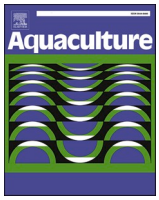
Aquaculture 543 (2021) 736890

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Effects of carbohydrate addition methods on Pacific white shrimp (*Litopenaeus vannamei*)

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ARTICLE INFO

Keywords:
Biofloc
Carbohydrate addition method
Litopenaeus vannamei
Microbial communities
Nutrient budget
Periphyton

ABSTRACT

The addition of external carbohydrate in Pacific white shrimp (*Litopenaeus vannamei*) culture has shown positive effects on water quality and shrimp performance. However, this practice requires additional skills and labor, and therefore may not be widely adopted. This research investigated the potential to combine the carbon source and the feed in one pellet in shrimp culture. The carbon source used in this experiment was corn starch. The experiment was executed in 6 indoors mesocosms tanks, with full control of the water source, temperature, and light intensity and duration. The three treatments including COM-Feed (commercial feed), COM-Feed + CHO (commercial feed with separate corn starch addition), and CHO-Feed (pelleted diet made by incorporating additional corn starch into the commercial feed) were randomly assigned to the mesocosms tanks, and run two times consecutively. Adding corn starch separately resulted in higher shrimp biomass gain and protein efficiency ratio ($P < 0.05$), compared to dietary corn starch addition and no corn starch addition. Carbohydrate addition caused a significant drop in inorganic nitrogen and orthophosphate concentrations in the water. The treatments had no effect on the biofloc and periphyton growth, and the proximate composition of shrimp, biofloc, and periphyton, however increased the eukaryotic microbial diversity in the bioflocs. Meanwhile, the carbohydrate addition reduced the dietary energy and carbon utilization efficiencies regardless of the carbohydrate addition methods.

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is an important farmed species, accounting for more than 52% of global crustaceans production in 2018 (FAO, 2020). Intensive farming of this species relies heavily on protein rich feed because the natural productivity of the pond food web is insufficient to sustain the desired high productivity. Xia et al. (2010) shows that Pacific white shrimp performs best with feed of 38–40% dietary protein levels. However, the use of high protein feed in large quantity is often associated with ammonia and nitrite accumulations in water. Exposure to high concentrations of these substances may negatively affect the metabolic responses of Pacific white shrimp (Racotta and Hernández-Herrera, 2000).

Manipulating the C/N ratio in the system through organic carbohydrate addition directly into the water provides energy for bacteria to assimilate nitrogenous compounds into microbial biomass (Avnimelech, 1999). By increasing the C/N ratio in the system, the total ammonia, nitrite, and nitrate nitrogen concentrations are significantly reduced (Panigrahi et al., 2019). This practice improves water quality and Pacific white shrimp production (Panigrahi et al., 2019; Ren et al., 2019). The beneficial effects of carbohydrate additions into the water were demonstrated in several fish culture systems (e.g. Magondu et al., 2013; Pérez-Fuentes et al., 2016) and shrimp culture systems (Asaduzzaman et al., 2008; Hari et al., 2004; Panigrahi et al., 2019; Xu and Pan, 2012). In a minimal water exchange system for Pacific white shrimp, a C/N ratio of 15 was optimal for shrimp survival, growth, and immune activity (Panigrahi et al., 2019).

Adding extra carbohydrate into the water in addition to feeding requires additional skills and labor which may not be favored by farmers. If the additional carbohydrate can be mixed in the feed to produce pellets that provide jointly carbohydrates for the microbes in the pond and nutrients for the shrimp, system management is simplified and more easily adopted. However, according to Xia et al. (2010), the inclusion of carbohydrate in the feed at the expense of protein increases the overall

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https://doi.org/10.1016/j.aquaculture.2021.736890
Received 17 February 2021; Received in revised form 10 April 2021; Accepted 6 May 2021
Available online 8 May 2021

feed digestibility, but reduces the protein digestibility. This may increase nutrient excretion and reduce shrimp growth, at the same time provide more substrate for microbial growth, and promote the development of bioflocs. Guo et al. (2006) also demonstrated that the dietary corn starch inclusion levels affects the feed digetibility, shrimp growth rate, survival and feed conversion ratio (FCR), being optimal at 10–15% dietary content(Guo et al., 2006)(Guo et al., 2006)(Guo et al., 2006)(Guo et al., 2006)(Guo et al., 2006)(Guo et al., 2006). It is unknown how the inclusion of carbohydrate source in the diet will affect the shrimp culture system, compared to biofloc system. This research therefore investigated the effect of carbon supplementation as well as the potential of including the carbohydrate in the feed for biofloc culture of Pacific white shrimp.

2. Materials and methods

2.1. Biofloc stimulation

Four weeks prior to the experimental start, biofloc growth was stimulated in three indoor tanks (with a working volume of 0.8 m^3 per tank) stocked with Nile tilapia (*Oreochromis niloticus*). The fish, which were obtained from the Carus Experimental Station of Wageningen University, were stocked at a density of 0.5 kg/m^3, corresponding to 400 g of fish per tank. Each tank was provided with continuous aeration, and 12 h/12 h dark/light regime. The fish were fed a diet containing 33% protein, twice daily at 08.00 and 16.00 h, at 2.5% body weight per day, assuming an FCR of 1.0. Native corn starch was added one hour after each feeding to maintain a C/N ratio of 20 of the combined feed and carbohydrate input to stimulate the biofloc development.

Tilapia tanks were first filled with 160 L of freshwater. The water salinity in these tanks was gradually increased by addition of salt water to reach the target salinity of 25 ppt after 4 weeks to a final volume of 800 L/tank. At the start of the shrimp experiment, tilapia were removed and the biofloc water from these three tanks was pooled (total 2.4 m^3) and well mixed. The mixed biofloc water was equally distributed over the six experimental tanks (400 L per tank). Saline and tap water were added to bring the water volume to 600 L per tank and salinity of 25 ppt.

2.2. Experimental design

The experiment consistent of three different treatments. The first treatment ("COM-Feed") consisted of feeding a pelleted diet that mimicked a commercial diet having a C/N ratio of 7.6 g/g. For the second treatment ("CHO-Feed"), consisted of feeding a pelleted diet with a high C/N ratio (14.6 g/g). The pelleted "CHO-Feed" consisted of 50% "COM-Feed" and 50% gelatinized corn starch. For the third treatment ("COM-Feed + CHO") the same amount of "COM-Feed" and corn starch were added to the tanks, but starch was directly added to the tanks instead of being included into the pelleted diet. At the tank level also for the "COM-Feed + CHO" treatment the C/N ratio was 14.6 g/g.

In order to increase the number of replicates (i.e., tanks) per treatment, the experiment consisted of two trials, and data from both trials were combined for statistical analysis. Each trial lasted 4 weeks and was performed with 6 tanks (i.e., 6 experimental units). Within each trial, each treatment was randomly assigned to the experimental units in duplicate. The experimental conditions were identical between both trials. All tanks were connected to one central air blower, which continuously aerated through a round plastic pipe positioned at the bottom of each tank. The pipe was 0.6 m in diameter with eight 0.5 cm holes, allowing equal air diffusion in the water column and maintaining an oxygen concentration above 6 mg/L. A lighting regime of 12 h light and 12 h dark was applied, and the temperature was kept constant with a heater at 27 °C.

