SD*C:N	FL*C:N	SD*FL*C:N
Body length	**0.000**	**0.000**	**0.000**	0.783	0.335	0.490	0.880
Body mass	**0.000**	**0.000**	**0.000**	0.271	0.496	0.329	0.966
Total biomass	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.705	0.498
Survival	0.304	**0.004**	0.998	0.310	**0.033**	0.392	0.418
FCR	**0.000**	**0.007**	**0.000**	**0.047**	0.147	0.758	0.484
AGR	**0.000**	**0.000**	**0.000**	0.271	0.496	0.329	0.966
SGR	**0.000**	**0.000**	**0.000**	0.721	0.104	0.239	0.938

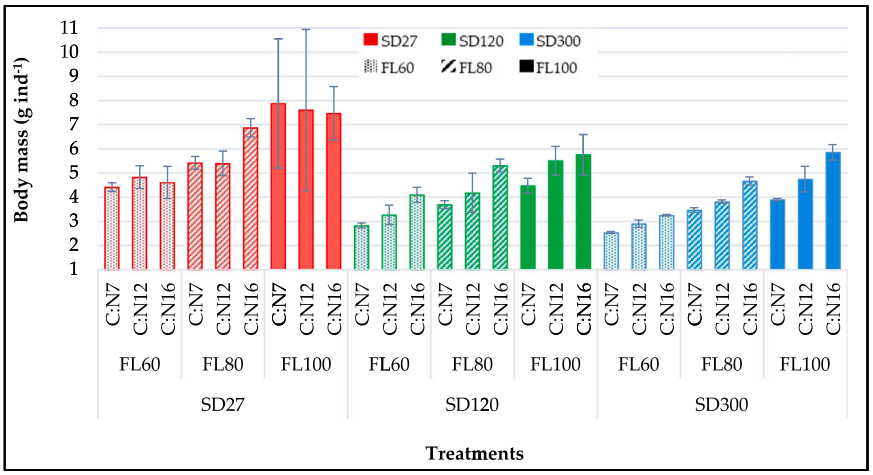

Fig. 2. Individual shrimp body mass at harvest. Column heights are mean ± standard deviation of *3* replicate tanks per treatment (Stocking density by Feeding level by C:N ratio), except for treatment SD120-FL100-C:N7 where the value is mean ± standard deviation of 9 replicate tanks. The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio.

reference Silva database (138 version) (McLaren, 2020). Owing to the variation in sequence depths between samples, all samples were normalized to the lowest depth by subsampling.

2.8. Data analysis

Statistical analysis was performed with IBM SPSS Statistics 25 software (IBM Corporation, NY, USA). One-way ANOVA analysis was used to compare the shrimp production among treatments in each grow-out trial. Three-way ANOVA analysis was performed on shrimp production parameters, combining the 3 grow-out trials. Repeated measure three-way ANOVA was performed with bi-weekly shrimp growth data, and water quality parameters. A probability value (P value) of less than 0.05 indicated a significant effect.

For the microbial community alpha-diversity analysis, Shannon H′ diversity, richness (observed taxa), and rare taxa abundance were

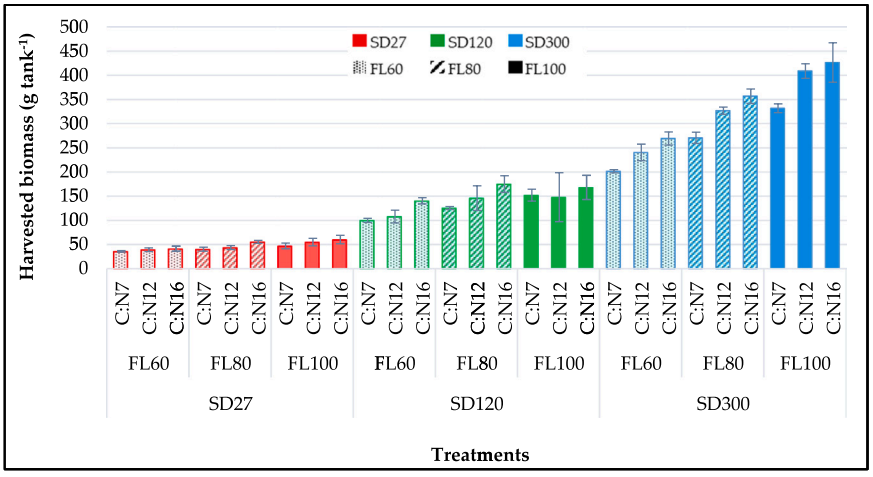

Fig. 3. Total shrimp biomass per tank at harvest. Column heights are mean ± standard deviation of 3 replicate tanks per treatment (Stocking density by Feeding level by C:N ratio), except for treatment SD120-FL100-C:N7 where the value is mean ± standard deviation of 9 replicate tanks. The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio.

