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Supplementation of Ex-Situ Biofloc to Improve Growth Performance and Enhance Nutritional Values of the Pacific White Shrimp Rearing at Low Salinity Conditions

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Featured Application: This ex situ biofloc technology has high potential to be a strategy for climate change adaptation and mitigation, which is useful for improving shrimp farming practice in the Asia Pacific region and for enhancing food security with high quality of shrimp to serve consumers worldwide.

Abstract: Shrimp is an important food source consumed worldwide. An intensive aquaculture system with overuse of feed in combination with detrimental effects from climate change are serious problems leading to mass mortality of cultured shrimp. Biofloc technology is an approach to managing water quality and controlling the disease to counter the negative side of intensive culture system; however, most of the biofloc applications are naturally formed, which could be inconsistent. In this study, we employed an established optimal ratio of microbial consortium called "ex-situ biofloc (BF)" to be used as a feed supplement in shrimp cultured in a zero-water discharged system at low salinity conditions. Three feeding groups (100%commercial pellet (C), 95%C+BF, 90%C+BF) of shrimp were cultured for six weeks. The effect of an ex-situ biofloc supplement with commercial pellet reduction showed that levels of ammonium, nitrite, nitrate and phosphate were significantly decreased in water culture. Shrimp fed with ex-situ biofloc supplement with commercial pellet reduction exhibited significantly increased shrimp weight and survival, and significantly expressed growth-related genes involving lipolysis and energy metabolism higher than those fed with 100% commercial pellet. Nutritional analysis indicated a significant increase of docosahexaenoic acid (DHA) and eicosenoic acid (C20:1) concentrations in the ex-situ biofloc supplemented shrimp. This finding revealed the potential of ex-situ biofloc to manage water quality, improve shrimp growth performance and enhance shrimp nutritional value under intensive culture at low salinity conditions. The beneficial effects of the ex-situ biofloc in shrimp culture system make it a promising alternative strategy to mitigate climate change effects leading to the sustainable production of high-quality shrimp in the future.

Keywords: ex-situ biofloc; Pacific white shrimp; *Litopenaeus vannamei*; trace mineral; amino acid profile; fatty acid profile; nutritional value; feed supplement; growth performance

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1. Introduction

Shrimp is an economically important aquatic animal due to an increasing demand from human consumption worldwide. Capturing wild shrimp presents serious problems

of the shortage of wild shrimp and overexploitation of the ecological system. The high demand from global consumption makes shrimp aquaculture one of the fastest growing industries in the world. An intensive culture system with high stock density and overuse of feed is mostly used to increase the production yield [1,2]. However, this system has many drawbacks, especially the side effect of uneaten feed accumulation [3]. It has been estimated that 70–80% remains in the pond, in the water or the sediment [4]. The uneaten feed in the bottom of the pond is consumed by heterotrophic bacteria, including pathogenic ones such as *Vibrio* sp. that can naturally be found in water column. If a high level of these pathogenic bacteria is present, it can result in mass mortality of cultured shrimp [5,6]. Uneaten feed also releases ammonia to water and in higher concentrations can disturb shrimp metabolism [7]. The uneaten feed becomes an unnecessary cost that the intensive culture system has to bear [8].

In addition, FAO stated the negative impacts of climate change on aquaculture affecting the water system in shrimp ponds (temperature, pH and salinity) and increasing disease outbreaks (bacterial and viral pathogen infection) [9,10]. These are threats to global food security as they cause not only production loss but also the reduction of shrimp quality. Previously, to overcome these problems, the management of water systems to control environmental factors from climate change and the prophylactic use of antibiotics to prevent disease outbreaks were explored. However, the water management system results in high costs for the farming industry, while the use of antibiotics is not sustainable and environmentally-friendly and prohibited in many countries.

Biofloc technology presents a promising alternative to managing water systems and could potentially reduce the risk of diseases to counter the negative side of intensive closed culture systems [11]. Biofloc is an aggregate of microorganism—bacteria, microalgae and protozoa—and organic matter that has high porosity [12]. Water with high nitrogen (N) and phosphorus (P) compounds can pass through the pores in biofloc, allowing the nutrient exchange. Biofloc can reduce the external organic input by microbial activities and convert it into biomass, which can be used as a natural feed source for shrimp. This natural feed can decrease the use of commercial feed in the intensive culture system, thereby reducing the production cost [12].

Benefits of biofloc technology have been shown to improve water quality [13–15], increase growth in shrimp culture [16,17], protect against pathogenic *Vibrio* [18–20] and provide supplementary nutritional intake to many aquatic animals [12,21,22]. Generally, one of the microbial communities in biofloc, genera of *Bacillus*, also has the potency to help in the digestion and absorption process in the gut of shrimp by breaking down the larger particles into smaller ones and results in better growth [19]. Nonetheless, the most implemented biofloc technology is in situ biofloc which is naturally formed in the water column. The weakness of the in-situ biofloc is the unknown composition and function of the biofloc. Therefore, the production and application of ex-situ biofloc are needed in order to optimize the performance of biofloc technology with the possibility to predict and customize the function of the biofloc. The ex-situ biofloc can be produced in suspended growth bioreactors, and the dried (powdered) biofloc can be used as a feed supplement to improve growth performance, immune responses, and metabolic activities of shrimp such as *Penaeus monodon* [23,24] and *Litopenaeus vannamei* [25].

Previously, we established ex-situ biofloc containing probiotic bacteria (*B. cereus*, *B. megaterium*), microalgae (*Chaetoceros calcitrans*), and a consortium of nitrifying bacteria according to their beneficial effects as aforementioned. The ex-situ biofloc using an inoculum percentage (% *v*/*v*) (*B. megaterium:B. cereus*:*C. calcitrans*: Nitrifying bacteria) of 1:1:6:9 was found to be an optimal ratio based on biofloc porosity and volume (unpublished data). Despite its benefits, there is still limited knowledge on how biofloc technology affects the shrimp growth performance and enhances shrimp nutritional value for food security, especially at the molecular levels.

Therefore, in this study, we aim to evaluate the effects of the established ex-situ biofloc supplementation in Pacific white shrimp (*L. vannamei)* cultured on water quality

management, the shrimp growth performance and nutritional properties, and growth-related genes to shed light on the molecular mechanism of the benefits of the ex-situ biofloc on shrimp.

2. Materials and Methods

2.1. Preparation of Ex-Situ Biofloc Production

A consortium of *B. megaterium*:*B. cereu*:*C. calcitrans*: a mixture of nitrifying bacteria (*Nitrosomonas* sp. and *Nitrobacter* sp.) (1:1:6:9 v/v) was prepared as an ex-situ biofloc for shrimp supplementation. Each component was inoculated to modify the medium that was made from molasses and ZA (zwitter ammonium) fertilizer at C/N ratio of 15 with a final pH of 7.5 ± 1.0. The medium was enriched with 0.1% v/v Na_2SiO_3 to optimize the growth of *C. calcitrans*. The components were incubated for eight days using an Imhoff cone at 27 ± 2 °C, a light intensity around ±2500 lux, photoperiodism 24:0 and salinity 12 ± 2 ppt.

2.2. Supplementation of Ex-Situ Biofloc to Shrimp Grow-Out Culture at Low Salinity Condition

Nine tanks containing 70 L of 10 ppt water were used as the experimental culture units. A group of fifteen-day old post-larval shrimp (PL15) with initial average body weight of 0.0057 ± 0.003 g was distributed randomly to the experiment tanks at a density of 500 shrimps/m^3. Commercial feed (Feng Li shrimp feed, PT. Matahari Sakti, Indonesia) was provided four times daily for six weeks of the culture period. Temperature, pH, salinity, alkalinity, dissolved oxygen and biofloc volume were maintained at the optimum range for shrimp growth [14].

Three treatment groups were tested with three replicate tanks per treatment: (1) commercial pellet treatment without biofloc supplementation (100%C); (2) 5% (w/w) feed reduction supplemented with 0.3% (v/v) biofloc (95%C+BF); and (3) 10% (w/w) feed reduction supplemented with 0.3% (v/v) biofloc (90%C+BF) (Figure 1). The ex-situ biofloc was delivered every week and freshwater was regularly added to make up for water loss due to evaporation. Water quality (DO, pH, salinity and alkalinity) was measured weekly using a portable DO meter, a pH meter, a refractometer and titration, respectively. Total ammonium nitrogen, NO_2-N, NO_3-N, and orthophosphate were measured daily following the procedures in the Standard Methods for the Examination of the Water and Wastewater [26].