2.3. Experimental animals and feeding

Pacific white shrimp (*Litopenaeus vannamei*) were obtained from CreveTec bvba, Ternat, Belgium. From a base population, 300 shrimp of similar initial weight (3.2 ± 0.04 and 3.4 ± 0.05 g for trial 1 and 2, respectively) were selected and randomly stocked into the experimental tanks (50 shrimp per tank). Two experimental diets were produced by steam pelleting using a die size of 2 mm resulting in sinking pellets by Research Diet Services (Wijk bij Duurstede, The Netherlands). The ingredient and analyzed nutrient composition of both diets are shown in Table 1. During the experiment, shrimp were fed with commercial feed or corn starch-mixed feed according to their assigned treatment twice daily at 08.00 and 16.00 h. The amount of feed for treatments COM-Feed and COM-Feed + CHO was calculated using a feeding rate of 5% BW/day, an assumed feed conversion ratio (FCR) of 1.3 and a survival rate of 95% for the whole experimental period. Additionally, the treatment COM-Feed + CHO received the same amount of corn starch after every feeding as the amount of COM-feed administered. Treatment CHO-Feed received double the amount of feed to ensure similar protein input among treatments.

2.4. Water quality monitoring

During the experiment, temperature, salinity and pH in experimental tanks were measured daily using a multi-parameter electronic meter (WTW Multi3630IDS™). Water quality parameters including NH_4^+, NO_2^-

Table 1
Ingredients and nutrient composition of the experimental diets used in this experiment.

	Commercial feed (COM-Feed)	Corn starch-mixed feed (CHO-Feed)	Corn starch (CHO)
Ingredient composition (%):			
Gelatinized corn starch	–	50	100
Wheat	20.58	10.29	
Wheat flour	15	7.5	
Wheat gluten	10	5	
Wheat bran	15	7.5	
Soybean meal	12	6	
Fishmeal LT (CP > 680)	20	10	
Fishmeal hydrolysate (CPSP)	1	0.5	
Soy lecithin	0.7	0.35	
Fish oil	2	1	
Cholesterol	0.3	0.15	
Calcium carbonate	0.5	0.25	
Mono calcium phosphate	1.2	0.6	
L-lysine	0.2	0.1	
DL-Methionine	0.3	0.15	
L-Threonine	0.2	0.1	
Premix	1	0.5	
Yttrium oxide	0.02	0.01	
Total	100	100	
Nutrient composition (g/kg DW):			
Dry matter (g/kg WW)	934	928	873
Crude protein	394	200	1
Crude fat	70	39	6
Ash	72	37	1
Energy (kJ/g)	20	19	17
Total carbon (g/kg WW)	447	433	398
Total nitrogen (g/kg WW)	59	30	1
C/N ratio	7.6	14.6	294

and NO_3^- were monitored by sensitivity test using the MColortest™ kit, while the biofloc volume was measured with Imhoff cones.

2.5. Sample collection and analysis

Water samples were collected weekly, starting on the day of stocking. Plastic bottles of 2 L were held in the middle of the water column, and then opened to fill with culture water. A magnet was inserted in each bottle for magnetic stirring at the speed of 350 rpm to keep the biofloc in suspension during the sub-sampling process. Sub-samples of 100 mL were taken from each bottle, and filtered through 1.5 μm pore size filter paper. The filtrates were acidified to pH of 2–3 with HCl and immediately submitted to CBLB laboratory (Chemical Biological Soil Laboratory, Wageningen University, The Netherlands) for the following analyses: total carbon (TC), inorganic carbon (IC), total nitrogen (TN), total ammonia nitrogen (TAN), nitrite and nitrate (NO_x), phosphate phosphorus (PO_4-P) using a segmented flow analyzer (SAN++, Skalar Analytical B.V., The Netherlands). The chlorophyll *a* contents were determined every two weeks using unfiltered water samples (APHA, 1995).

Biofloc samples were collected at the start, middle and end of the experiment. The samples were taken by filtering 100 mL of culture water through 1.5 μm pore size filter paper, and rinsing with 100 mL demineralized water to eliminate the salt content. Filter papers containing biofloc were used for determination of biofloc biomass in terms of total suspended solids (TSS) and volatile suspended solids (VSS) (APHA, 1995), proximate composition (AOAC, 2000), and TC and TN contents (using LECO CN 628 Dumas analyzer, LECO Instrumente GmbH., Germany).

A hundred shrimp were collected at the start as a composite sample and all remaining shrimp in each experimental unit were collected at the end of the experiment. Periphyton samples were collected from each treatment tank only at the end by carefully scratching the tank walls. All shrimp and periphyton samples were preserved at -20 °C until further analysis. These samples were freeze-dried for one week prior to analysis of their proximate composition (AOAC, 2000), TC and TN contents (using LECO CN 628 Dumas analyzer, LECO Instrumente GmbH., Germany). During the experiment, roughly 10 g of each feed and CHO were taken daily. These daily samples were pooled per diet and at the end of the experiment analyzed for their proximate composition, and TC and TN contents employing the same methods as for shrimp and periphyton, without prior freeze-drying.

For microbiota analysis, DNA was extracted from biofloc samples. The biofloc samples, were collected at the end of the experiment by filtering through a 0.45 (particulate part) and a 0.22 μm (seston part) pore size sterile filter. The samples were subjected to lysis by lysozyme buffer and proteinase K before DNA extraction using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). The harvested DNA was quantified using the nano drop spectrophotometer. Sequencing of the PCR-amplified V4 region of the 16S rRNA (prokaryotic microbial communities), using primers 515 F (5′-CTAGTGCCAGCMGCCGCGGTAA −3′) and 806 R (5′-CTAGGACTACHVGGGTWTCTAAT-3′), and of the 18S SSU, using primers 3NDf-CS1F (5′ - GGCAAGTCTGGTGCCAG - 3′) and V4-Euk-CS2R (5′ - ACGGTATCTRATCRTCTTCG - 3′) (eukaryotic microbial communities) 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.

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 16S gene reference Silva database (138 version) (McLaren, 2020) and the 18S gene reference Silva database (128 version) (Morien and Parfrey, 2018). Owing to the variation in sequence depths between samples, all samples were normalized to the lowest depth by subsampling.

For the alpha-diversity analysis, Shannon H′ diversity, richness (observed taxa) and rare taxa abundance were calculated for both the prokaryotic and the eukaryotic microbial communities. 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.

2.6. Calculation and data analysis

The specific growth rate (SGR) was calculated as follows, in which ln ($BW_{initial}$) and ln(BW_{final}) are the natural logarithm of the shrimp individual body weight at the beginning and end of the experiment, respectively:

$$SGR\ (\%BW/day) = \frac{ln\left(BW_{final}\right) - ln(BW_{initial})}{Days\ of\ culture} \times 100\% \quad (1)$$

The feed conversion ratio was calculated with (FCR-CHO) or without taking into consideration CHO addition (FCR) as follows:

$$FCR + CHO = \frac{Feed\ fed\ (g) + Carbohydrate\ added\ (g)}{Biomass\ gain\ (g)} \quad (2)$$

$$FCR = \frac{Feed\ fed\ (g)}{Biomass\ gain\ (g)} \quad (3)$$

Protein efficiency ratio (PER), nitrogen and energy use efficiencies were calculated as follows:

$$PER = \frac{Gain\ in\ shrimp\ biomass\ (g)}{Protein\ input\ from\ feed\ and\ carbohydrate\ (g)} \quad (4)$$

$$Nitrogen\ use\ efficiency\ (\%) = \frac{Nitrogen\ retained\ in\ shrimp\ (g)}{Nitrogen\ input\ from\ feed\ and\ carbohydrate\ (g)} \times 100\% \quad (5)$$

$$Carbon\ use\ efficiency\ (\%) = \frac{Carbon\ retained\ in\ shrimp\ (g)}{Carbon\ input\ from\ feed\ and\ carbohydrate\ (g)} \times 100\% \quad (6)$$

$$Energy\ use\ efficiency\ (\%) = \frac{Energy\ retained\ in\ shrimp\ (g)}{Energy\ input\ from\ feed\ and\ carbohydrate\ (g)} \times 100\% \quad (7)$$

The treatment effects on shrimp, water, biofloc, and periphyton were analyzed using IBM SPSS Statistics 23 software (IBM Corporation, NY, USA). Water quality parameters were analyzed using repeated-measure ANOVA to account for sampling dates. Parameters relating to shrimp, biofloc, and periphyton were analyzed using one-way ANOVA. Due to significant difference in initial shrimp body weight among phases, the treatment effects on shrimp growth and nutrient utilization were analyzed with one-way ANCOVA, considering initial biomass as a covariate. The probability (P) value <0.05 indicates a significant effect, while P value <0.1 indicated a trend toward a significant effect. Least Significant Difference (LSD) post-hoc test was performed when a

significant effect was found.