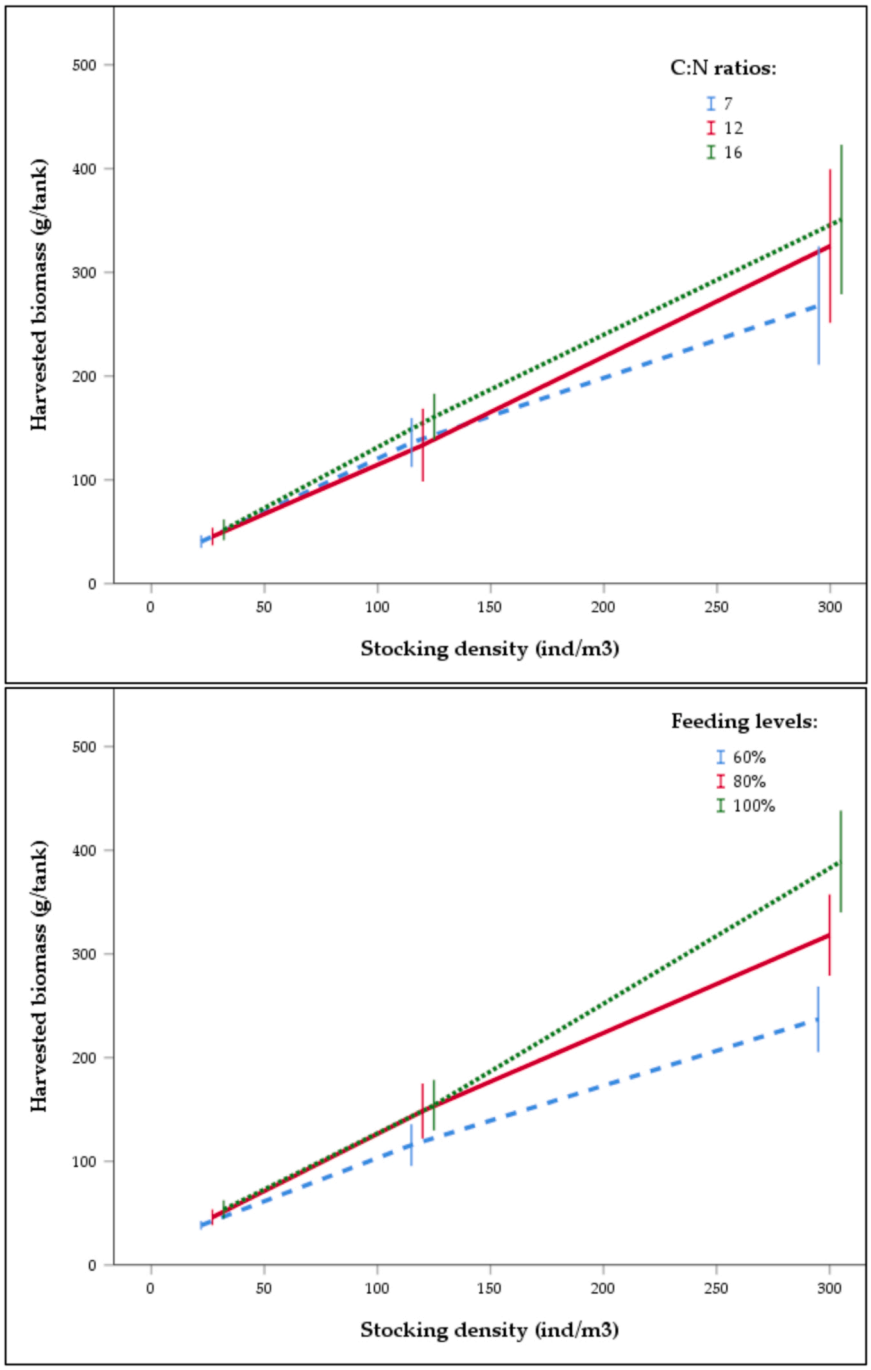

Fig. 4. Harvested shrimp biomass at three stocking densities (SD27, SD120, and SD300 ind m^{-3}) with varied feeding levels (top) and C:N ratios (bottom). Values are mean ± standard deviation of 9 tanks per treatment, except for treatments SD120-FL100 and SD120-C:N7, where values are mean ± standard deviation (SD) of 15 tanks. The lines were shifted backward and forward on the X axis to increase the visibility of the SD error bars.

calculated. Non-parametric tests (Wilcoxon test) and linear mixed-effect models (nlme R package (Pinheiro and Bates, 2007) were used to assess alpha-diversity. Adonis implementation of Permanova (Anderson, 2001) (non-parametric permutational multivariate analysis of variance) was used for comparison between groups. Exact sequent variants that had a differential abundance between feeding level and C:N ratio treatments were detected using the Deseq2 tool (Love et al., 2014).

3. Results

3.1. Inter-phase controls

The shrimp production parameters were not different among different grow-out trial repeats of the inter-phase control ($P > 0.05$) (Table 2; Fig. 1). This showed that the experimental procedure and culture conditions were similar among the grow-out trials. Therefore, data among inter-phases were combined and analyzed with three-way ANOVA.

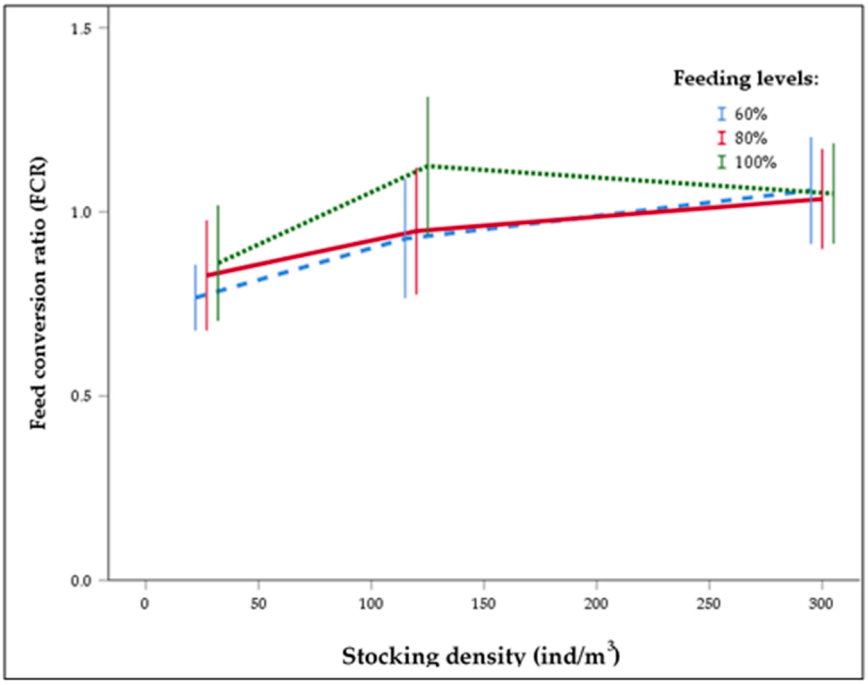

Fig. 5. Shrimp feed conversion ratio (FCR) at different stocking densities (SD27, SD120, and SD300 ind m^{-3}) with varied feeding levels. Values are mean ± standard deviation of 9 tanks per treatment, except for treatment SD120-FL100, where the value is mean ± standard deviation of 15 treatment tanks. The lines were shifted backward and forward on the X axis to increase the visibility of SD error bars.