Shrimp growth performance was evaluated. The final biomass was calculated as the total weight of live shrimp at the end of cultivation. Gained weight and length were measured by calculating the difference between final weight/length and initial weight/length. Specific growth rate (SGR) was calculated with this formula:

$$\text{SGR } (\%/\text{day}) = \frac{\ln(\text{final weight}/\text{initial weight}) \times 100\ \%}{\text{Day of cultivation (days)}}$$

At the end of the experiment, survival was measured as the percentage of live shrimp on the final day relative to the total initially stocked shrimp. Shrimp muscle was dissected, quickly frozen in liquid nitrogen and stored at −80 °C prior to growth-related gene expression and nutritional analyses.

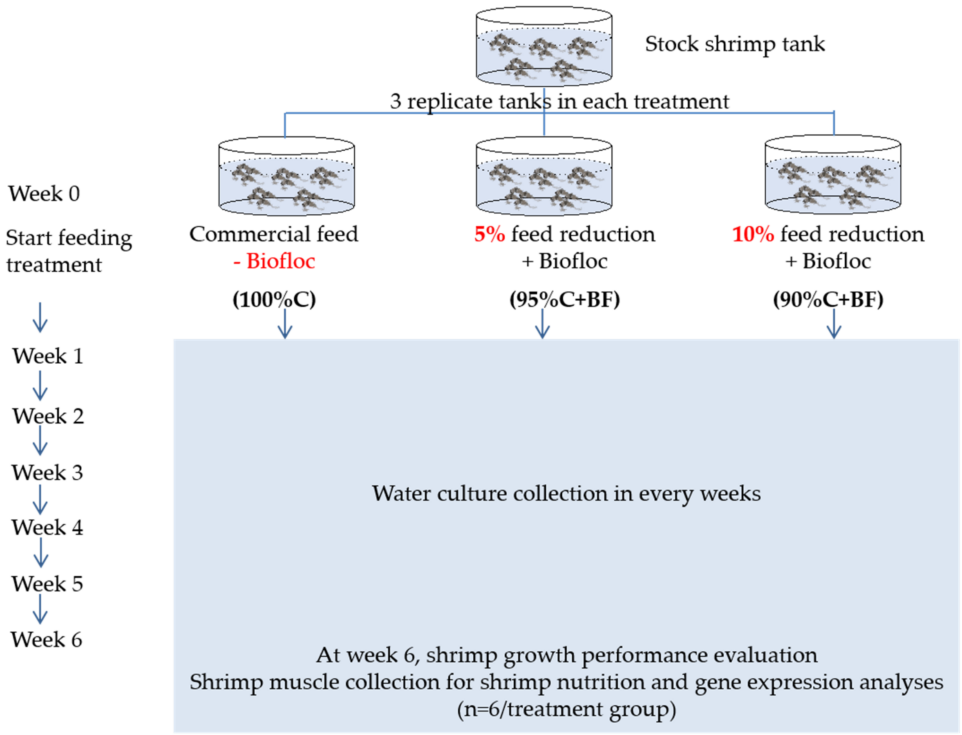

Figure 1. Experimental design in this study.

2.3. Growth-Related Gene Expression Profiles in Shrimp Using Quantitative Realtime PCR

Total RNA was extracted from individual shrimp muscle with TriReagent® (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions and was subsequently treated with 0.5 unit/μg RQ1 RNase-free DNase (Promega, Madison, WI, USA) for 30 min at 37 °C to remove any contaminated genomic DNA. The concentration of total RNA was measured using a NanoDrop ND8000 spectrophotometer. Total RNA was used for cDNA synthesis with an ImProm-II® Reverse Transcriptase System kit (Promega, Madison, WI, USA) following the company's instruction.

In brief, 1.5 mg total RNA was reverse transcribed to cDNA by priming with an oligo(dT) primer. The transcript levels of six growth-related genes containing *fatty acid synthase* (*Fas*) [27], *lipase* [28], *carnitine palmitoyltransferase* (*Cpt-1*) [27], *cytochrome C oxidase* (*Cox*) [29], *ATP synthase* (*ATPase*) [29], and *NADH dehydrogenase* (*NADH*) [30] were determined. *Elongation factor 1 alpha* (*EF1a*) was used as the internal control for quantitative real-time PCR (qPCR) analysis. A qPCR reaction (20 μL) composing of cDNA (0.5 μL), specific primers (200 nM each) and SsoAdvanced™ Universal SYBR® Green supermix (Bio-Rad, Irvine, CA, USA) according to the company's instruction. The thermal cycling parameters were 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s and 72 °C for 30 s. The melting curve analysis was performed from 65 °C to 95 °C with a continuous fluorescent reading with a 0.5 °C increment. The threshold cycle (Ct) was analyzed using BioRad CFX Manager 2.1 software (Bio-Rad, Irvine, CA, USA). Relative mRNA abundance of each gene in the sample group was calculated based on $2^{-\Delta\Delta Ct}$ method [31].

2.4. Assessment of Nutritional Properties in Feeds and Shrimp

The frozen ex-situ biofloc, frozen shrimp and frozen water samples were lyophilized using a lyophilizer (Christ, Harz, Germany). The freeze-dried samples were pulverized to fine powder using ball mill grinder (Retsch, Haan, Germany). The commercial pellet, which is an instant dried powder, was collected. All the fine powder samples were subject to the following nutritional analysis.

2.4.1. Proximate and Trace Mineral Analyses

Proximate analyses of protein, crude fat, carbohydrate, moisture and ash were analyzed using reference AOAC methods at Central Laboratory Co., Ltd. (Song Khla, Thailand). Trace minerals containing copper, manganese, zinc, iron, calcium, magnesium, sodium and potassium were analyzed at the Environmental Research Institute, Chulalongkorn University (Bangkok, Thailand).

2.4.2. Determination of Amino Acid Profiles in Feeds and Shrimp Using Gas Chromatography-Mass Spectrometry (GC-MS)

Samples (50 mg) were mixed with a hydrolysis solution (5 mL; 6 N HCl, 5% thioglycolic acid and 0.1% phenol). The mixture was incubated at 110 °C for 18 h in a hot air oven (UF110, Memmert, Büchenbach, Germany). Hydrolysate sample (1 mL) was transferred into a new microtube and centrifuged at 10,000× *g* for 10 min (5810R, Eppendorf, Hamburg, Germany). Then, the supernatant (100 µL) was added with 1 M sodium carbonate solution (310 µL) to adjust a final pH to 1–2. The mixture (25 µL) was transferred into a GC vial including an internal standard (50 µL; 200 nmol/mL norleucine) and dried using a speed vacuum concentrator (concentrator plus, Eppendorf) at 60 °C for 1 h. The dried samples were reconstituted with dichloromethane (50 µL) and dried in a speed vacuum concentrator at 60 °C for 30 min. The dried samples were derivatized using silylation reagent (50 µL; N-tert-Butyl dimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) in 1% tert-Butyldimethyl-chlorosilane (TBDMSCl), in acetonitrile (50 µL) and mixed for 30 s. The derivatized sample was incubated in the hot air oven at 100 °C for 4 h prior to injection onto a GC-MS instrument (7890B, Agilent, Santa Clara, CA, USA).

The chromatographic analysis was performed on a gas chromatography (Agilent 7890B) equipped with a mass spectrometer (Agilent 7000D) and a PAL auto sampler system (CTC Analytics AG, Zwingen, Switzerland). Aliquots of the derivatized amino acids (2 µL) were injected by using pulsed split mode at 1:5 split ratio at 280 °C into a HP-5MS column (30 m, 0.25 mm i.d., Agilent J&W GC column). Helium was used as a carrier gas with a constant flow rate of 1.4 mL/min. The GC oven was programmed as follows: ramp from 130 °C to 190 °C (6 °C/min) and to 230 °C (30 °C/min), held at 230 °C for 5 min, then ramp to 325 °C, and held at 325 °C for 6 min. Temperatures of transfer line, ion source (EI), and quadrupole were set at 325 °C, 240 °C, and 180 °C, respectively. The mass spectrometer was acquired in selected ion monitoring (SIM) mode. The calibration curves of 20 amino acids mixtures were serially diluted in a linear range of 25–400 nmol/mL. Mass spectral data of the samples were quantified by external calibration curve using Quantitative Analysis B.07.00 (Agilent Technologies, Manchester, UK).