3. Results

3.1. Shrimp performance

The treatments had significant effects on the harvested biomass, feed conversion ratios, and protein, nitrogen and energy utilization efficiencies ($P < 0.05$) (Table 2). Specifically, the harvested biomass and protein efficiency ratio was highest when carbohydrate (CHO) was added separately (treatment COM-Feed + CHO) ($P < 0.05$). Treatment COM-Feed had higher harvested biomass than treatment CHO-Feed ($P = 0.05$). Meanwhile, the energy efficiency was the best when additional carbohydrate was not used (treatment COM-Feed). The FCR and nitrogen efficiency in treatments COM-Feed and COM-Feed + CHO were not different from each other ($P > 0.05$), but were higher than those in treatment CHO-Feed ($P < 0.05$). The survival rate was not different among treatments ($P > 0.05$), but tended to be higher in treatment COM-Feed + CHO than in treatments CHO-Feed, and COM-Feed respectively ($P < 0.1$). Similarly, the final body weight, weight gain, and growth rate of the shrimp were not different among treatments ($P > 0.05$), but tended to be higher in treatments COM-Feed and COM-Feed + CHO than in treatment CHO-Feed ($P < 0.1$).

3.2. Water quality

During the experiment, the water temperature, pH, salinity and dissolved oxygen were 27 ± 2 °C, 8.2 ± 0.1, 24 ± 1 ppt, and above 6 mg/L, respectively, and similar among treatments ($P > 0.05$). Most measured parameters did not change during the experimental period ($P_{Time} > 0.05$) (Table 3). The TN, IN, and NOx changed with time but the changes were dependent on the treatment effects ($P_{Treatment\ x\ Time} < 0.05$). The fluctuation of TN during the experiment is shown in Fig. 1. The fluctuation of IN and NOx (Figure not shown) showed similar pattern with that of TN. Orthophosphate and inorganic carbon ($P_{Time} < 0.05$) change with time. In all treatments, TC and OC increased during the experiment.

The treatments had significant effects on most water quality parameters ($P_{Treatment} < 0.05$), except total ammonia nitrogen, organic nitrogen, and inorganic carbon ($P_{Treatment} < 0.05$). The TAN concentration was similarly low in all treatments ($P > 0.05$), but was numerically lower when carbohydrate was added ($P > 0.05$). Total and inorganic nitrogen, nitrite and nitrate nitrogen (NO_x), and phosphate were lower, whereas total and organic carbon were higher when CHO was added ($P < 0.05$), irrespective how. The chlorophyll *a* and chemical oxygen demand (COD) increased during the experiment ($P_{Time} < 0.05$), and were similar among treatments ($P_{Treatment} > 0.05$) (Fig. 2).

3.3. Biofloc growth

Biofloc growth evaluated using total suspended solids (TSS) and volatile suspended solids (VSS) is shown in Fig. 3. During the experiment, both TSS and VSS increased with time ($P_{Time} < 0.05$). One-way ANOVA with data on each sampling date showed that the TSS and VSS were not statistically different among treatments at any of the sampling times ($P_{Treatment} > 0.05$). However, both parameters were numerically higher in treatment CHO-Feed at the end of the experiment than in other treatments.

3.4. Proximate composition of shrimp, biofloc, and periphyton

Shrimp proximate composition was not affected by treatments ($P > 0.05$) (Table 4). Similarly, biofloc proximate composition was not different among treatments ($P_{Treatment} > 0.05$), but changed with time ($P_{Time} < 0.05$) except for phosphorus content ($P_{Time} > 0.05$) (Table 5). Specifically, the ash, calcium, and magnesium contents of biofloc decreased, whereas the crude protein, energy, total carbon and nitrogen contents increased during the culture period. Regarding periphyton, the total biomass, and dry matter and ash contents were not affected by treatment ($P > 0.05$), although the ash content tended to be higher in treatment COM-Feed than in treatments CHO-Feed, and COM-Feed + CHO respectively ($P < 0.1$) (Table 6). The periphyton in treatment COM-Feed + CHO had statistically the highest crude protein, energy, and total carbon and nitrogen contents ($P < 0.05$), while these values are similar among the other two treatments ($P > 0.05$).

3.5. Nutrient balances

The absolute amounts of carbon and nitrogen in each experimental unit at stocking and harvesting of the experiment are shown in Figs. 4 and 5, respectively. The carbon in shrimp, water, and biofloc at stocking and in shrimp, water, biofloc, and periphyton at harvesting were similar among treatments ($P > 0.05$). The total carbon at harvest in COM-Feed + CHO and CHO-Feed treatments was similar ($P > 0.05$), but higher than that in treatment COM-Feed ($P < 0.05$). The carbon in water at harvesting was not different among treatments ($P > 0.05$), but tended to be higher in treatment CHO-Feed than in treatments COM-Feed + CHO, and COM-Feed respectively ($P < 0.1$). Of the total input carbon 58% was lost at the treatments COM-Feed + CHO and CHO-Feed during the experiment, while at the COM-Feed treatment this was only 39%.

The total nitrogen at stocking and harvesting was similar among treatments ($P > 0.05$). The nitrogen from shrimp, water, and biofloc at stocking were similar among treatments ($P > 0.05$). At the end of the experiment, the nitrogen content in shrimp of treatment COM-Feed + CHO was similar to the content in treatment COM-Feed ($P > 0.05$), but higher than that of treatment CHO-Feed ($P < 0.05$). The nitrogen in biofloc at harvesting was not different among treatments ($P > 0.05$), but tended to be higher in treatment CHO-Feed than in treatments COM-Feed + CHO and Feed, respectively ($P < 0.1$). All treatments tanks retained more than 92% of the total input nitrogen.

Table 2
Production performance of L. *vannamei* juveniles subjected to different feeding treatments in the 4-week experiment.

Parameters	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	*P* values
Feed fed (g/tank)	358	356	708	–	–
Cornstarch added (g/tank)	–	359	–	–	–
Harvested biomass (g)	421[b]	445[a]	403[c]	4.210	**0.011**
Final BW (g/ind)	9.1	9.0	8.4	0.129	0.097
Weight gain (g/ind)	5.8	5.8	5.1	0.875	0.097
Survival rate (%)	93	99	96	0.129	0.089
SGR (% BW/day)	3.5	3.5	3.2	0.051	0.090
FCR	1.4[a]	1.3[a]	3.0[b]	0.051	**0.000**
FCR + CHO	–	2.6[a]	3.0[b]	0.052	**0.000**
Protein efficiency ratio	1.9[b]	2.1[a]	1.8[b]	0.032	**0.010**
Nitrogen efficiency (%)	43[a]	46[a]	38[b]	0.784	**0.009**
Carbon efficiency (%)	21	12	10	1.554	**0.000**
Energy efficiency (%)	21[a]	13[b]	11[c]	0.203	**0.000**

Values are adjusted means of four replicate tanks per treatment and standard error of the means (SEM) when body weight (BW) at the start is considered as a covariate. SGR = specific growth rate; FCR = feed conversion ratio; FCR-CHO = feed conversion ratio accounting carbohydrate as feed. Probability (P) values in bold indicate significant treatment effects ($P < 0.05$). Adjusted means with different superscripts are statistically different. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

Table 3
Summary of selected water quality parameters among different feeding treatments of the 4-week experiment.

