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Different protein levels in a super-intensive culture of juvenile Pacific white shrimp (*Litopenaeus vannamei*) in biofloc systems during the early rearing phase

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ABSTRACT

The determination of crude protein levels in diets of aquatic organisms allows maximizing growth, decreasing feed costs, and improving water quality. A rearing culture of *Litopenaeus vannamei* was carried out in superintensive biofloc systems using five diets with different crude protein (CP) levels (320 g·kg^{-1} CP, 360 g·kg^{-1} CP, 400 g·kg^{-1} CP, 440 g·kg^{-1} CP, and 480 g·kg^{-1} CP); each diet was used in quadruplicates. The experimental period lasted 35 days, and the shrimps (0.59 ± 0.001 g) were stocked at the density of 600 PL·m^{-3}, using 20 experimental units (50 L of useful volume). There were no significant differences in animal survival between treatments ($p > 0.05$). The animals fed with 480 g·kg^{-1} presented higher final weight and specific growth rate when compared to the treatments fed with 320 g·kg^{-1} ($p < 0.05$). Furthermore, feeding shrimp with 32% CP diets results in a reduction in feed utilization and nutrient retention, thus increasing feed costs. Overall, according to the broken line analysis, 38% CP would be ideal, and according to the quadratic regression 46% CP would result in the best growth of *L. vannamei* at this growth stage.

Keywords: Antioxidant system; Feed conversion ratio; Feeding cost; Growth.

Diferentes níveis proteicos no cultivo superintensivo de juvenis de camarões-brancos *Litopenaeus vannamei* em sistema de bioflocos durante berçário secundário

Resumo

A determinação dos níveis de proteína bruta em dietas de organismos aquáticos permite maximizar o crescimento, reduzir os custos com alimentação e melhorar a qualidade da água. Foi realizado o cultivo de *Litopenaeus vannamei* em sistema superintensivo com bioflocos utilizando cinco dietas com diferentes níveis de proteína bruta (PB) (320 g·kg^{-1} PB, 360 g·kg^{-1} PB, 400 g·kg^{-1} PB, 440 g·kg^{-1} PB e 480 g·kg^{-1} PB). Cada dieta foi utilizada em quadruplicata. O período experimental durou 35 dias, e os camarões (0,59 ± 0,001 g) foram estocados na densidade de 600 PL·m^{-3}, utilizando 20 unidades experimentais (50 L de volume útil). Não houve diferenças significativas na sobrevivência dos animais entre os tratamentos ($p > 0,05$). Os animais alimentados com 480 g·kg^{-1} apresentaram maior peso final e taxa de crescimento específico quando comparados aos tratamentos alimentados com 320 g·kg^{-1} ($p < 0,05$). Além disso, alimentar camarões com dietas com 32% de PB resultou em redução na utilização de ração e retenção de nutrientes, aumentando assim os custos com ração. No geral, de acordo com a análise *broken line*, 38% de PB seria o ideal, e de acordo com a regressão quadrática 46% de PB resultaria no melhor crescimento de *L. vannamei* nessa fase de crescimento.

Palavras-chave: Sistema antioxidante; Fator de conversão alimentar; Custos de alimentação; Crescimento.

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INTRODUCTION

Marine shrimp farming is one of the most important aquaculture activities worldwide, due to factors such as price and the supply of high-quality protein (Cai et al., 2019). The main species used, the Pacific white shrimp, *Litopenaeus vannamei*, stands out as the most produced species in the world, mainly in semi-intensive and intensive culture systems (FAO, 2022; Strebel et al., 2023). In addition, the culture of *L. vannamei* in intensive systems with biofloc has received attention in recent decades, in terms of reduced water consumption, higher stocking densities, increased productivity, and improvement of the immune and antioxidant systems of the animals, besides its use as a supplementary feed for farmed organisms (Emerenciano et al., 2017; Panigrahi et al., 2018; Krummenauer et al., 2020; Silveira et al., 2022a; Khanjani et al., 2023). However, a too large increase in stocking density can cause a stressful effect on the animals, known as the crowding effect, which reduces growth and food utilization; impairs the immune and antioxidant systems of shrimps; and, in extreme cases, can affect the survival of the organisms (Lin et al., 2015; Liu et al., 2017; Silveira et al., 2022a).

Nutrition plays an important role in the face of stressors, as it is one of the main tools to increase the well-being of aquatic organisms through the balance of nutrients. In this sense, shrimp should be provided with a nutritionally complete diet specific to each stage and culture condition to ensure proper growth and profitability of culture (Braga et al., 2016; Xu et al., 2018). Li et al. (2017) reported changes in the nutritional requirements of *L. vannamei* cultured at low and high salinity. However, these requirements for superintensive farming are still unknown (Emerenciano et al., 2022).