3.2. Shrimp production

The stocking density, feeding level, and C:N ratio showed independent effects on most shrimp production parameters (Table 3). The stocking density was negatively correlated with shrimp body length and mass, absolute and specific growth rate, and feed conversion ratio, while the feeding level and C:N ratio were positively correlated with those parameters. The shrimp individual body mass and total biomass at harvest are illustrated in Figs. 2 and 3, respectively. The effects of feeding level and C:N ratio on shrimp harvested biomass varied with stocking densities (P_{SD*FL}, $P_{SD*C:N} < 0.05$), and were more pronounced at high stocking density (Fig. 4). Increasing the feeding level increased the final body mass. In contrast, the feeding level profoundly affected the FCR at low stocking density (Fig. 5). The shrimp survival rate was above 75% and the average was 93.1% across all the experimental units (data not shown) and was only affected by the feeding level ($P > 0.05$).

3.3. Biofloc growth and proximate composition

Biofloc mass in terms of total suspended solids (TSS) and volatile suspended solids (VSS) increased significantly during the experiment, here shown for days 42 and 84 of the grow-out trials (Figs. 6 and 7, respectively) (repeated measure three-way ANOVA, $P_{Time} < 0.05$). There was no significant three-way interaction among factors on biofloc TSS and VSS, however, significant interactions between stocking density and feeding level, and stocking density and C:N ratio on both TSS and VSS, and feeding level and C:N ratio on VSS were observed (Table 4). Overall, the TSS and VSS increased with increasing stocking density, feeding level and C:N ratio.

The three factors had independent effects on biofloc proximate composition, except for biofloc protein content ($P_{SD*FL*C:N} < 0.05$) (Table 5). Specifically, increasing the stocking density reduced the biofloc ash content, and increased the biofloc fat and NFE contents ($P < 0.05$) (Table 6). The feeding level showed no effect on biofloc proximate composition. Meanwhile, increasing the C:N ratio significantly reduced the biofloc ash content and increased the biofloc NFE content (Table 6).

3.4. Water quality

The repeated measure three-way ANOVA showed that the chlorophyll a and ammonia nitrogen levels significantly increased, while the nitrite nitrogen levels decreased during the experiment ($P_{Time} < 0.05$). The stocking density, feeding level, and C:N ratio showed independent effects on the chlorophyll a concentrations in the water column (Table 7). Specifically, the chlorophyll a concentration was not affected by the feeding level, but increased with increasing stocking densities and C:N ratios. There were significant interactions among the three factors on the TAN levels ($P_{SD*FL*C:N} < 0.05$). Meanwhile, nitrite nitrogen was significantly affected by stocking density and C:N ratio interactions, but not the feeding level.

3.5. Microbial communities

Linear mixed effects model analysis for the biofloc microbial community (prokaryotes) alpha-diversity at the high stocking densities (richness, Shannon H′ and rare taxa abundance) revealed significant effects coming from C:N ratio (Fig. 8B; $P_{Shannon} = 0.0280$) and feeding level (Fig. 8C; $P_{rare} = 0.0206$), respectively, showing an increase with increasing levels of those variables.

Looking at the beta-diversity (microbial composition), the microbial communities were affected by both the C:N ratio and the feeding level (Two-way Permanova analysis; $P = 0.001$; Table 8). Significant differences were mainly found between C:N ratio 7 and 16 ($P = 0.003$) and feeding level 60 and 100 ($P = 0.006$), as indicated by Permanova (Bray-Curtis metric) and Principal component Analysis (Fig. 9).

The relative abundance of the most abundant prokaryotic phyla and genera are presented in Fig. 10. Among the phyla, Proteobacteria was the most abundant, followed by Bacteroidota, Actinobacteria, Planctomycetote and Chloroflexi. Regarding genera, a large portion of the microbial communities was not assigned to a genus (60% of the relative abundance), while the most abundant genera were *Pirelulla (4.3%)*,

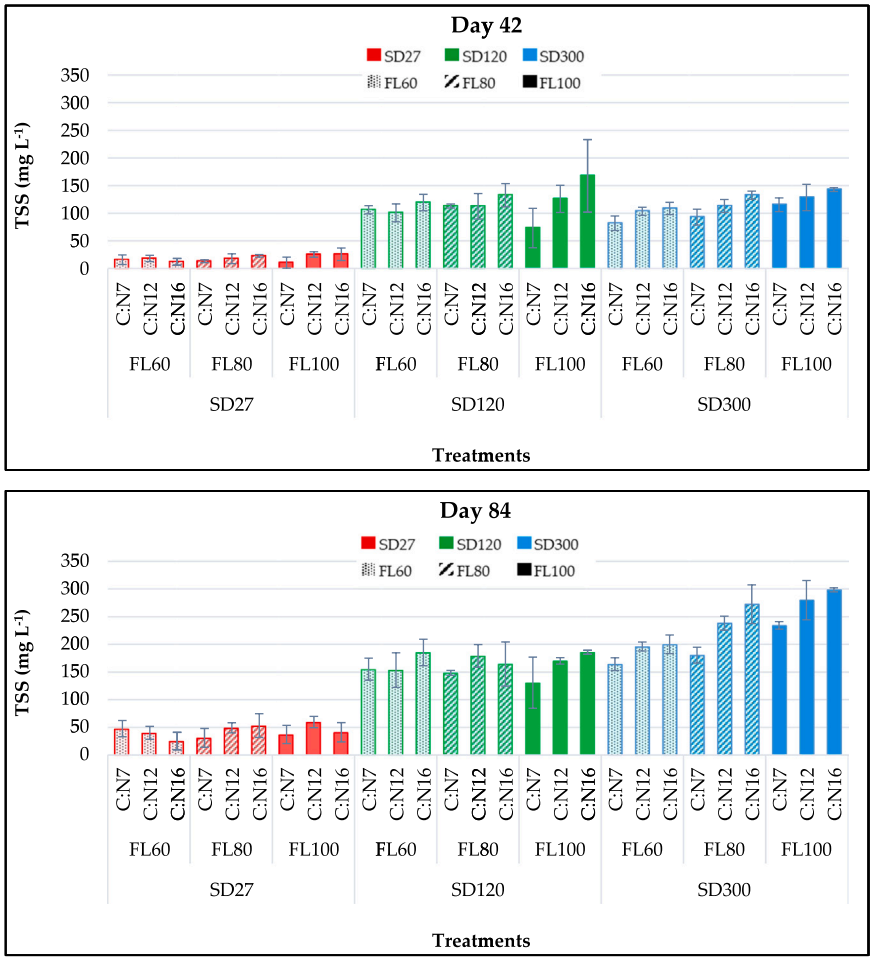

Fig. 6. The total suspended solids (TSS) on days 42 (top) and 84 (bottom) of the experiment. Column heights are mean ± standard deviation of 3 replicate tanks, except for treatment SD120-FL100-C:N7 with 9 replicate tanks per treatment (stocking density by feeding level by C:N ratio). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio.