2.4.3. Determination of Fatty Acid Analysis in Feeds and Shrimp Using GC-MS

Powder shrimp samples (50 mg) were mixed with an ice-cold Chloroform:MeOH (2 mL; 1:1 *v*/*v*) for 10 min using multi-tube vortexer (Allsheng, Hangzhou, China), sonicated for 15 min at 4 °C and then centrifuged at 3500× *g* for 20 min at 4 °C. The supernatant was collected in a sterile glass tube and dried under a constant stream of nitrogen (TurboVap, Caliper life science, Hopkinton, MA, USA) at 35 °C for 30 min. The dried samples were transesterified into FAME by adding 1% H_2SO_4-MeOH (1 mL) and heating at 50 °C for 2 h. The samples were left at room temperature to cool before being vigorously mixed with 15% NaCl (1 mL) and hexane (1 mL) and centrifuged at 3500× *g* for 5 min. The upper phase was collected into a 1 mL crimp top vial (SU860064, Supelco, Bellefonte, PA, USA) and dried under a constant stream of nitrogen for 10 min at 45 °C. The dried samples dissolved with hexane containing 5 ppm of internal standard, methyl nonanoate (9:0) and injected onto a GC (7890A, Agilent Technologies, Santa Clara, CA, USA).

GC-MS analysis was performed on an Agilent 7890A GC system coupled with Agilent 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The FAME extracts (1 µL) were separated on a 30 m × 0.250 mm × 0.25 µm film thickness DB-FastFAME capillary column (G3903–63011, Agilent Technologies, Santa Clara, CA, USA) under the

following program: an initial temperature at 50 °C, ramp 35 °C min^{-1} to 175 °C, ramp 3 °C min^{-1} to 185 °C, ramp 1.5 °C min^{-1} to 190 °C and ramp 3 °C min^{-1} to 230 °C. Pulsed Split injection was conducted with a 15:1 split ratio, helium was used as the carrier gas and the injector temperature was 60 °C. The MS detection conditions were as follows: an interface temperature, 240 °C; ionization mode, EI+; electron energy, 70 eV; full/selected ion monitoring (SIM) scan acquisition mode; mass range, 55–382 amu. Samples were analyzed with the addition of the FAME standard injections to ensure the reproducibility of the chromatography. The processed chromatograms were identified by comparison of their retention time and mass spectrum using the FAME standard mixture and an online NIST mass spectral library. The peak areas of each FAME in the samples were normalized to the peak area of the internal standard (9:0) prior to analysis. The calibration curves were performed using the 37-component FAME mix with different concentrations ranging from 0.13 to 122.12 µg/mL. Fatty acids in the samples were quantified based on the external calibration.

2.5. Statistical Analysis

Survival data were normalized using the arcsine transformation prior to statistical analysis. In order to evaluate the differences between all treatment groups, data on growth and survival parameters of all treatments were analyzed using one-way analysis of variance (One-way ANOVA) followed by Duncan Post-Hoc Test with 95% confidence intervals using statistical software SPSS® Version 24.0.

Student *t*-test was used to compare nutritional properties between two different feeds (commercial pellet and ex-situ biofloc), whereas one-way analysis of variance (One-way ANOVA) followed by multiple comparison test (Tukey; $p < 0.05$) was used to compare among shrimp samples-fed with three different treatments by SPSS® Version 15.0.

Relative fold change of gene expression among shrimp samples-fed with three different treatments was analyzed using one-way analysis of variance (One-way ANOVA) followed by Duncan Post-Hoc Test ($p < 0.05$) using SPSS® Version 15.0.

3. Results

3.1. Supplementation of Ex-Situ Biofloc in Shrimp Grow-Out Culture at Low Salinity Conditions

3.1.1. Water Quality Parameters

All water quality parameters, including temperature, DO, pH, salinity and alkalinity were maintained at the optimum range for shrimp growth (Table 1), while the biofloc concentration was kept at 0.3% (*v*/*v*) for 95%C+BF and 90%C+BF treatment groups.

Table 1. Water quality parameters for six weeks shrimp grow-out culture.

Parameter	Optimum Range	100%C	95%C+BF	90%C+BF
Temperature (°C)	28–32	27.4–30.3	27.8–30.8	27.8–31.0
Salinity (ppt)	0.5–35	11.0–15.7	10.7–16.0	11.7–16.7
DO (mg/L)	5.0–9.0	5.8–6.4	6.0–6.4	5.9–6.5
pH	7.0–8.3	7.2–7.5	7.3–7.5	7.3–7.5
Alkalinity ($CaCO_3$/L)	50–150	50.0–70.7	50.0–83.3	53.3–80.0
Biofloc volume (mL/L)	2.0–15.0	0.6–0.7	3.8–4.4	3.4–3.8

Ammonia, nitrite, nitrate and phosphate levels in the treatments supplemented with biofloc were lower than control (100%C) (Figure 2a–d). The levels of ammonia, nitrite, nitrate and phosphate in 100%C were dramatically increased along the culture period while those in 95%C+BF and 90%C+BF were slightly up until week 4 and were then stable. Ammonium concentrations in biofloc-supplemented groups were significantly lower than the control group ($p < 0.05$) at week 4 (Figure 2a). Nitrite and nitrate concentrations in biofloc-supplemented groups were significantly lower than the control group ($p < 0.05$) at week 5 (Figure 2b,c). Phosphate concentration in biofloc-supplemented groups were significantly lower than the control group ($p < 0.05$) at week 2 (Figure 2d). The results

showed that microbial community in the ex-situ biofloc may play a role in managing and stabilizing inorganic nitrogen and phosphate molecules in the shrimp culture.

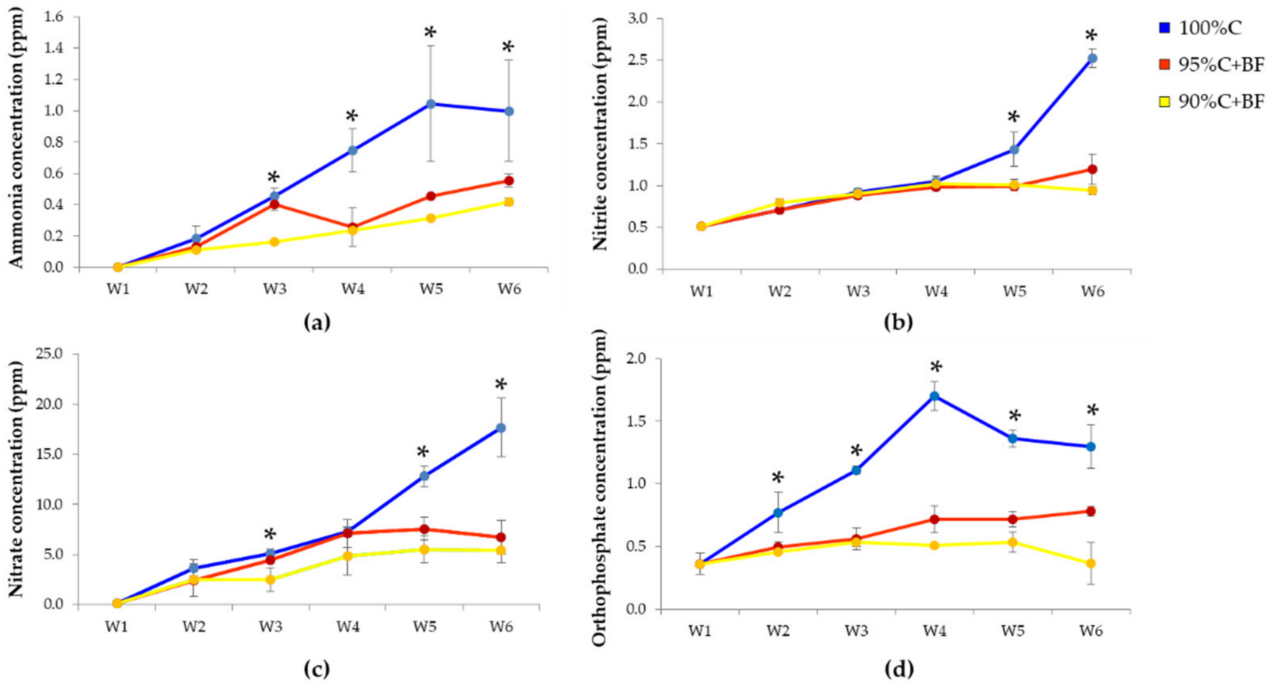

Figure 2. Concentrations of ammonia (**a**), nitrite (**b**), nitrate (**c**) and orthophosphate (**d**) concentrations during 6 weeks (W1-W6) of experiment using commercial pellet treatment without biofloc supplementation (100%C; blue), 5% (*w*/*w*) feed reduction supplemented with 0.3% (*v*/*v*) biofloc (95%C+BF; red) and 10% (*w*/*w*) feed reduction supplemented with 0.3% (*v*/*v*) biofloc (90%C+BF; yellow). Error bars represent a value of ±standard derivation. An asterisk represents a significant difference among the three groups at particular time point ($p < 0.05$).