Parameters	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	P values		
					Treatment	Time	Treatment*Time
TAN (mg/L)	0.4	0.1	0.1	0.1	0.238	0.328	0.173
NO_x (mg/L)	20^a	15^b	15^b	1.0	**0.009**	0.083	**0.046**
PO_4 (mg/L)	2.0^a	1.3^b	1.3^b	0.2	**0.029**	**0.032**	0.231
TC (mg/L)	8^a	11^b	12^b	0.9	**0.019**	0.525	0.224
IC (mg/L)	0.9	0.7	0.8	0.1	0.202	**0.044**	0.197
OC (mg/L)	7^a	10^{ab}	12^b	1.0	**0.032**	0.270	0.121
TN (mg/L)	21^a	17^b	16^b	1.0	**0.018**	0.292	**0.044**
IN (mg/L)	20^a	15^b	15^b	0.9	**0.005**	0.119	**0.030**
ON (mg/L)	0.7	1.6	1.4	0.2	0.062	0.209	0.462

Values are means of four sampling times of four replicate tanks per treatment. Starting values of each parameter were used as covariates in ANCOVA analysis of the respected parameters. TAN = total ammonia nitrogen, NO_x = nitrite and nitrate nitrogen, TC = total carbon, IC = inorganic carbon, OC = organic carbon, TN = total nitrogen, IN = inorganic nitrogen, ON = organic nitrogen. Probability (P) values in bold indicate significant treatment effects ($P < 0.05$). Means with different superscripts are statistically different. Treatments include COM-Feed (diet only, C/*N* = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/*N* = 14.6), and CHO-Feed (corn starch included diet, C/*N* = 14.6).

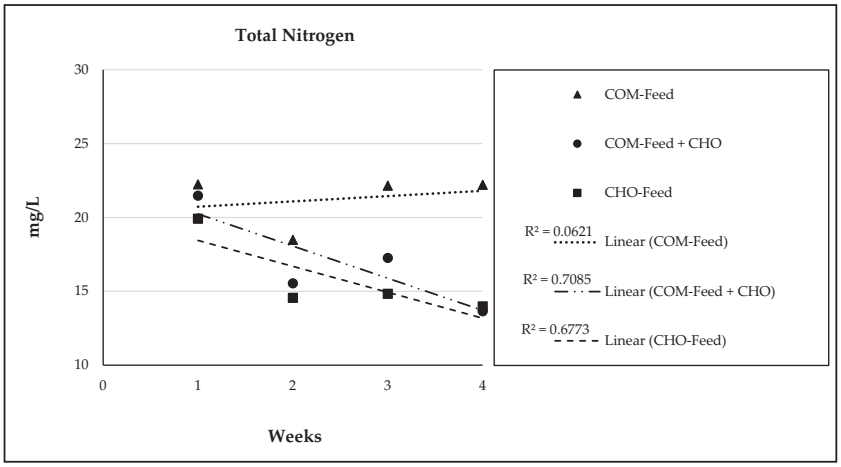

Fig. 1. Fluctuation of total nitrogen in the water during the 4-week experiment. Data points are means of four replicate tanks per treatment. Treatments include COM-Feed (diet only, C/*N* = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

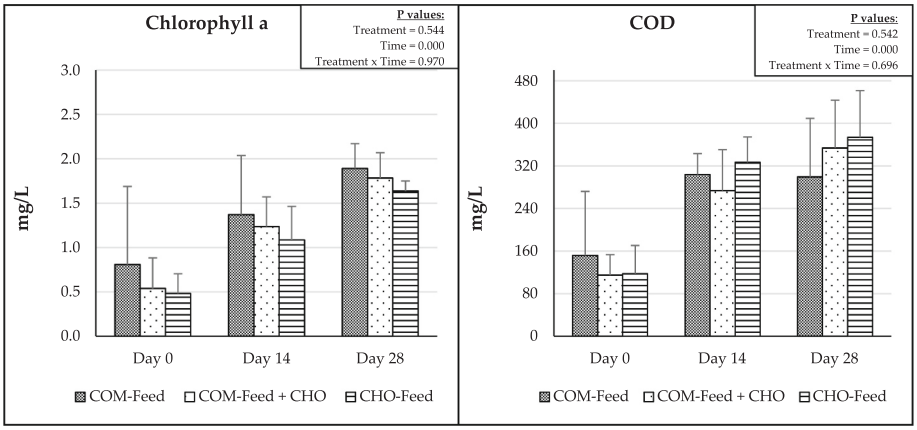

Fig. 2. Fluctuation of chlorophyll *a* and chemical oxygen demand (COD) in water among different feeding treatments. Column heights are means with error bars showing standard deviation of four replicate tanks per treatment. Treatments include COM-Feed (diet only, C/*N* = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/*N* = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

3.6. Microbiota analysis

Diversity analysis (alpha-diversity) of the prokaryotic microbial communities (16S rRNA) in the two types of biofloc samples, the particulate and the seston communities, did not revealed significant changes between the different treatments (Fig. 6; $P > 0.05$). However, diversity analysis of the eukaryotic microbial communities (18S SSU) indicated significant difference in the richness between treatments between COM-Feed and CHO-Feed, and on the abundance of rare taxa between COM-Feed and COM-Feed + CHO (Fig. 6; $P < 0.05$). Overall,

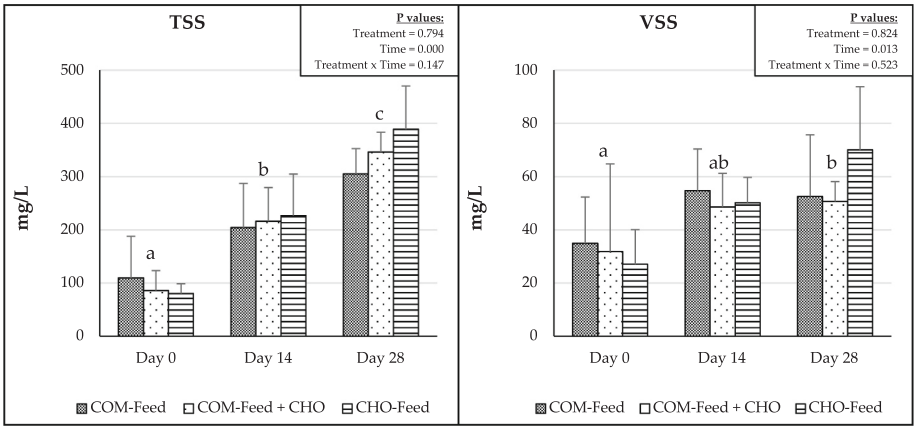

Fig. 3. Fluctuation of total suspended solids (TSS) and volatile suspended solids (VSS) in water among different feeding treatments. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6). Column heights are means with error bars showing standard deviation of four replicate tanks per treatment. Sampling days with different letters on the top are statistically different ($P < 0.05$).