Robiginitalea (5%), and *Muricauda (2%)*. Looking at specific taxa that were affected by feeding level and C:N ratio, we detected several taxa using the DESeq2 and SIMPER analysis ($P < 0.05$; Fig. 11). *Pirellula* and *Maricauda* genus increased with feeding level and C:N ratio (Figs. 11B, 11C), while on the other hand *Pseudoalteromonas* decreased with feeding level, but increased with C:N ratio (Fig. 11A).

4. Discussion

4.1. Shrimp

Shrimp exhibited a better growth with low stocking density and with high feeding level and C:N ratio. The inverse relationship between stocking density and shrimp growth and survival was previously reported in conventional ponds (Sandifer et al., 1987; Sookying et al., 2011), recirculating systems (Williams et al., 1996), and biofloc systems in seawater (Krummenauer et al., 2011) or freshwater (Araneda et al., 2008). Reductions in growth and survival of cultured animals at high stocking density could be related to increased competition for space and feed, and reduced water quality (Araneda et al., 2008; Esparza-Leal et al., 2020; Liu et al., 2017; Williams et al., 1996). Moreover, previous research indicated that at high stocking density, cultured shrimp show depressed digestive enzyme activities, antioxidant status and immunology parameters (Esparza-Leal et al., 2020; Liu et al., 2017; Said et al., 2022), which could potentially explain our results.

In systems with high primary production (e.g. flow-through system), the feeding level was reported to not significantly affect growth and survival, while the FCR significantly increased with increasing feeding level (Roy et al., 2012). Similar results were found in biofloc systems with a TSS concentration of 246 ± 67 mg/L SD (Lara et al., 2017). The biofloc might contribute up to 29% of daily food intake (Burford et al., 2004). Guemez-Sorhouet et al. (2019) demonstrated that in biofloc systems, shrimp store a surplus of glycogen and carbohydrates in their hepatopancreas, which may increase their resistance to stress. In this research, promoting the biofloc development by increasing the C:N ratio also showed positive effects on shrimp growth. However, lowering the feeding level significantly reduced shrimp growth and survival. This could be due to low biofloc concentrations in most treatment tanks during the experiment, only at the end reaching 300 mg/L in some tanks, which was not able to compensate the effects of reduced feeding level. Schveitzer et al. (2013b) and da Silveira et al. (2020) suggested a suitable TSS concentration of 400–600 mg/L for Pacific white shrimp culture. Higher TSS concentration may negatively affect shrimp survival and final biomass (Schveitzer et al., 2013a; Prates et al., 2023). Overall, in systems with abundant natural food, the daily feed ration can be lowered to as low as 60% without affecting the shrimp growth, while enhancing profitability (Roy et al., 2012). In our results, we observed that 20% feed reduction can be compensated by biofloc (C:N increase), thus supporting existing literature.

Shrimp culture systems are dynamic, and interactions among variables in the systems have been of interest to many researchers. Moss and Moss (2004) reported significant effects of both stocking density and culture condition (i.e. substrate addition) on shrimp growth, but no significant density, substrate effect, or density and substrate interaction

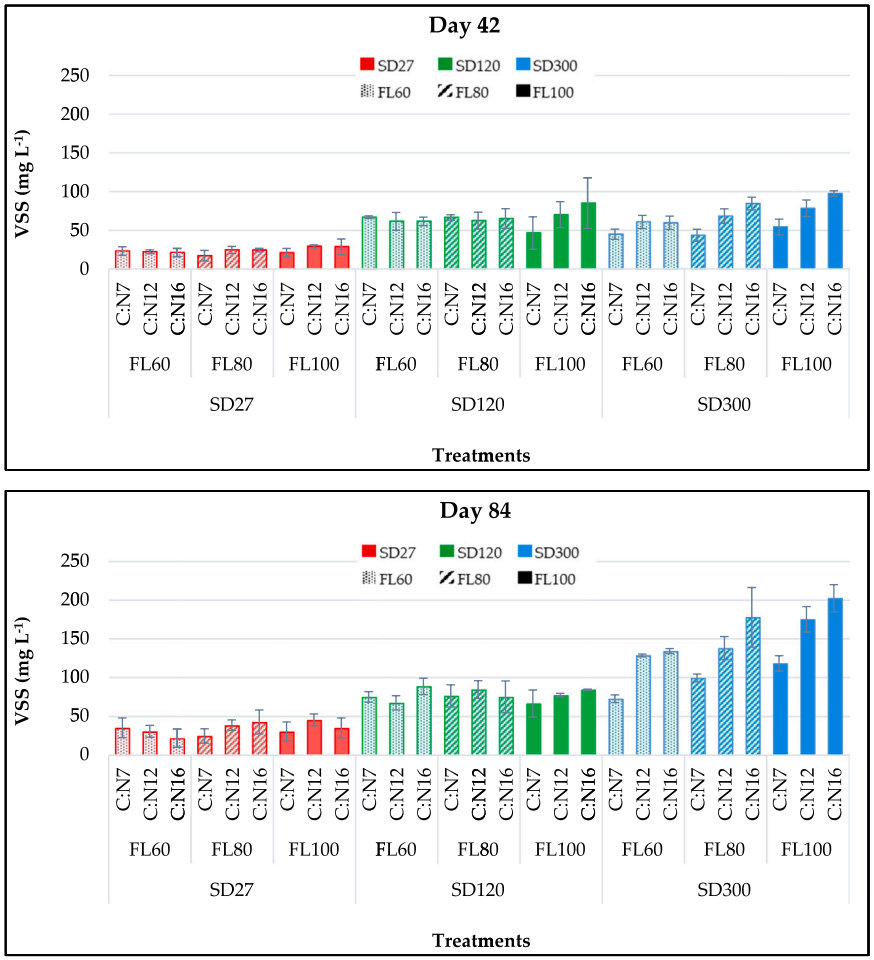

Fig. 7. The volatile suspended solids (VSS) on days 42 (top) and 84 (bottom) of the experiment. Column heights are mean ± standard deviation of 3 replicate tanks, except for treatment SD120-FL100-C:N7 with 9 replicate tanks per treatment (stocking density by feeding level by C:N ratio). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio.