3.1.2. Shrimp Growth Performance

At six weeks culture period, supplementation with ex-situ biofloc significantly increased the survival and growth of the shrimp (Table 2). Survival rates of shrimp-fed with commercial pellet (100%C) were significantly lower than shrimp-fed with 5% and 10% pellet reduction supplemented with 0.3% ex-situ biofloc (95%C+BF and 90%C+BF). In addition, gained weight, gained length, and specific growth rate of shrimp-fed with 95%C+BF and 90%C+BF were significantly higher than those of shrimp-fed with 100%C. Results indicated that microbial community in the ex-situ biofloc may have a role as a feed supplement to improve shrimp growth performance.

Table 2. Shrimp growth performance with and without supplementation of ex-situ bioflocs (100%C) and with different feeding regimes in which 95%C+BF were shrimp fed with 5% (*w*/*w*) feed reduction supplemented with 0.3% (*v*/*v*) biofloc, and 90%C+BF were shrimp fed with 10% (*w*/*w*) feed reduction supplemented with 0.3% (*v*/*v*) biofloc. Different letters above the values indicate significant differences among the treatments ($p < 0.05$).

Shrimp Growth Performance	100%C	95%C+BF	90%C+BF
Survival (%)	53.33 ± 5.94^{a}	83.81 ± 3.30^{b}	85.71 ± 7.56^{b}
Gained weight (g)	0.86 ± 0.15^{a}	1.97 ± 0.14^{b}	1.89 ± 0.29^{b}
Gained length (cm)	4.57 ± 0.52^{a}	6.82 ± 0.13^{b}	6.92 ± 0.22^{b}
Specific Growth Rate (SGR) (%/day)	6.59 ± 0.50^{a}	8.31 ± 0.28^{b}	8.45 ± 0.71^{b}
Biomass (g/m^3)	246.81 ± 67.17^{a}	851.63 ± 86.79^{b}	826.55 ± 44.00^{b}

3.2. Nutritional Analysis between Commercial Pellet Diet and Ex-Situ Biofloc

Four nutritional analyses were measured, including proximate analysis, trace mineral analysis, amino acid profile and fatty acid profile analyses. Proximate analysis showed that fat, protein and carbohydrate contents of commercial pellets were higher than those of ex-situ biofloc for 11.88, 2.93 and 2.00 folds, respectively (Table 3). On the other hand, ash and moisture contents of commercial pellet were lower than those of the ex-situ biofloc for 0.24 and 0.47 folds, respectively. The result indicated that commercial pellet had a higher major energetic nutrients than ex-situ biofloc, but the ex-situ biofloc had a larger amount of inorganic substances, which might be trace elements supplementing the energetic nutrients.

Table 3. Proximate analysis between commercial pellet and ex-situ biofloc.

Parameter	Unit	Commercial Pellet (C)	Ex-Situ Biofloc (BF)	Fold Change (C/BF)	Reference Method
Ash	g/100 g	11.61	48.46	0.24	[a]
Carbohydrate	g/100 g	33.92	16.99	2.00	[b]
Energy	Kcal/100 g	334.84	126.81	2.64	[b]
Crude fat	g/100 g	3.92	0.33	11.88	[c]
Moisture	g/100 g	9.58	20.25	0.47	[d]
Protein	g/100 g	40.97	13.97	2.93	[e]

[a] AOAC (2016) 942.05. [b] Journal of AOAC International; 1993. p. 106. [c] AOAC (2016) 920.39. [d] AOAC (2016) 930.15. [e] In-house method TE-CH-012 based on AOAC (2016) 981.10.

Trace mineral profiles analysis revealed that copper (8.49-fold increase), manganese (2.55-fold increase), and potassium (1.28-fold increase) contents in the commercial pellet were higher than those in ex-situ biofloc whereas zinc (0.27-fold decrease), iron (0.26-fold decrease), calcium (0.54-fold decrease), magnesium (0.08-fold decrease) and sodium (0.03-fold decrease) contents in the commercial pellet were lower than those in ex-situ biofloc (Table 4). Results indicated that a large number of essential trace minerals (zinc, iron, calcium, magnesium and sodium) for shrimp growth were observed in ex-situ biofloc.

Table 4. Trace mineral analysis between commercial pellet and ex-situ biofloc.

Mineral (mg/kg)	Commercial Pellet (C)	Ex-Situ Biofloc (BF)	Fold Change (C/BF)
Copper	50.150	5.908	8.49
Manganese	30.680	12.020	2.55
Zinc	50.340	184.000	0.27
Iron	477.00	1857.00	0.26
Calcium	2064	3824	0.54
Magnesium	1928	25,084	0.08
Sodium	3898	129,067	0.03
Potassium	9956	7758	1.28

In-depth analysis of amino acid and fatty acid profiles showed higher contents of 19 detected amino acids in commercial pellet than those in ex-situ biofloc in a range of 2.8–9.1 folds (Table 5). Glutamic acid, proline, glycine and arginine contents were found to be dominant in commercial pellet, whereas glutamic acid, aspartic acid, alanine, and leucine contents were found to be abundant in ex-situ biofloc. Proline and glycine found predominantly in the commercial pellet were higher than those in ex-situ biofloc for 9.08 and 5.90 folds, respectively. All essential amino acids were found to be significantly higher in the commercial pellet than those in ex-situ biofloc: threonine (4.94-fold increase), histidine (6.29-fold increase), isoleucine (3.23-fold increase), leucine (4.19-fold increase), valine (3.38-fold increase), lysine (3.50-fold increase), methionine (3.00-fold increase) and phenylalanine (3.37-fold increase).

Table 5. Amino acid profiles between commercial pellet and ex-situ biofloc. Different letters above the values indicate significant differences between the feeds. Letters [a] and [b] represent statistical analysis using student-*t*-test. Different letters comparing between commercial pellet and ex-situ biofloc represent significant difference ($p < 0.05$).

Amino Acids (g/100 g DW)	Commercial Pellet (C)	Ex Situ-Biofloc (BF)	Fold Change (C/BF)
Alanine	2.29 ± 0.04 [a]	0.73 ± 0.06 [b]	3.14
Glycine	2.95 ± 0.09 [a]	0.50 ± 0.09 [b]	5.90
Valine	1.76 ± 0.06 [a]	0.52 ± 0.02 [b]	3.38
Leucine	2.68 ± 0.09 [a]	0.64 ± 0.02 [b]	4.19
Isoleucine	1.55 ± 0.06 [a]	0.48 ± 0.01 [b]	3.23
Proline	4.63 ± 0.12 [a]	0.51 ± 0.04 [b]	9.08
Methionine	0.69 ± 0.01 [a]	0.23 ± 0.01 [b]	3.00
Serine	1.80 ± 0.13 [a]	0.29 ± 0.09 [b]	6.21
Threonine	1.68 ± 0.12 [a]	0.34 ± 0.08 [b]	4.94
Phenylalanine	1.55 ± 0.06 [a]	0.46 ± 0.02 [b]	3.37
Aspartic acid	2.63 ± 0.20 [a]	0.77 ± 0.27 [b]	3.42
Hydroxyproline	0.77 ± 0.01 [a]	0.144 ± 0.0004 [b]	5.35
Cysteine	0.54 ± 0.04 [a]	0.053 ± 0.003 [b]	10.19
Glutamic acid	5.22 ± 0.24 [a]	1.44 ± 0.39 [b]	3.63
Lysine	1.19 ± 0.02 [a]	0.34 ± 0.10 [b]	3.50
Arginine	2.89± 0.06 [a]	0.660 ± 0.126 [b]	4.38
Histidine	2.61 ± 0.01 [a]	0.415 ± 0.011 [b]	6.29
Tyrosine	1.27 ± 0.05 [a]	0.44 ± 0.03 [b]	2.89
Tryptophan	1.03 ± 0.03 [a]	0.170 ± 0.0003 [b]	6.06

Note: Blue-colored text indicated that these calculated values were out of limit of quantification.