Table 4
Proximate composition on dry weight basis of L. *vannamei* juveniles at the end of the 4-week experiment.

Parameters	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	P values
DM (g/kg WW)	271	271	271	0.3	0.524
Ash (g/kg)	131	138	129	2.6	0.429
CP (g/kg)	767	777	763	3.5	0.260
Fat (g/kg)	38	41	42	1.7	0.648
Energy (kJ/g)	20	21	21	0.1	0.474
TC (g/kg)	475	470	470	3.4	0.854
TN (g/kg)	127	126	123	0.9	0.854

Values are means of four replicate tanks per treatments and standard error of the means (SEM). DM = dry matter, WW = wet weight, CP = crude protein, TC = total carbon, TN = total nitrogen. Treatments include COM-Feed (diet only, C/*N* = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/*N* = 14.6), and CHO-Feed (corn starch included diet, C/*N* = 14.6).

the eukaryotic microbial community diversity and richness was numerically higher in the particulate biofloc samples, while CHO-Feed was found to have higher alpha-diversity compared to the rest of the treatments. This observation was confirmed by linear mixed-effects model analysis, which indicated that treatment and sample type (particulate versus seston) were the major factors shaping eukaryotic microbial community richness and abundance of rare taxa, respectively ($P < 0.05$).

Looking at the beta-diversity (microbial composition), both prokaryotic and eukaryotic microbial communities were affected by time (Permanova analysis; $P < 0.001$), while no significant difference were observed by the different samples types or treatments ($P > 0.05$). The relative abundance of the most abundant prokaryotic and eukaryotic phyla and genera are presented in Figs. 7 and 8, respectively. In the prokaryotic communities, the most abundant microbial phyla was Proteobacteria (35% of the relative abundance), followed by Planctomycetote (15%), Bacteroidota (15%), Cyanobacteria (12%) and Chloroflexi (10%). With regard to genera, a large portion of the microbial communities was not assigned to a genus (57% of the relative abundance), while the most abundant genus was *Phaeodactylibacter* (4%), followed by *Maricauda* (2%), *Planctomicrobium* (2%), *Pseudoalteromonas* (1%) and *Ruegeria* (1%). In the eukaryotic communities, Chlorophyta phylum occupied the higher proportion of the relative abundance (45% of the relative abundance), followed by Rotifera (5%), Perkinsidae (4%), Nematoda (2%) and Dinoflagellates (1.2%). Among the Chlorophyta phylum, *Nannochloropsis* (20% of the relative abundance) was the most abundant genera.

4. Discussion

The carbohydrate (CHO) addition to the Pacific white shrimp culture systems improves the production, FCR, and protein efficiency. This practice promotes bacterial growth which assimilates inorganic nitrogen and improves water quality (Avnimelech, 1999; Panigrahi et al., 2019; Ren et al., 2019; Tong et al., 2020). Beside this, the bioflocs also serve as supplemental food, and increase the nitrogen retention (Ekasari et al., 2014a; Hari et al., 2004; Schneider et al., 2005). Manipulating C/N ratio increases the abundance of zooplankton, and potentially beneficial bacteria and bioactive metabolites (Gao et al., 2012; Guo et al., 2020). Adding CHO to the culture system has also been related with enhanced digestive enzyme activities (Xu and Pan, 2012), immune cellular response (Xu and Pan, 2013), and disease resistance (Ekasari et al., 2014b; Hostins et al., 2019; Panigrahi et al., 2019) of the Pacific white shrimp. The benefits of adding CHO on water quality and natural food availability were demonstrated in numerous species including Pacific white shrimp (Liu et al., 2014), black tiger prawn (Anand et al., 2013;

Table 5
Proximate composition on dry weight basis of biofloc in different feeding treatments in the 4-week experiment.

Parameters	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	P values		
					Treatment	Time	Treatment*Time
Ash (g/kg)	263	230	238	48	0.881	**0.018**	0.969
CP (g/kg)	338	382	379	32	0.581	**0.001**	0.966
Energy (kJ/g)	13	16	16	2.2	0.716	**0.012**	0.824
TC (g/kg)	328	338	335	9.1	0.754	**0.004**	0.985
TN (g/kg)	54	54	55	2.7	0.959	**0.001**	0.924
P (g/kg)	16	18	16	3.0	0.850	0.569	0.613
Ca (g/kg)	118	132	119	23	0.888	**0.005**	0.712
Mg (g/kg)	30	31	29	3.7	0.901	**0.001**	0.870

Values are means of three sampling times of four replicate tanks per treatment and standard error of the means (SEM). CP = crude protein TC = total carbon, TN = total nitrogen, P = phosphorus, Ca = calcium, Mg = magnesium. Probability (P) values on bold indicate significant treatment effects. Treatments include COM-Feed (diet only, C/*N* = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/*N* = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

Table 6
Proximate composition on dry weight basis of periphyton at the end of the 4-week experiment.

	COM-Feed	COM-Feed + CHO	CHO-Feed	SEM	P values
Biomass (g WW)	85	149	139	23	0.507
DM (g/kg WW	891	860	913	26	0.747
Ash (g/kg)	409	361	384	8.7	0.055
CP (g/kg)	292[a]	327[b]	303[a]	6.1	**0.040**
Energy (kJ/g)	12.7[a]	14.4[b]	13.3[a]	0.2	**0.013**
TC (g/kg)	298[a]	337[b]	315[a]	5.9	**0.005**
TN (g/kg)	47[a]	55[b]	49[a]	1.1	**0.001**

Values are means of four replicate tanks per treatment and standard error of means (SEM). DM = dry matter, WW = wet weight, CP = crude protein; TC = total carbon and TN = total nitrogen. Probability (P) values in bold indicate significant treatment effects. Means with different superscripts are statistically different. Treatments include COM-Feed (diet only, C/*N* = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/*N* = 14.6).

Hari et al., 2004), giant freshwater prawn (Asaduzzaman et al., 2008), Japanese tiger prawn (Zhao et al., 2012), Nile tilapia (Avnimelech, 1999; Hu et al., 2014) and integrated culture of three carp species (Zhao et al., 2014).

In this research, the inclusion of additional CHO in the feed as one pellet (treatment CHO-Feed) resulted in poorer shrimp production and FCR compared to the separate CHO addition (treatment COM-Feed + CHO) although all treatment tanks were fed similar amounts of protein. This could be a result of reduced feed ingestion due to doubling of feed amount and diluting of feed attractant in treatment CHO-Feed. However, since the feed ingestion was not monitored in any treatment and the water quality was similar among COM-Feed + CHO and CHO-Feed treatments, this hypothesis was not yet validated. According to Xia et al. (2010), increasing the dietary CHO content at the expense of protein may increase amylase activity of the culture animal, and the digestibility of the feed. However, this reduces the protease activity in the digestive system of the culture animal, and possibly the deposition of protein in shrimp body. The CHO-Feed, which had high carbohydrate and low protein composition (Table 1), assumingly resulted in lower protein absorption in the shrimp, and therefore lower shrimp growth compared to the COM-Feed. Both assumptions on ingestion and absorption of CHO-Feed remain to be proven. Nevertheless, future research on similar topics should improve the experimental design, for example splitting daily feed ration into more frequent feeding when a low protein feed is used. The Pacific white shrimp performs best with feed of 39–43% protein (Xia et al., 2010). In the biofloc system where CHO is separately added, lowering the dietary protein level from 35% to 30% or 25% did not significantly affect the growth, FCR, survival, and