Table 4
Probability (P) values for the main and interaction (*) effects of the three independent factors on biofloc growth. P values in bold indicate significant effects ($P < 0.05$). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio, TSS = total suspended solids, VSS = volatile suspended solids.

Parameters	P values						
	SD	FL	C:N	SD*FL	SD*C:N	FL*C:N	SD*FL*C:N
TSS (mg/L)	**0.000**	**0.000**	**0.000**	**0.000**	**0.011**	0.135	0.277
VSS (mg/L)	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.032**	0.250

Table 5
Probability (P) values for the main and interaction (*) effects of the three independent factors on biofloc proximate composition. P values in bold indicate significant effects ($P < 0.05$). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio, NFE = nitrogen-free extract.

Parameters	P values						
	SD	FL	C:N	SD*FL	SD*C:N	FL*C:N	SD*FL*C:N
Ash	**0.000**	0.655	**0.003**	0.161	0.209	0.261	0.080
Fat	**0.012**	0.686	0.892	0.220	0.348	0.980	0.861
Protein	**0.000**	0.112	0.351	0.460	0.145	0.169	**0.002**
NFE	**0.000**	0.528	**0.010**	0.298	0.070	0.330	0.371

on survival or water quality. Meanwhile, Guemez-Sorhouet et al. (2019) demonstrated stocking density and culture system interactions on shrimp growth. In biofloc and biofloc with substrate systems, shrimp growth was negatively correlated with stocking density, while such correlation was not found in clear water systems where nitrite and nitrate concentrations were higher than in other systems (Guemez-Sorhouet et al., 2019). Interactions among stocking density, feeding level, and C:N ratio on shrimp growth were not observed in this research. Nevertheless, we found interactions between the stocking density, feeding level, and C:N ratio on total biomass, survival, and FCR of the shrimp.

Table 6
Biofloc proximate composition at different stocking densities of L. vannamei, and C:N ratios of nutrient inputs. Values are mean ± standard deviation of 27 tanks for the stocking densities 27 and 300 ind m^{-3}, and of 33 tanks for the stocking density 120 ind m^{-3}. Within rows, different superscripts indicate significant differences among stocking densities or C:N ratios ($P < 0.05$). NFE = nitrogen free extract.

Parameters	Stocking densities (ind m^{-3})			C:N ratios		
	27	120	300	7	12	16
Ash (%)	53 ± 5^{a}	43 ± 11^{b}	26 ± 13^{c}	47 ± 15^{a}	39 ± 15^{b}	36 ± 15^{b}
Fat (%)	0.6 ± 0.6^{a}	0.8 ± 0.8^{ab}	1.2 ± 0.6^{b}	0.9 ± 0.8	0.9 ± 0.5	0.8 ± 0.8
Protein (%)	17 ± 2^{a}	21 ± 4^{b}	12 ± 5^{c}	17 ± 6	17 ± 6	18 ± 5
NFE (%)	30 ± 3^{a}	35 ± 11^{a}	61 ± 12^{b}	35 ± 15^{a}	43 ± 17^{b}	45 ± 14^{b}

4.2. Water

In Moss and Moss (2004), the stocking density, substrate, or their interaction showed no effect on DO concentration and pH, while treatment effects on TAN and nitrite were not reported. Interestingly, Krummenauer et al. (2011) found similar nitrite concentrations among stocking densities, while TAN levels were lower at high stocking density. Contrasting results were reported by Tong et al. (2020), in which the TAN concentration was not affected by the feeding level, and the nitrite concentration increased with feeding level.

The positive effects of increasing the C:N ratio on TAN, nitrite and nitrate concentration reduction were demonstrated in numerous research (Asaduzzaman et al., 2008; Dauda et al., 2018; Hari et al., 2006). However, results were also reported, in which the addition of carbohydrate showed no significant effects on TAN, nitrite and nitrate (Pérez-Fuentes et al., 2016; Xu and Pan, 2012; Xu et al., 2016). This could be due to the different carbon sources used (Table 9). Tinh et al. (2021a) demonstrated that at the same C:N ratio, corn starch and molasses affected the shrimp culture system differently. In this research,

Table 8
Permanova results for biofloc microbiota based on Bray–Curtis distances.

	d.f.	SS	MS	PseudoF	R^2	*P*-value
Feeding level	1	0.3920	0.39195	2.7103	0.09534	0.001 * *
C:N	1	0.4625	0.46254	3.1984	0.11251	0.001 *
Feeding level x C:N	1	0.2198	0.21975	1.5195	0.05345	0.056
Residuals	21	3.0369	0.14462		0.73870	
Total	24	4.1112			1.00000	

* and * * Statistical significance at $P < 0.05$ and 0.001, respectively. Permutations n = 999. d.f., degrees of freedom; SS, sum of squares; MS, mean sum of squares.

Table 7
Probability (P) values for the main and interaction (*) effects of the three independent factors stocking density, feeding level and C:N ratio on water quality. P values in bold indicate significant effects ($P < 0.05$). The abbreviations are SD = stocking density, FL = feeding level, C:N = carbon:nitrogen ratio, TAN = total ammonia nitrogen, NO_2-N = nitrite nitrogen.