Unlike amino acid profiles, not all of the fatty acid content of the commercial pellet was higher than that of ex-situ biofloc (Table 6). A total of 32 fatty acids (6–24) were detected in the commercial pellet, whereas all except for erucic acid (22:1n9) and Cis-13,16-docosadienoic acid (22:2) were detected in ex-situ biofloc. While both feeds share some common major fatty acids (Palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:1n9c) and eicosapentaenoic acid (20:5n3; EPA)), other major fatty acids were different. Considering amounts of fatty acid between the commercial pellet and ex-situ biofloc, 18 fatty acids showed significantly higher levels in the commercial pellet ($p < 0.05$); of these, four, namely oleic acid (18:1n9c, 11.69-fold increase), δ-linolenic acid (18:3n6, 31.62-fold increase), cis-11-Eicosanoic acid (20:1, 24.22-fold increase) and DHA (22.38-fold increase) were >10 folds higher. On the other hands, only four fatty acids in ex-situ biofloc showed significantly higher levels than in the commercial pellet ($p < 0.05$): myristoleic acid (14:1, 0.38-fold decrease), palmitoleic acid (16:1, 0.51-fold decrease), cis-8,11,14-eicosatrienoic acid (20:3n6, 0.14-fold decrease) and cis-11,14,17-eicosatrienoic acid (20:3n3, 0.71-fold decrease).

Table 6. Fatty acid profiles between commercial pellet and ex-situ biofloc. Different letters above the values indicate significant differences between the feeds. Letters [a] and [b] represent statistical analysis using student-*t*-test. Different letters comparing between commercial pellet and ex-situ biofloc represent significant difference ($p < 0.05$).

Fatty Acid (mg/100 g DW)	Symbol	Commercial Pellet (C)	Ex-Situ Biofloc (BF)	Fold Change (C/BF)
Methyl Hexanoate	6:0	1.23 ± 0.14 [a]	1.08 ± 0.07 [a]	1.14
Methyl Octanoate	8:0	2.03 ± 0.01 [a]	2.04 ± 0.04 [a]	0.99
Methyl Decanoate	10:0	ND	ND	ND
Methyl Undecanoate	11:0	ND	ND	ND
Methyl Laurate	12:0	2.70 ± 0.45 [a]	0.91 ± 0.10 [b]	2.98
Methyl Tridecanoate	13:0	ND	ND	ND
Methyl Myristate	14:0	36.39 ± 7.85 [a]	9.44 ± 2.43 [b]	3.86
Myristoleic Acid Methyl Ester	14:1	1.21 ± 0.27 [b]	3.20 ± 0.81 [a]	0.38
Methyl Pentadecanoate	15:0	6.55 ± 1.42 [a]	2.90 ± 0.68 [b]	2.25
Cis-10-Pentadecanoic Acid Methyl Ester	15:1	61.17 ± 45.90 [a]	90.38 ± 53.40 [a]	0.68
Methyl Palmitate	16:0	215.46 ± 30.88 [a]	91.74 ± 53.35 [b]	2.35
Methyl Pamitoleate	16:1	48.82 ± 11.91 [b]	95.06 ± 25.59 [a]	0.51
Methyl Heptadecanoate	17:0	16.63 ± 4.51 [a]	3.76 ± 0.92 [b]	4.42
Cis-10-Heptadecanoic Acid Methyl Ester	17:1	3.64 ± 0.77 [a]	2.13 ± 0.57 [b]	1.71
Methyl Stearate	18:0	79.60 ± 25.93 [a]	8.17 ± 2.06 [b]	9.75
Trans-9-Elaidic Methyl Ester	18:1n9t	67.15 ± 15.97 [a]	5.59 ± 1.48 [b]	12.01
Cis-9-Oleic Acid Methyl ester	18:1n9c	158.45 ± 16.28 [a]	13.55 ± 3.52 [b]	11.69
Linolelaidic Acid Methyl Ester	18:2n6t	ND	ND	ND
Methyl Linoleate	18:2n6c	14.05 ± 4.95 [a]	4.18 ± 3.99 [b]	3.36
Gamma-Linolenic Acid Methyl Ester	18:3n6	42.49 ± 10.24 [a]	1.34 ± 0.13 [b]	31.62
Methyl Linolenate	18:3n3	42.93 ± 10.35 [a]	7.29 ± 1.81 [b]	5.89
Methyl Arachidate	20:0	6.40 ± 1.18 [a]	1.94 ± 0.30 [b]	3.31
Methyl cis-11-Eicosanoate	20:1	18.46 ± 3.50 [a]	0.76 ± 0.06 [b]	24.22
Cis-11,14-Eicosadienoic Acid Methyl Ester	20:2	5.61 ± 1.07 [a]	0.87 ± 0.08 [b]	6.45
Methyl Heneicosanoate	21:0	1.36 ± 0.12 [a]	0.71 ± 0.04 [b]	1.92
Cis-8,11,14-Eicosatrienoic Acid Methyl Ester	20:3n6	3.03 ± 0.46 [b]	22.43 ± 6.12 [a]	0.14
Cis-11,14,17-Eicosatrienoic Acid Methyl Ester	20:3n3	15.54 ± 3.10 [a]	21.77 ± 6.02 [a]	0.71
Methyl Cis-5,8,11,14-Eicosatetraenoic Acid Methyl Ester	20:4n6	2.56 ± 0.43 [a]	1.73 ± 0.26 [b]	1.48
Methyl Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	20:5n3	43.44 ± 9.43 [a]	29.29 ± 7.89 [a]	1.48
Methyl Behenate	22:0	2.44 ± 0.35 [a]	1.90 ± 0.30 [a]	1.28
Methyl Erucate	22:1n9	14.03 ± 4.67	ND	ND
Methyl Tricosanoate	23:0	0.84 ± 0.09 [a]	0.75 ± 0.11 [a]	1.12
Cis-13,16-Docosadienoic Acid Methyl Ester	22:2	11.44 ± 2.85	ND	ND
Methyl Lignocerate	24:0	4.45 ± 0.78 [a]	2.86 ± 0.53 [b]	1.55
Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	22:6n3	69.92 ± 14.57 [a]	3.12 ± 0.74 [b]	22.38
Methyl Nervonate	24:1	3.77 ± 1.14 [a]	0.94 ± 0.37 [b]	4.01

Note: Blue-colored text indicated that these calculated values were out of limit of quantification. ND: not detectable.

3.3. Nutritional Analysis in Shrimp under Different Feeding Regime

Commercial pellets and ex-situ biofloc were fed to shrimp with a different ratio for six weeks. Nutritional contents of the shrimps (trace mineral content, amino acid profile and fatty acid profile) in each treatment group were determined to evaluate nutrients uptake into the shrimp. Trace mineral contents in shrimp-fed with 5% commercial pellet reduction and supplement with 0.3% ex-situ biofloc (95%C+BF) showed no significant differences to the control group (100%C), whereas calcium, manganese and magnesium contents in shrimp-fed with 10% commercial pellet reduction and supplement with 0.3% ex-situ biofloc (90%C+BF) were significantly lower than the control group (Figure 3a,b).

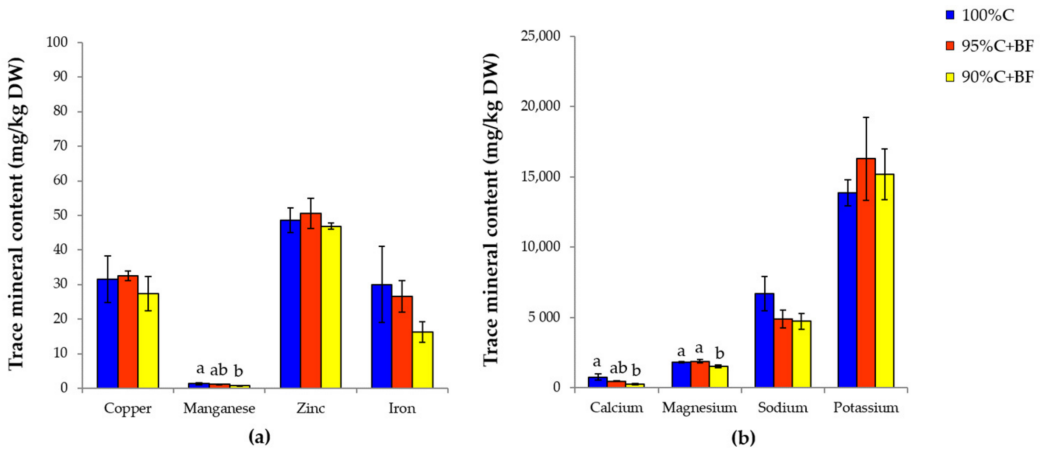

Figure 3. Trace mineral analysis of shrimp-fed with commercial pellet (100%C; blue) and shrimp-supplemented with ex-situ biofloc in different commercial pellet reduction ratios (95%C+BF, red; 90%C+BF, yellow). (**a**) Copper, Manganese, Zinc and Iron, and (**b**) Calcium, Magnesium, Sodium, and Potassium. Error bars represent a value of ± standard derivation. Different letters above bars (a and b) represent a significant difference among the three groups.