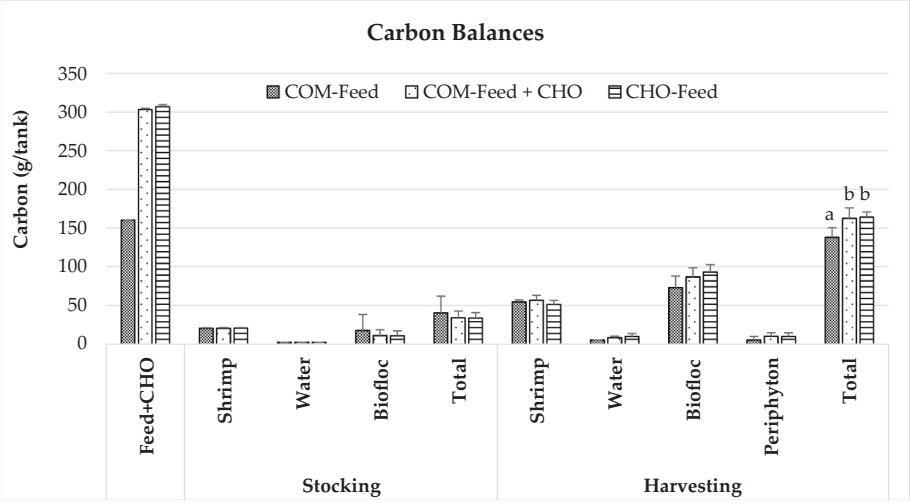

Fig. 4. Carbon balances among different feeding treatments. Column heights are means with error bars showing standard deviation of four replicate tanks per treatment. Treatments include COM-Feed (diet only, C/*N* = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/*N* = 14.6), and CHO-Feed (corn starch included diet, C/*N* = 14.6). Bars in the same group with different letters on the top are statistically different among others ($P < 0.05$). The carbon input from Feed+CHO was controlled, and therefore was not statistically analyzed.

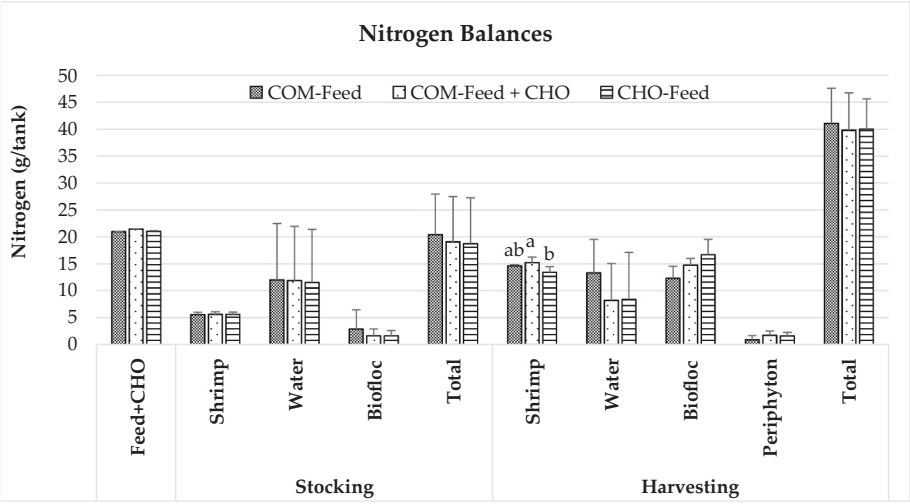

Fig. 5. Nitrogen balances among different feeding treatments. Column heights are means with error bars showing standard deviation of four replicate tanks per treatment. Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6). Bars in the same group with different letters on the top are statistically different among others ($P < 0.05$). The nitrogen input from feed and corn starch was controlled, and therefore was not statistically analyzed.

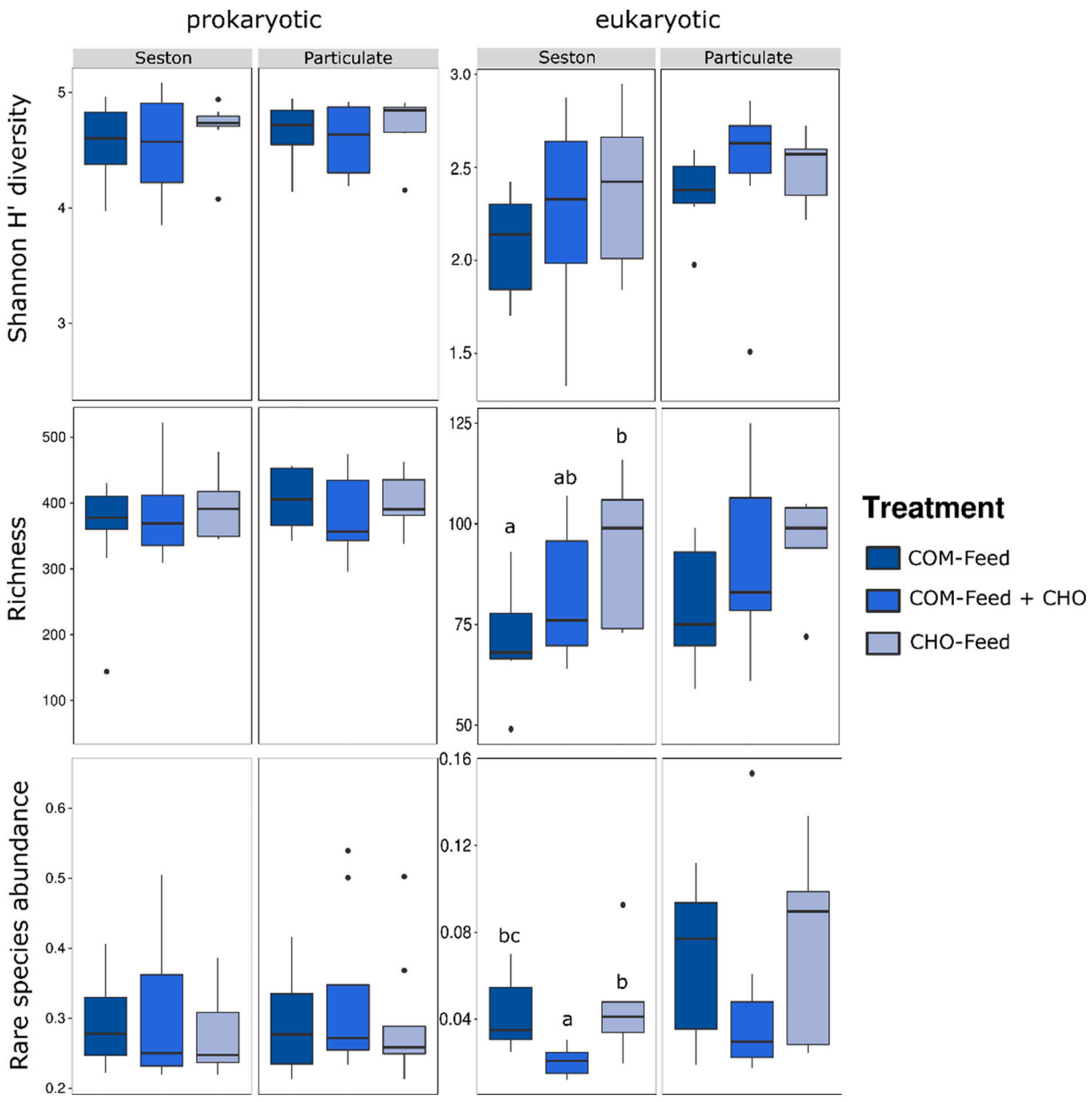

Fig. 6. Alpha diversity of the prokaryotic and eukaryotic microbial communities in the different treatments. Data are shown as box plots; the horizontal line indicates the median and the whiskers indicate the lowest and highest points within 1.5× the interquartile ranges of the lower or upper quartile, respectively. All data were included in the analysis. Significance was tested with Wilcoxon rank-sum two way test at $P < 0.05$. Treatments include COM-Feed (diet only, C/$N = 7.6$), COM-Feed + CHO (diet + separate corn starch addition, C/$N = 14.6$), and CHO-Feed (corn starch included diet, C/N = 14.6).

immune response of the Pacific white shrimp (Xu and Pan, 2014a). The further decrease of dietary protein level to 20% showed negative consequences on shrimp health and growth. Besides, the effects of dietary protein levels and C/N ratios on shrimp growth and water quality are independent of each other (Xu and Pan, 2014b).