Parameters	P values						
	SD	FL	C:N	SD*FL	SD*C:N	FL*C:N	SD*FL*C:N
Chlorophyll a (μg/l)	**0.000**	0.910	**0.037**	0.680	0.766	0.205	0.296
TAN (mg/L)	**0.000**	**0.029**	0.143	**0.002**	0.220	0.951	**0.044**
NO_2-N (mg/L)	**0.000**	0.199	0.305	0.064	**0.014**	0.284	0.075

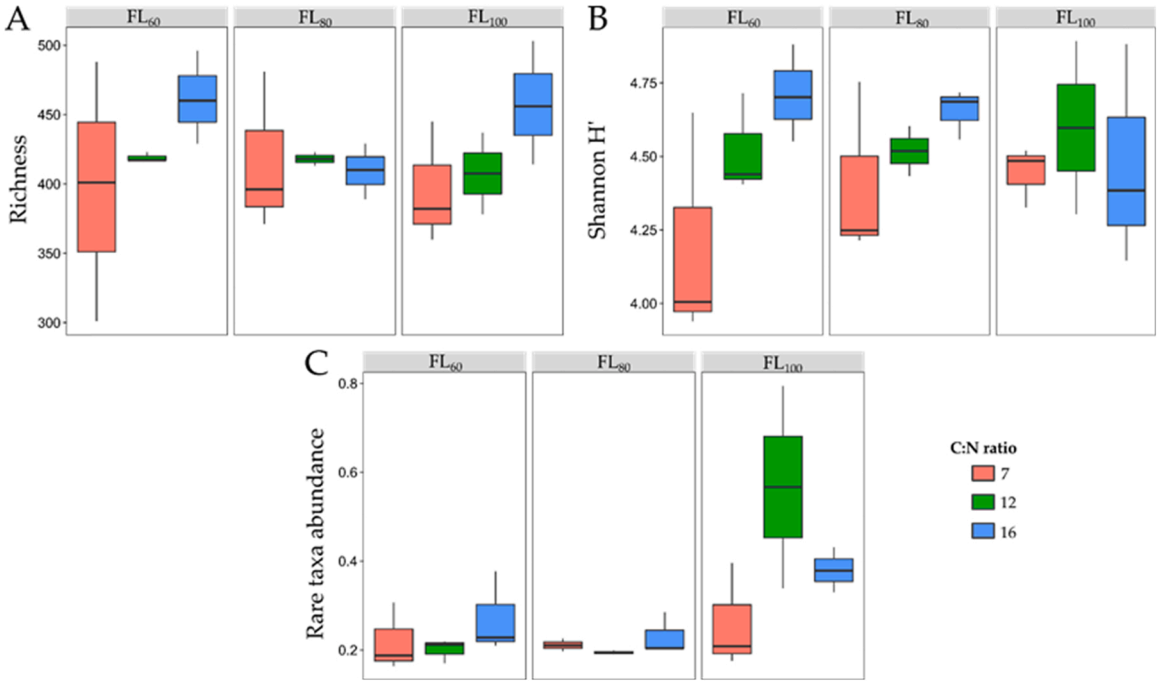

Fig. 8. Alpha diversity of the biofloc microbial communities in the different treatments, referring to feed levels FL60, FL80 and FL100 and carbon:nitrogen ratios C:N7, C:N12 and C:N16. Data are shown as box plots; the horizontal line indicates the median and the whiskers indicate the lowest and highest points within 1.5 × the interquartile ranges of the lower or upper quartile, respectively.

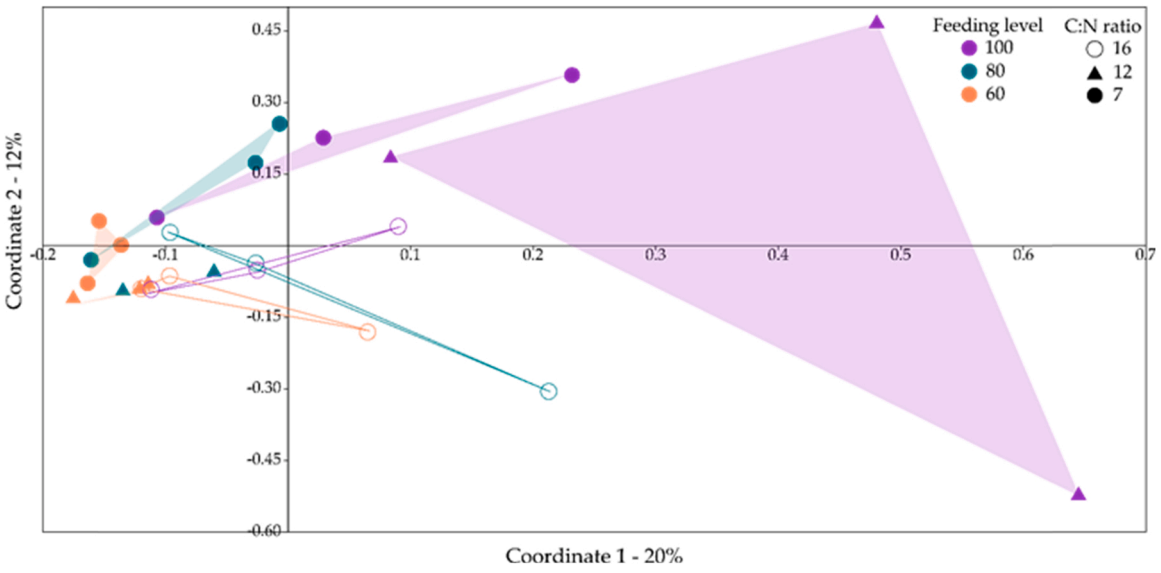

Fig. 9. Principal coordinate analysis (PCoA) using Bray-Curtis metric. There was a significant clustering (Two-way Permanova, P = 0.001) affected by both the C:N ratio and the feeding level.