For amino acid profiles, most amino acid contents in 95%C+BF- and 90%C+BF-fed shrimp were not significantly different from the control shrimp (100%C), except alanine, glycine and leucine, whose levels in 95%C+BF- fed shrimp were significantly lower than those in 100%C. In addition, valine, and isoleucine whose levels in both 95%C+BF- and 90%C+BF-fed shrimp were significantly lower than those in 100%C (Figure 4).

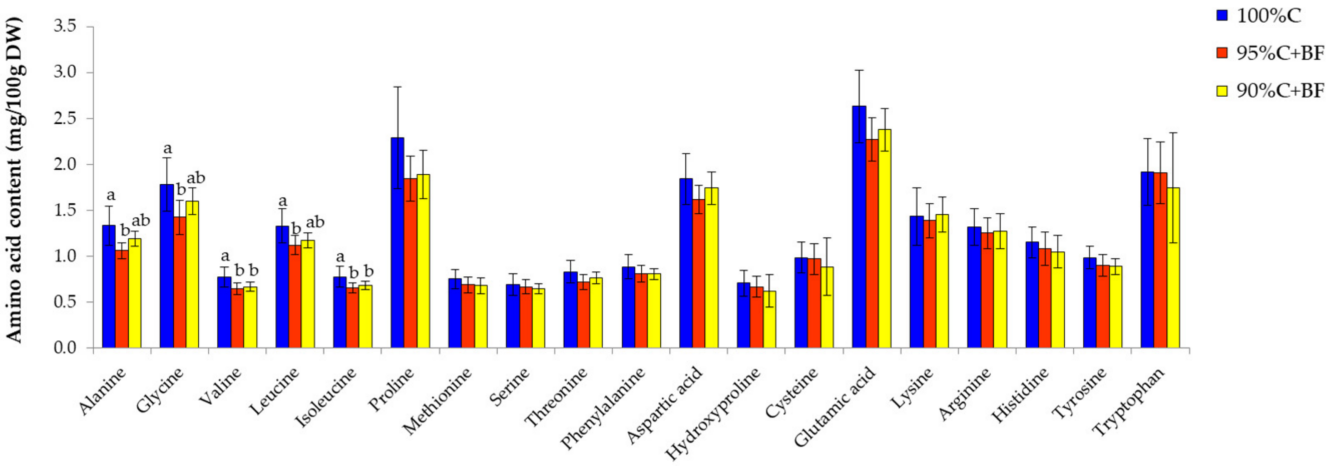

Figure 4. Amino acid profiles analysis of shrimp-fed with commercial pellet (100%C, blue) and shrimp-supplemented with ex-situ biofloc in different commercial pellet reduction ratios (95%C+BF, red; 90%C+BF, yellow). Error bars represent a value of ±standard derivation. Different letters above bars (a and b) represent a significant difference among the three groups.

Fatty acid profiles in shrimp were predominant in saturated fatty acids (16:0 and 18:0), monounsaturated fatty acids (18:1n9c and 18:1n9t) and polyunsaturated fatty acids (18:2n6c, 20:3n6, 20:3n3, 20:5n3 and 22:6n3) (Figure 5a). Levels of 14:1, 16:0, 17:0 and 18:0 fatty acids in 95%C+BF- fed shrimp were significantly higher than those in control-fed shrimp, whereas the levels of 20:1 and 22:6n3 (DHA) were significantly higher in both 95%C+BF- and 90%C+BF-fed shrimp ($p < 0.05$) compared to the control group (Figure 5b). The rest of the detected fatty acids were not significantly different among the treatment groups.

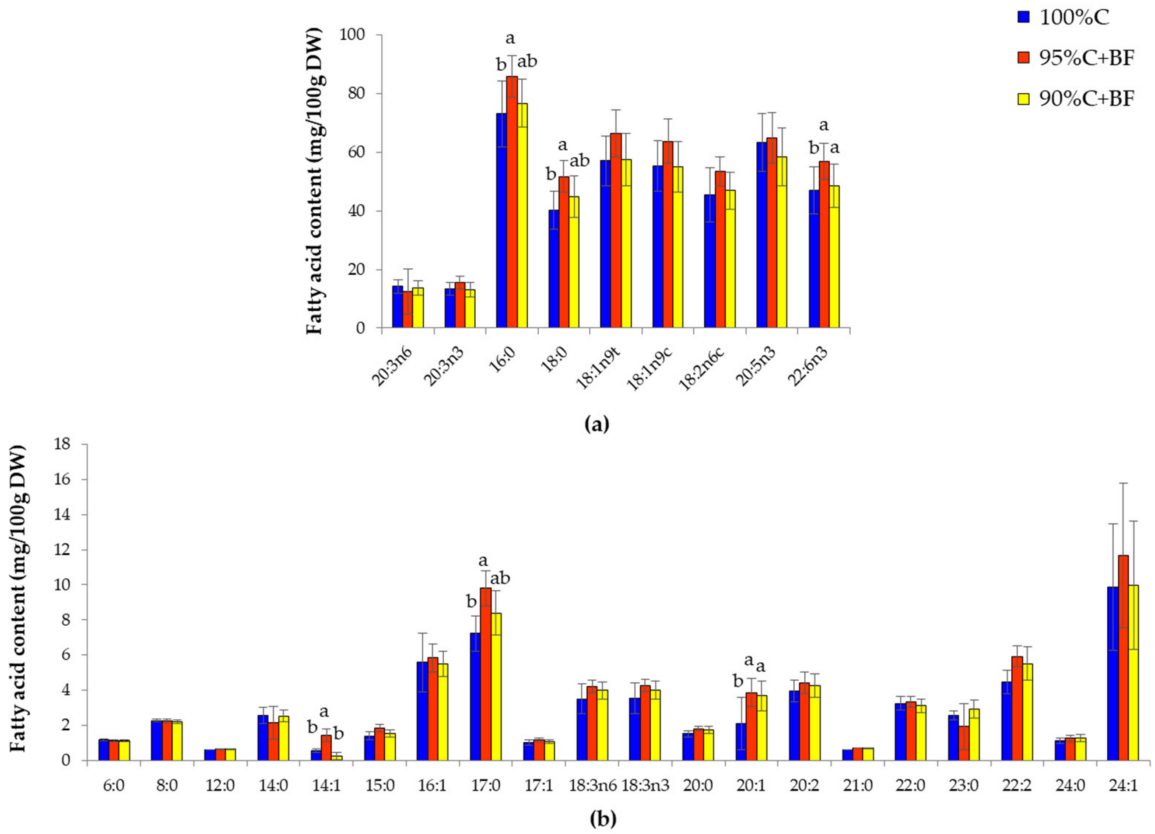

Figure 5. Fatty acid profiles analysis of shrimp-fed with commercial pellet (100%C, blue) and shrimp-supplemented with ex-situ biofloc in different commercial pellet reduction ratios (95%C+BF, red; 90%C+BF, yellow). Error bars represent a value of ±standard derivation. Different letters above bars (a and b) represent a significant difference among the three groups.

3.4. Gene Expression Analysis

Six growth-related genes containing *fatty acid synthase (Fas), Lipase, carnitine palmitoyltransferase (Cpt-1), cytochrome C oxidase (Cox), ATP synthase (ATPase)* and *NADH dehydrogenase (NADH)* were examined for molecular responses in shrimp among commercial pellet feeding, and 5% and 10% commercial pellet reduction with ex-situ biofloc supplement. The expression levels of all six genes were significantly higher in both shrimp fed with 5% and 10% commercial pellet reduction with ex-situ biofloc supplement (95%C+BF and 90%C+BF, respectively) than the control (100%C) (Figure 6).

For genes involved in lipid metabolism and the digestion process, *Fas* (2.15 and 3.40 folds), *Lipase* (2.21 and 3.13 folds), and *Cpt-1* (1.79 and 1.57 folds) transcripts exhibited slightly significant increases in shrimp-fed with 95%C+BF and 90%C+BF, respectively compared to the control (100%C). Comparing between shrimp-fed with 95%C+BF and 90%C+BF, *Fas* and *Lipase* expression levels in 90%C+BF-fed shrimp were significantly higher than those in 95%C+BF-fed shrimp for 1.58 and 1.42 folds, respectively. In contrast, there was no significant difference for *Cpt-1* transcript.

For genes involved in the energy metabolic process, the expression levels of *ATPase* (25.06 and 20.51 folds) and Cox (19.19 and 19.01 folds) were dramatically significantly higher in shrimp-fed with 95%C+BF and 90%C+BF, respectively, whereas *NADH* expression level was slightly significant higher (2.91 and 2.34 folds, respectively). Comparing between shrimp-fed with 95%C+BF and 90%C+BF, *ATPase* and *NADH* expression levels in 90%C+BF-fed shrimp were significantly lower than those in 95%C+BF-fed shrimp for 1.22 and 1.01 folds, respectively, while there was no significant difference for the *Cox* transcript.