The water quality and biofloc growth were similar in both methods of adding CHO. The TAN concentration was low in all treatments of this experiment irrespective of CHO addition. This may stem from the fact that the experiment started with a biofloc concentration of ≥100 mg/L TSS, at which an input C/N ratio of 6 was sufficient to maintain good water quality (Emerenciano, 2017; Martins et al., 2020). Nevertheless, the nitrite and nitrate (NO_x) concentrations significantly reduced when CHO was added, concurring with observations by Panigrahi et al. (2019) and Ren et al. (2019). Here, we report enhancements in shrimp production at the C/N ratio of 14.6 without negative effects on water quality. However, contradictory results were also reported in which increasing the C/N ratio above 12 showed no significant effects on shrimp growth, TAN and NO_2 (Tong et al., 2020). In some cases, C/N ratios above 12 caused water quality deteriorations, resulting in poorer shrimp growth, yield, and FCR (Xu et al., 2018, 2016).

Both biofloc and periphyton had a protein level above 30% DM (Tables 5 and 6), and were comparable among treatments and to previous reports (Tinh et al., 2021b; Xu and Pan, 2014b). The proximate composition of biofloc was not affected by the absence or presence of additional carbohydrate in this research (Table 5). In other research, adding carbohydrate reduced biofloc ash content (Tinh et al., 2021a; Xu and Pan, 2014b), and increased biofloc protein content (Xu and Pan, 2014b). The contribution of biofloc and periphyton to shrimp growth was not measured in this research, however both biofloc and periphyton are nutritious natural food for cultured shrimp. The biofloc contribution to nitrogen retention in Pacific white shrimp may range between 18% to 29% (Burford et al., 2004). In other shrimp species, the natural food

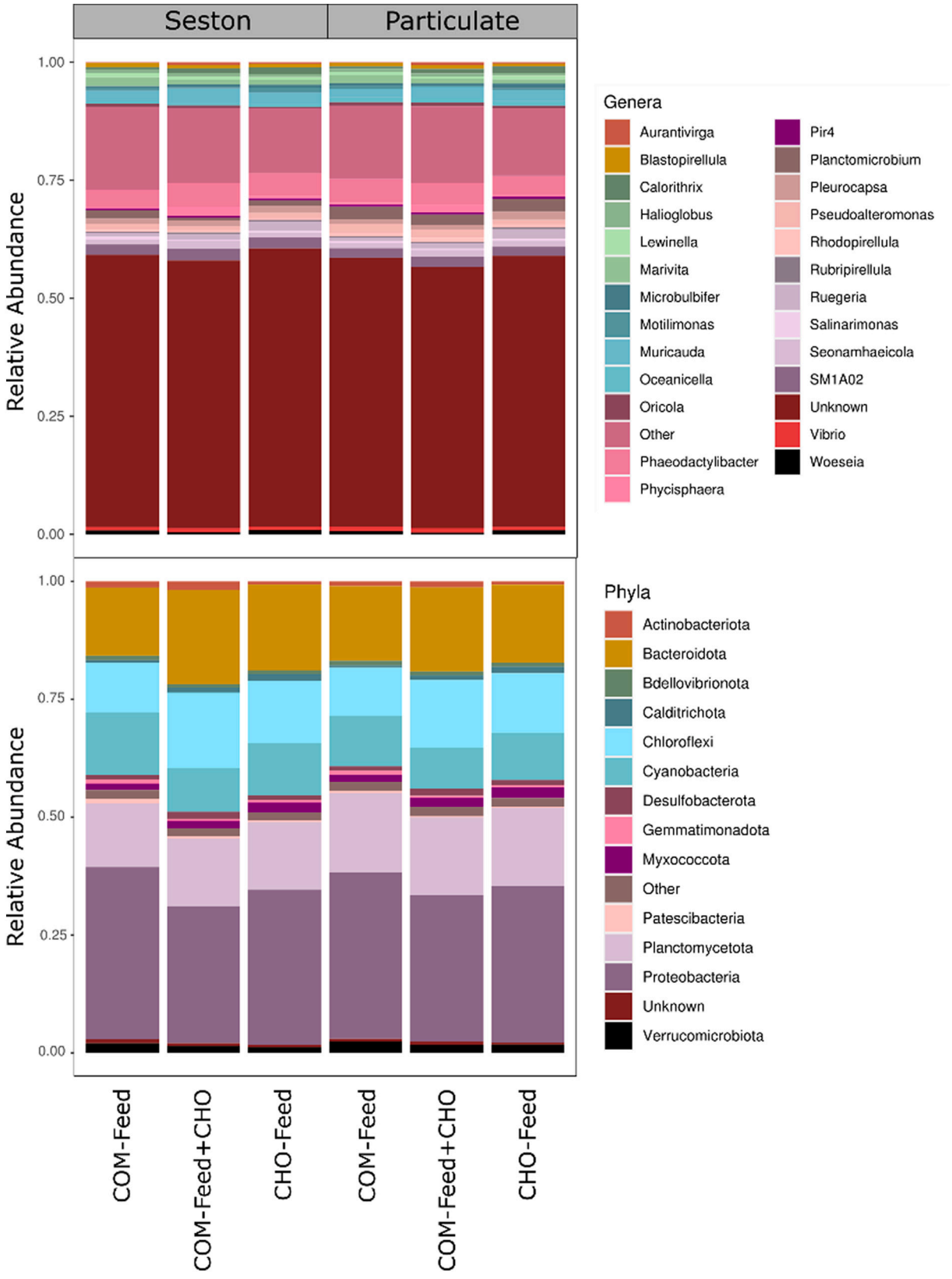

Fig. 7. Prokaryotic microbial composition of the biofloc samples between the different treatments at the phylum (bottom) and genus level (top). Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