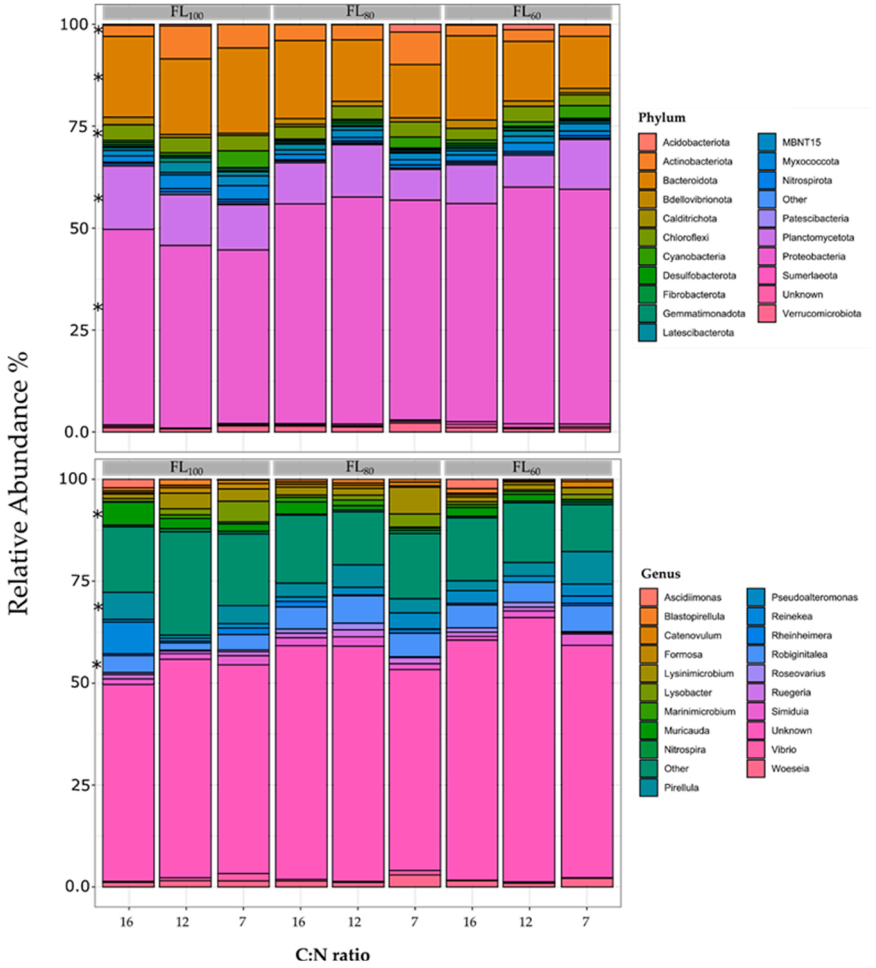

Fig. 10. Prokaryotic microbial composition of the biofloc samples at the phylum (top) and genus level (bottom) among different treatments, referring to feed levels FL60, FL80 and FL100 and carbon:nitrogen ratios C:N7, C:N12 and C:N16. Stars indicate the most abundant phyla and genera.

we found significant three-way interactions between stocking density, feeding level, and C:N ratio on TAN concentrations. In addition, the nitrite concentrations were not affected by the feeding level, but significantly influenced by the stocking density x C:N ratio interaction. Therefore, differences in the experimental design, for example tested species, stocking densities, experimental durations, or biofloc

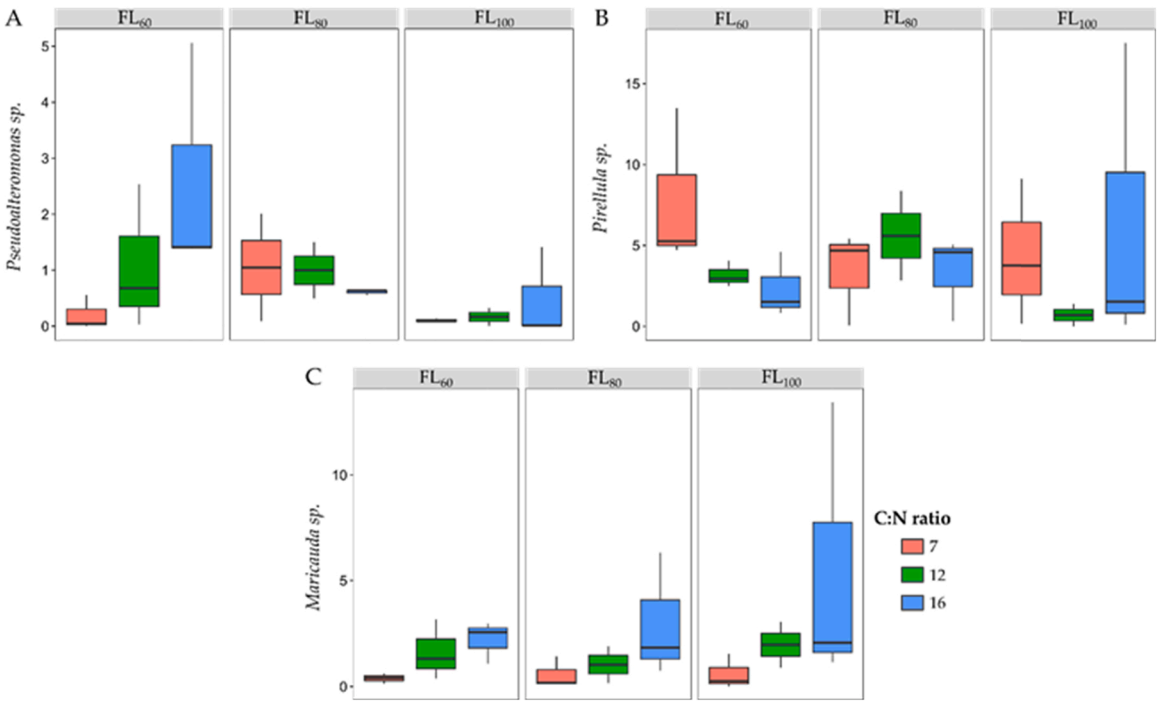

Fig. 11. Relative abundance of significantly affected microbial taxa within the biofloc communities, as indicated by DESeq analysis ($P < 0.05$), referring to feed levels FL60, FL80 and FL100 and carbon:nitrogen ratios C:N7, C:N12 and C:N16 . Data are shown as box plots; the horizontal line indicates the median and the whiskers indicate the lowest and highest points within 1.5 × the interquartile ranges of the lower or upper quartile, respectively. On the Y-axis, relative abundance is expressed in %.

Table 9
Some parameters of the experimental design among research on C:N ratios supporting or not supporting significant effects on TAN, nitrite and nitrate.