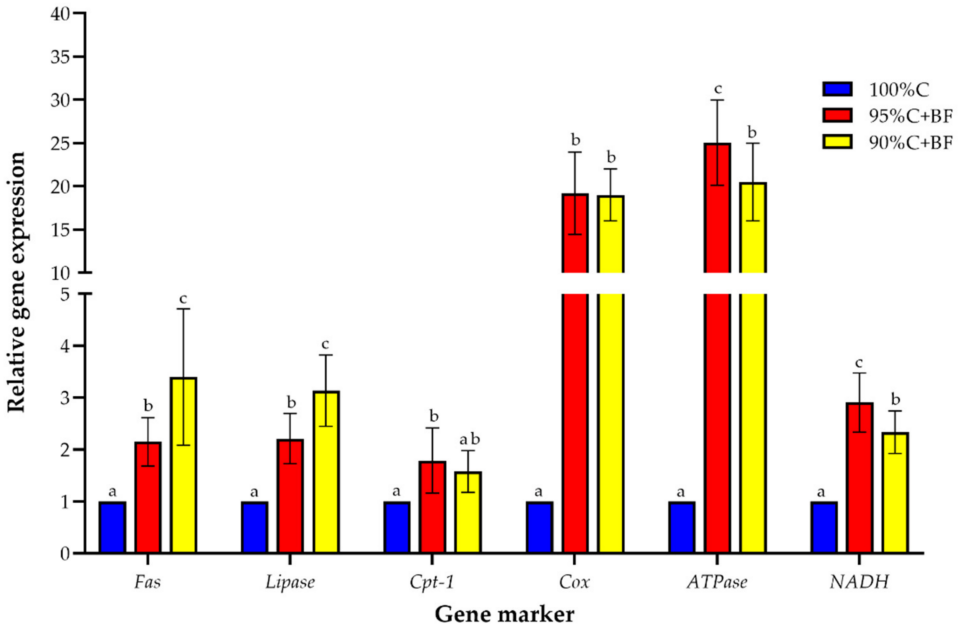

Figure 6. Relative gene expression levels (*fatty acid synthase (Fas), Lipase, carnitine palmitoyltransferase (Cpt-1), cytochrome C oxidase (Cox), ATP synthase (ATPase)*, and *NADH dehydrogenase (NADH))* in shrimp muscle from the shrimp-fed with commercial pellet (100%C, blue) and shrimp-supplemented with ex-situ biofloc in different commercial pellet reduction (95%C+BF, red; 90%C+BF, yellow). Error bars represent a value of ± standard derivation. Different letters above bars (a, b and c) indicate statistical difference ($p < 0.05$) among three different groups.

4. Discussion

This study applied the established ex-situ biofloc containing probiotic bacteria (*B. cereus*, *B. megaterium*), microalgae (*Chaetoceros calcitrans*), and a consortium of nitrifying bacteria as a feed supplement in Pacific white shrimp cultured upon a grow-out phase at low salinity condition. The biofloc volume used in this study is within the ranges previously suggested: 5 to 15 mL/L (or equal to 0.5% to 1.5% v/v) [15], 0.5, 1% and 2% (v/v) [25] and 3 to 4 mL/L (or equal to 0.3% to 0.4% v/v) [32], which resulted in a higher specific growth rate in shrimp post-larval culture. Thus, in this study, we tested the supplementation of ex-situ biofloc along with a reduction of feed amount of 5% and 10% to evaluate the potential application of ex-situ biofloc as partial replacement of artificial diets, and to evaluate the effect of ex-situ biofloc supplementation on feed utilization as well as shrimp growth performance. Considering our application, the ex-situ biofloc as a feed supplementation in the shrimp culture revealed the potential ability to maintain water quality in the ponds, improve shrimp growth performance and also enhance shrimp nutritional value. The effect of the ex-situ biofloc on the three different aspects was discussed below.

4.1. Effect of Ex-Situ Biofloc on Water Quality Maintenance

The ex-situ biofloc applied into the shrimp culture indicated lower ammonia, nitrite, nitrate and phosphate concentrations than the control (no ex-situ biofloc supplement). Lower ammonia concentration could be due to the activity of heterotrophic bacteria *B. cereus* and *B. megaterium*, a consortium of nitrifying bacteria, as well as diatom *C. calcitrans*. It has been reported that heterotrophic bacteria and diatom uptake ammonia and metabolize it into biomass [33,34], while nitrifying bacteria convert ammonia to nitrite then nitrate [15]. The nitrite and nitrate concentrations in bioflocs treatment were lower than the control, suggesting that the reduction of ammonia in the system was dominated by the activity of heterotrophic or diatom. A lower concentration of phosphate was predicted as the result of diatom activity that metabolizes orthophosphate to biomass [35]. Thus, the ex-situ biofloc

revealed that water quality maintenance activity should be used as a good microbe starter for aquaculture.

4.2. Effects of Ex-Situ Biofloc as Feed Supplement to Improve Shrimp Growth Performance

Interestingly, when supplementing ex-situ biofloc with 5% and 10% commercial pellet reduction, the survival rates and the shrimp body weights were significantly higher than the control, suggesting that ex-situ biofloc might play a role as a feed supplement to enhance shrimp growth performance. From proximate analysis, commercial pellet contained a high amount of macromolecule nutrients (e.g., carbohydrate, fat and protein), while ex-situ biofloc was comprised of the high amount of minerals (e.g., zinc, iron, calcium, magnesium and sodium).

In addition, the amounts of fatty acids (stearic acid (18:0), oleic acid (18:1n9c), linoleic acid (18:2n6), linolenic acid (18:3n3), arachidonic acid (20:4n6), eicosapentaenoic acid (20:5n3; EPA) and DHA (22:6n3)) from the commercial pellet were significantly higher than those from the ex-situ biofloc. These fatty acids are essential nutrients for growth and development in animals and humans [36–38] and also play important roles in shrimp nutrition and shrimp growth and reproduction [39]. For amino acids, overall detectable amino acids (nine essential amino acids and ten non-essential amino acids) in commercial pellet were significantly higher than those in ex-situ biofloc. Proline, glutamic acid and glycine were predominant in the commercial pellet. They are involved in collagen synthesis for strengthening connective tissues such as bone, skin, cartilage and blood vessel in mammals, livestock and aquatic animals [40], maintaining digestive function and protecting the integrity of the intestinal mucosa of human [41]. This finding suggests that macromolecule nutrients from commercial pellet were the major source of nutrients feeding to shrimp; however, feed utilization efficiency may be improved by minerals obtained from ex-situ biofloc.

According to the following supported evidence, the microbial community that mainly contributes to the water quality control process was possibly from microalgae. Microalgae biomass is a well-known source of essential vitamins and minerals (e.g., Na, K, Ca, Mg, Fe, and Zn) [42,43]. Microalgae can also serve as "prebiotic-like" when interacting with heterotrophic bacteria in the biofloc system. This symbiosis can maintain the nutrient cycle and energy flow, resulting in balancing C and N compounds in aquaculture and supporting the aquaculture production yields [44]. Using a diatom, *Chaetoceros calcitrans* as feed replacement to protein, vitamins and minerals of commercial pellet in black tiger shrimp revealed increasing feed conversion efficiency, growth performance and survival [45].

Considering the roles of minerals, calcium (Ca) and magnesium (Mg) play an important role in molting and new shell formation [46]. Ca also support the growth of *L. vannamei* in low salinity conditions [47]. Sodium, potassium and Mg are essential for maintaining proper physiological function and osmoregulation in biological organisms [48]. Mg supplementation showed increasing survival of *L. vannamei* in low salinity conditions [48,49]. Zinc (Zn) is an essential micronutrient required for plants, animals and humans for growth and reproduction [50]. Zinc also plays a pivotal role as a regulatory co-factor of different enzymes and proteins in many important biochemical pathways [51]. Zn supplementation to shrimp indicated enhancing the growth and induction of immune responses [52,53]. Iron (Fe) plays an important role in energy metabolism, homeostasis and co-factor/activator of the enzyme system [54,55]. It has been proven to support the growth and the survival of juvenile giant river prawns [56]. Improvement of shrimp growth performance and survival suggests that mineral contents (Zn, Fe, Ca, Mg and Na) from the ex-situ biofloc were essential for biological processes such as osmoregulation, structural components of shell, co-factors of enzyme system for nutrient absorption and energy production, leading to enhanced shrimp survival and growth performance.