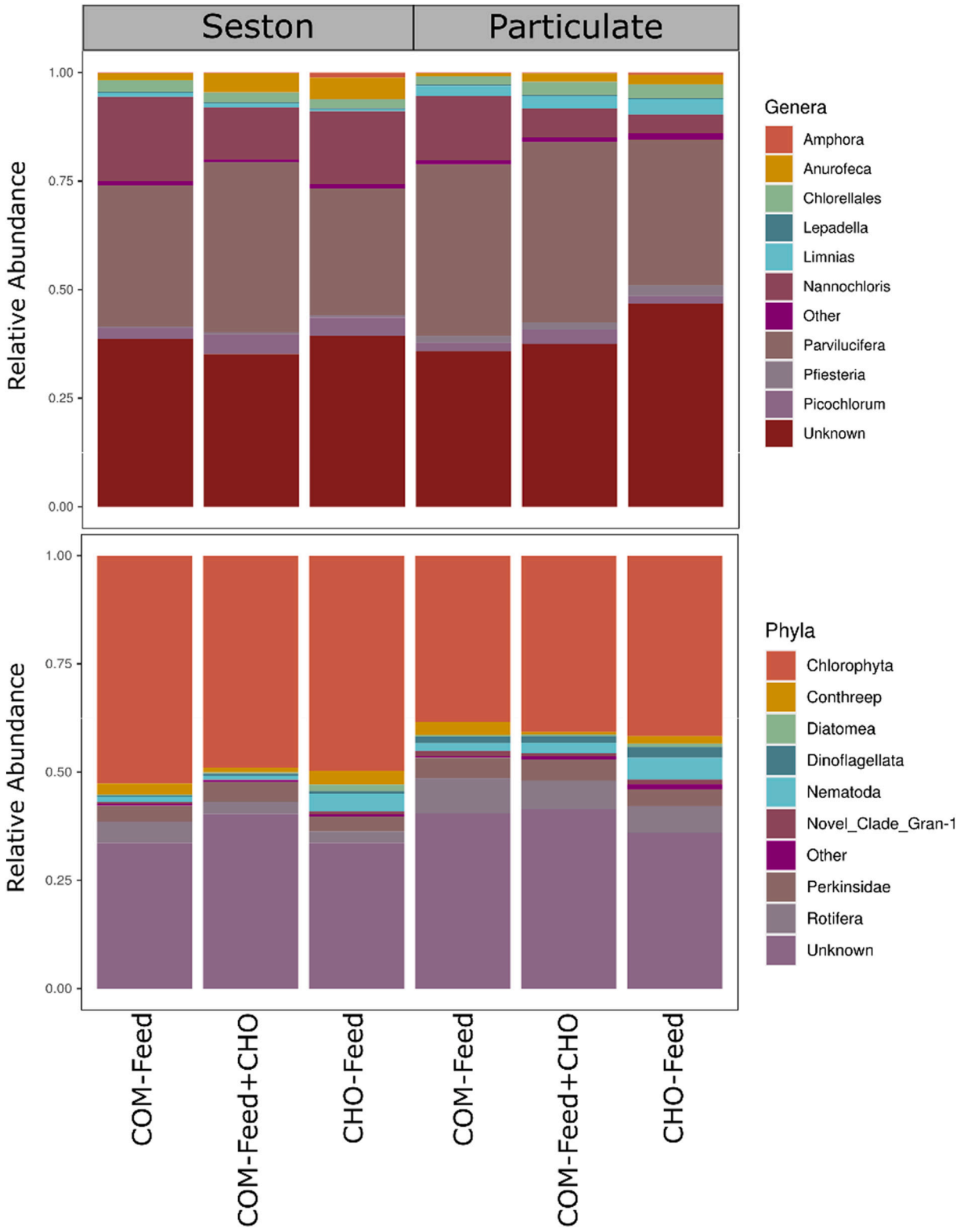

Fig. 8. Eukaryotic microbial composition of the biofloc samples between the different treatments at the phylum (bottom) and genus level (top). Treatments include COM-Feed (diet only, C/N = 7.6), COM-Feed + CHO (diet + separate corn starch addition, C/N = 14.6), and CHO-Feed (corn starch included diet, C/N = 14.6).

production may contribute up to 37% nitrogen and 40% carbon in the shrimp growth (Cardona et al., 2015). In our study, the biofloc contained a larger amount of carbon and a similar amount of nitrogen compared to shrimp at the end of the research. All this information suggests that the biofloc utilization efficiency in our study was not yet optimal. In order to increase the nutrient use efficiency, further efforts are required to make use of the remaining biofloc.

The microbial diversity within the bioflocs, composed of heterotrophic bacteria, microalgae, zooplankton and rotifers (De Schryver et al., 2008), has been reported to be affected by the carbon source type and C/

N ratio (Deng et al., 2018; Panigrahi et al., 2018). Recent studies using different carbon sources in the water were reported to select for certain phyla, while this could be a way to modulate the developing heterotrophic communities (Gutiérrez et al., 2016; Vargas-Albores et al., 2019). Addition of corn starch as a carbon source either as an external source or in the feed did not significantly affect the biofloc prokaryotic microbial communities in the present study. Despite that the major microbial prokaryotic microbial communities identified were similar to what was previously reported, with Proteobacteria as the most abundant phylum, followed by Bacteroidetes, Planctomycetes and Actinobacteria, and genera such as Maricauda, Ruegeria and Phaeodactilibacter (Gutiérrez et al., 2016; Huang et al., 2020). However, carbon addition increased the eukaryotic microbial community diversity and richness, which increased further when the carbon source was added in the feed. More specifically, diatoms and protozoa were found to be more abundant when carbon was added in the diet. Such an increase may be associated with a higher carbon availability in those treatments. Eukaryotic communities have been reported to alter according to the availability of nitrogenous compounds and suspended solids (Gallardo-Collí et al., 2019). Although not significant, higher TSS and VSS were observed in the carbohydrate diets which could potentially explain such findings. Besides, studies comparing carbon sources with different degradation rates showed significant implication on the microbial communities (Jiang et al., 2020; Serra et al., 2015; Wei et al., 2016). The inclusion of corn starch in the feed which likely went through shrimp digestive tract may have altered corn starch bioavailability, and therefore its effects on the microbial communities compared to separate addition in this research. Further research is necessary to improve knowledge of the eukaryote and prokaryote microbial communities present in biofloc systems.

With an isoprotein feeding level at tank level, the overall nitrogen retention in the system was similarly high in all treatments. A previous report shows that the nitrogen retention was not different between the conventional and biofloc systems, accounting for 77% and 79–87% of the input nitrogen respectively, excluding periphyton (Tinh et al., 2021a). In this experiment, more than 92% of the added nitrogen was retained in the system at the end of the experiment. However, the addition of extra carbon, either mixed within the feed pellet or added separately, did not significantly improve the nitrogen retention in shrimp, nor the overall retention in the system compared to the control. In fact, most of the additional carbon was lost from the system. The conventional COM-Feed treatment retained 69% of the total carbon input, while in carbon-added treatments this was 48% (Fig. 4). Tinh et al. (2021a) reported a 62% total carbon retention for conventional non-carbon-added systems, but only 26–31% total carbon retention for biofloc systems. A similar doubling of C loss was reported by Hu et al. (2014) rearing tilapia in a biofloc system when increasing the C/N ratio to 16 compared to the input of feed only (Hu et al., 2014). The carbon retention in biofloc systems seems to be affected by the carbon source used (Tinh et al., 2021b). To reduce the carbon emission from aquaculture systems, and its effects on global warming, research to increase carbon use efficiency in biofloc systems is needed.

5. Conclusions

The separate carbohydrate addition was better than its inclusion in the feed for biofloc culture of Pacific white shrimp as high dietary carbohydrate inclusion may result in poorer feed ingestion and absorption by the shrimp. The additional carbohydrate enhanced the water quality during culture. Adding carbohydrate did not significantly increase the nitrogen retention and resulted in more than 50% carbon loss. Besides, the carbohydrate addition did not affect the prokaryotic microbial community diversity, but increased the eukaryotic microbial community diversity.

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.

Acknowledgements

The authors would like to express their sincere gratitude to NWO-WOTRO Science for Global Development (Grant W 08.250.101), WorldFish Organization, Skretting Company, and Can Tho University for their financial support of this project. We would like to thank NIOO Bioinformatic ICT services for providing the servers for sequencing data analysis and bioinformatic support. We are also thankful to Wageningen University for providing research facilities. Our appreciations are given to colleagues at Animal Sciences Group, Wageningen University for their kind help in preparing and conducting the experiment, as well as correcting this manuscript.

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