Carbon addition	References	Tested species	Density (ind/m^3)	Duration (weeks)	Tested C:N ratios	Carbon sources
Supported	Asaduzzaman et al. (2008)	*M. rosenbergii*	2	16	10, 15, 20	tapioca
	Dauda et al. (2018)	*C. gariepinus*	250	6	10, 15, 20	glycerol
	Hari et al. (2006)	*P. monodon*	7	16		tapioca
Not supported	Pérez-Fuentes et al. (2016)	*O. niloticus*	75	24	10, 12, 15, 17, 21	molasses
	Xu and Pan (2012)	*L. vannamei*	224	4	7, 12, 15	sucrose
	Xu et al. (2016)	*L. vannamei*	300	6	9, 10, 12, 15, 18	molasses

concentration and composition (Table 9), could also be the reason for contradictory conclusions on the effect of increasing C:N ration. This again confirmed the dynamics of shimp culture systems, and that biofloc systems are influenced by numerous additional factors, for example dietary ingredient sources (Shao et al., 2017), carbohydrate types (Tinh et al., 2021a), and biofloc levels (Schveitzer et al., 2013b).

4.3. Biofloc

In numerous research, biofloc mass in terms of TSS and VSS are expected to increase with feeding level and C:N ratio (Asaduzzaman et al., 2010; Panigrahi et al., 2018; Pérez-Fuentes et al., 2016; Tong et al., 2020; Xu and Pan, 2012). It was also reported that the feeding level, C:N ratio, or their interaction showed no significant effects on both TSS and VSS (Xu and Pan, 2014). Meanwhile, Xu et al. (2016) found that while the TSS concentration was not correlated with C:N ratio, the VSS and C:N ratio were positively correlated. Interestingly, Liu et al. (2018) demonstrated that the biofloc mass increased more rapidly in a carbohydrate-added system, but reached a similar plateau in all treatments at the end of the experiment. In this research, biofloc mass was affected by various two-way interactions between stocking density, feeding level and C:N ratio. At higher stocking densities, positive correlations between biofloc mass and feeding level or C:N ratio are more likely to be seen.

The biofloc protein content, 17 ± 5% SD on average for all treatments, was low compared to most previous research. This could be due to the type of carbohydrate used (rice bran – tapioca mixture). Tinh et al. (2021a) reported a biofloc protein content of 39–43%, depending on whether corn starch or molasses was used as the carbon source. The biofloc proximate composition was mainly affected by the stocking density, and to a lesser extent by C:N ratio in this research, while the biofloc protein content was affected by the three-way interaction among factors.

4.4. Microbial communities

Microbial diversity within the biofloc, composed of heterotrophic bacteria, microalgae, zooplankton and rotifers (De Schryver et al., 2008), has been reported to be affected by the C:N ratio (Deng et al., 2018; Guo et al., 2020; Panigrahi et al., 2018). This was also observed in the present study, with diversity increasing with C:N ratio. How the feeding level affects the microbial diversity in bioflocs, to our knowledge, has not been reported before. In this research, the microbial diversity and abundance of rare taxa (non-core taxa) increased with feeding level. This could be potentially related to a higher presence of nutrients available to the biofloc microbiota due to either a higher waste

production by the shrimp in tanks receiving more feed or carbon. A similar finding with an increase in biofloc microbial diversity with increasing nutrients due to stocking density was reported in shrimp biofloc systems (Deng et al., 2019). Although we did not evaluate the effect of stocking density on the biofloc microbial communities in this research, higher stocking densities producing higher nutrient inputs from feed and waste excretion have been reported to lead to more diverse communities (Deng et al., 2019). Interestingly, high microbial diversity in water and shrimp gut have been reported to increase system stability and shrimp tolerance to diseases (De Schryver and Vadstein, 2014; Rungrassamee et al., 2016). In this study, we reported a higher diversity with feeling level and C:N ratio, but we did not explore effects on shrimp health; however, this could be an interesting direction for future studies.

Besides the amount of nutrients, the types of carbohydrate used play an important role in shaping the microbial community in biofloc systems (Deng et al., 2018; Li et al., 2018). With regard to the microbial community composition, our results are in accordance with previous studies, with Proteobacteria being the most abundant phylum, followed by Bacteroidetes, Planctomycetes and Actinobacteria, and genera such as *Maricauda* and *Ruegeria* (Deng et al., 2019; Gutiérrez et al., 2016; Huang et al., 2020; Tinh et al., 2021b). We observed a significant increase on the abundance of *Maricauda sp*. and *Pseudoalteromonas sp*. taxa with increasing C:N ratio. The marine bacteria *Pseudoalteromonas* and *Muricauda* sp., which have been previously identified as core species in biofloc (Deng et al., 2019; Huang et al., 2020; Tinh et al., 2021b) while at high C:N ratios both have been reported to increase their biomass (López-Alcántara et al., 2022; Zhang et al., 2022), which is in agreement with our data. Interestingly, these two genera have been also reported to exhibit antimicrobial properties against pathogenic microbes (i.e *Staphylococcus aureus*) and quorum quenching properties, respectively (López-Alcántara et al., 2022; Zhang et al., 2022), indicating a potential beneficial effect for shrimp health; however as mentioned before such an effect was not explored in the current study. These altogether show that the microbiota in biofloc systems are sensitive to variations in the system management, and more research is needed to fully understand its mechanism and direction of changes, especially if these are connected to health-promoting communities.

5. Conclusions

To conclude, in the current study we show that shrimp growth and biofloc biomass gain (TSS, VSS) can be affected in a similar way by stocking density, feeding level, and C:N ratio. Meanwhile, the biofloc proximate composition was mainly affected by stocking density, and to a lesser extent by C:N ratio. On the other hand, the microbial community composition was altered by increasing C:N ratio and feeding levels, showing an increasing diversity and enrichment of core biofloc taxa. Future research should focus on how the type of carbohydrates and methods of administration affect shrimp growth and biofloc formation, and how this may promote health benefits through the microbial community manipulation.

CRediT authorship contribution statement

Tran Huu Tinh: Software, Formal analysis, Methodology, Investigation, Validation, Data curation, Writing – original draft, Visualization, **Fotini Kokou:** Data curation, Visualization, Writing – review & editing, **Tran Ngoc Hai:** Writing – review & editing, **Johan A. J. Verreth:** Writing – review & editing, Funding acquisition, **Marc C. J. Verdegem:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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