Most of trace mineral concentrations in shrimp flesh had no significant differences, except for calcium, magnesium and manganese, whose levels were significantly lower in the shrimp-fed with 90% commercial pellet reduction supplemented with ex-situ biofloc

(90%C+BF) than those in the control group ($p < 0.05$) (Figure 3a,b). Considering trace mineral contents in feeds (Table 4), manganese concentration was higher in commercial pellets than that of ex-situ biofloc. Consequently, a lower manganese level in shrimp flesh might be correlated to the reduced level of commercial pellets. Nevertheless, our observation suggests that a lower level of manganese concentration in shrimp-fed with 90%C+BF was sufficient for biological processes, as there were no negative effects on shrimp survival and growth. In contrast, magnesium and calcium concentrations, which were higher in ex-situ biofloc than those of commercial pellets, were found to be significantly lower in shrimp flesh in the 90%C + BF group. Magnesium and calcium are a major part of shrimp exoskeletons and also play an important role for osmoregulatory purposes [46,57]. Calcium and magnesium are mostly found in the shells of Pacific white shrimp [58]. Thus, we speculated that magnesium and calcium could be mostly deposited in shells, hence lower amounts were observed in the flesh.

To elucidate the molecular mechanism, the expression levels of the selected genes related to lipid metabolism and energy metabolic process, namely *fatty acid synthase* (*Fas*), *Lipase*, *Carnitine palmitoyltransferase-1* (*Cpt-1*), *cytochrome C oxidase* (*Cox*), and *NADH dehydrogenase* (*NADH*), were examined and found to be correlated to shrimp growth performance and survival. The lipid metabolism genes (*Fas*, *Lipase* and *Cpt-1*) were expressed higher in shrimp-fed with 5% and 10% commercial pellet reduction and ex-situ biofloc supplement, which may suggest improvement in fatty acid synthesis. The encoded gene products play a crucial role in the digestion of dietary fat [59]. Previous studies reported that *Bacillus* spp. could be a probiotic to stimulate digestive enzyme activity in *Fenneropenaeus indicus* [60], and in *L. vannamei* [61–63]. *Cpt-1* has a primary function for energy production through the β-oxidation of fatty acids in the mitochondria in Gilthead Sea Bream fish [64,65]. *Cox*, *ATPase*, and *NADH* genes are relevant to energy metabolism. *Cox* plays a crucial role in the metabolic pathway associated with fatty acid β-oxidation of peroxisomal [66]. The higher expression levels of energy-related genes in the biofloc groups indicated that flux energy is increased during the feeding trail. The elevated expression levels of these transcripts suggest that the ex-situ biofloc aids in enhancing digestion of lipid, increasing feed absorption and energy production, resulting in improving feed utilization, growth performance and survival in shrimp.

4.3. Effects of Ex-Situ Biofloc as Feed Supplement to Enhance Shrimp Nutritional

Ex-situ biofloc supplementation in shrimp culture also affected nutritional value in shrimp as observed from amino acids and fatty acids profile in shrimp muscle. Amino acid concentrations in shrimp fed with 5% and 10% commercial pellet reduction plus ex-situ biofloc supplements were mostly found to be similar to those in the shrimp fed with only the commercial pellet, except alanine, glycine, leucine, valine and isoleucine. This result indicated that amino acid concentrations did not increase in the shrimp muscle when supplementing with ex-situ biofloc. This might be caused by the fact that excessive amounts of proteins, for example, glycine, essential for muscle development, are turned to fat molecules for storage like in humans [67] and in juvenile Pacific white shrimp [68]. Another possibility may be from gut microbial and/or probiotics absorption and utilization of amino acids into health benefit molecules to the host, resulting in amino acid homeostasis in the host cells [69]. This agreed with our observation that most of the amino acid concentrations in shrimp muscle were still similar between control and supplementing with ex-situ biofloc, containing probiotic bacteria. In addition, this finding correlated with metabolomics study of whiteleg shrimp cultured in different culturing systems (recirculating aquaculture system (RAS), hybrid zero water discharge-recirculating aquaculture system (hybrid) and outdoor earthen pond), revealing that alanine concentrations decreased in RAS and hybrid systems because super-intensive shrimp culture caused oxidative stress induction [70]. This suggests that amino acids were affected by different factors, such as stress, from super-intensive culture, microbial utilization, and nutrient storage metabolism. Therefore,

ex-situ biofloc supplementation may not affect amino acid content in shrimp muscle in terms of nutritional value.

Interestingly, the ex-situ biofloc supplement affected fatty acid contents in shrimp muscle. Docosahexaenoic acid (DHA; 22:6n3) and eicosenoic acid (20:1) concentrations were significantly higher in shrimp fed with the ex-situ biofloc supplement with 5% and 10% commercial pellet reduction than feeding with commercial pellet alone. These fatty acids were found in both the commercial pellet and ex-situ biofloc; however, they were predominant in the commercial pellet. DHA is an omega 3 polyunsaturated fatty acid, a well-known nutrient source found in fish, shrimp, and marine organisms, that benefits visual and brain development in humans [71–74]. Eicosenoic acid (20:1) is a monounsaturated fatty acid existing in three different forms: 9-eicosenoic acid (an omega-11 gadoleic acid), 11-eicosenoic acid (an omega-9 gondoic acid), and 13-eicosenoic acid (an omega-7 paullinic acid) [75]. An eicosenoic acid-rich marine oil diet showed decreasing risk factors for cardiovascular-related diseases and atherosclerosis in mice [76] and suppressing lipogenesis in mice [77]. These fatty acids were found to be beneficial to humans.

Bacillus megaterium and *B. cereus* contained in the ex-situ biofloc are well-known heterotropic bacteria that have been reported to be potential probiotic bacteria in other organisms. Previous studies reported that a probiotic *Lactobacillus acidophilus* and *Lactobacillus plantarum*-supplemented diet provided benefits to overall health, immunity and gut microbial composition in crayfish [78]. Brevibacillus and Fermicutes communities showed abilities to increase nutrients absorption and digestion in gut mammal [79] and in gut shrimp *p*. monodon [80]. The fact that genes related to lipid metabolism and energy metabolic process (e.g., *fatty acid synthase*, *triacylglycerol lipase*, *carnitine palmitoyltransferase*, *NADH dehydrogenase*, *ATPase* and *cytochrome b oxidase*) were expressed higher in the ex-situ biofloc shrimp also supports that ex-situ biofloc-supplement enhanced lipids digestion and absorption in shrimp muscle to boost the nutritional value. Thus, supplementation with the ex-situ biofloc influenced an increase in DHA and 20:1 concentrations in shrimp muscle, suggesting that *B. megaterium* and *B. cereus* in the ex-situ biofloc might play important roles in triggering gut microbiota to enhance nutrient absorption from the commercial pellet as well as digestion and storage of lipids, which mostly contained DHA and 20:1 fatty acids into the shrimp muscle. This mode of action not only enhanced shrimp growth performance, but also boosted the nutritional value of shrimp for human consumption.

5. Conclusions

This study showed that biofloc could be formed externally (ex-situ biofloc) using probiotics *B. megaterium* and *B. cereus*, diatom microalgae *C. calcitrans*, and consortium of nitrifying bacteria, with the ratio of 1:1:6:9 as a starter. Overall, the ex-situ biofloc supplementation in shrimp grow-out culture can increase the shrimp culture performance (shrimp growth, survival and total biomass), and provide inorganic substances such as trace minerals (calcium, manganese, zinc and iron) to support shrimp growth performance in combination with a major source of energetic nutrients (proteins and lipids) from the commercial pellet. The optimal feed formulation for the shrimp grow-out phase was the 5% reduction of the commercial pellet in supplement with 0.3% ex-situ biofloc (95%C+BF). Shrimp fed with 95%C+BF showed a significant increase of growth-related genes (e.g., *fatty acid synthase*, *triacylglycerol lipase*, *carnitine palmitoyltransferase*, *NADH dehydrogenase*, *ATPase* and *cytochrome b oxidase*) compared to the control (100%C). Together, this study sheds light on the potential modes of action of ex-situ biofloc to maintain water culture quality, enhance shrimp growth performance and survival, and enable shrimp nutritional value. All these beneficial effects of ex-situ biofloc as a feed supplement in an intensive shrimp culture at low salinity conditions can be feasible for the shrimp farming industry, especially in-land culture, leading to a reduction of the cost of shrimp production and to sustaining the quality of shrimp products in the future.

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Conflicts of Interest: The authors declare no conflict of interest.

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