In general, the protein level in diets is the most influential factor in shrimp farming (Mansour et al., 2022b), as it plays a primary role in tissue construction and repair, maintenance of vital functions, immune response, and as an energy source for animal metabolism (Dumas et al., 2007; Wang et al., 2015). In the scientific literature, it is possible to identify a wide variation in the protein demand of *L. vannamei* (300 to 447 $g \cdot kg^{-1}$); this was related to farming conditions, type of system, and animal life stage (Henriques et al., 2021; Strebel et al., 2023). In intensive systems, the importance of diet quality, formulation, and proper feed management are responsible for maximizing productive results since they are the main source of nutrients for the animals (Emerenciano et al., 2022; Strebel et al., 2023). In addition, protein levels are directly related to feed costs (the main effective operating cost) mainly due to the increased use of high-cost ingredients such as fishmeal, one of the main protein ingredients due to its high digestibility (Rego et al., 2017; Almeida et al., 2021; Ashour et al., 2021).

Strategies to reduce the protein content of diets and partially replace fishmeal with other ingredients of animal and plant origin have been discussed in recent years (Cummins Jr. et al., 2017; Qiu et al., 2018; Yao et al., 2020). One of these alternatives is the use of bioflocs, which can be used either as an ingredient in shrimp feed (Shao et al., 2017; Castro et al., 2021; Nethaji et al., 2022) or as a direct intake from culture water (Yun et al., 2016; Mansour et al., 2022b). For juveniles of *L. vannamei* (0.80 g), biofloc could provide 63–100% of carbon and 35–86% of nitrogen (Krummenauer et al., 2020).

In the other hand, proteins are directly correlated with the antioxidant system of shrimp, especially by increasing the activity of the superoxide dismutase (SOD) enzyme, that has a significant role against reactive oxygen species (ROS) (Panigrahi et al., 2019, 2020). In addition, shrimp that have fed on the microbial aggregates present in situ during biofloc technology system (BFT) cultivation has an increased antioxidant capacity, since bioflocs are capable of incorporating bioactive compounds such as carotenoids, chlorophylls, phytosterols, and bromophenols (Panigrahi et al., 2019; Miao et al., 2020; Colombo et al., 2023).

In view of the above, the present study aimed to investigate the effect of different protein levels in the diets of juvenile *L. vannamei* cultured in superintensive biofloc systems on shrimp growth, antioxidant capacity, and feeding cost.

MATERIALS AND METHODS

Origin of animal and facilities

The post-larvae (PL 10) were purchased from the commercial larviculture "Pós-larvas do Sul Larvicultura", located in Itapoá, Santa Catarina, Brazil, and reared in a nursery at the Marine Aquaculture Station, Oceanographic Institute of the Universidade Federal do Rio Grande (FURG), until they reached an average weight of 0.59 ± 0.003 g, when they were transferred and acclimatized to the experimental conditions. *L. vannamei* juveniles were stocked in 20 polypropylene boxes (50 L of useful volume) at the density of 600 $juveniles \cdot m^{-3}$. Each experimental unit had a heating system (Roxin HT1300, 100 W) for thermal control (27°C), and aeration was provided through a porous hose (15 cm, Aero-tube TM, Swan, Marion, OH) individually connected to a blower (CR8, 7.5 CV, Ibram). The tanks were filled with 80% (40 L) of chlorinated (20 $mg \cdot L^{-1}$ active chlorine) seawater (salinity 30.38 $g \cdot L^{-1}$) and dechlorinated with constant aeration, and subsequently with 20% (10 L) of previously matured biofloc inoculum, according to the method

proposed by Santos et al. (2019), in order to ensure the following initial conditions: 0.07 ± 0.04 mg·L^{-1} TAN, 0.05 ± 0.01 mg·L^{-1} NO_2^--N, 15.27 ± 2.77 mg·L^{-1} NO_3^--N, 164.1 ± 12.6 mg·L^{-1} $CaCO_3$, and 137.10 ± 28.70 mg·L^{-1} SST. In addition, the photoperiod was controlled and maintained at 12:12 light:dark.

Experimental design and feeding formulated

The experimental design adopted was composed of five treatments, consisting of variations in the levels of crude protein (CP) in diets (320 g·kg^{-1} CP, 360 g·kg^{-1} CP, 400 g·kg^{-1} CP, 440 g·kg^{-1} CP, and 480 g·kg^{-1} CP), isolipidic and isoenergetic (90 g·kg^{-1} and 19,8 kJ·g^{-1}, respectively). Each treatment had four replicates. Dry ingredients such as fishmeal, soybean meal, cornstarch, cellulose, yeast, calcium carbonate, premix, and methionine were pre-mixed in the exact proportions (Table 1). The gelatin was hydrated in heated water (80°C), and fish oil (Special fish oil 0560-0, Campestre, São Paulo, Brazil) and distilled water were later added to the mixture to produce a stiff dough. Then, the mixtures were pelletized using a meat grinder (Metalúrgica 9000, PC-22, São Paulo, SP, Brazil). The feeds were dried in an oven (60°C) for 24 h, and then the pellets were broken until they reached the desired diameters (0.500 to 1.25 mm). The diets were stored in plastic containers in a freezer (-20°C) until later use.

The juveniles were fed three times a day, at 8 a.m., 1 p.m. and 6 p.m., according to Peixoto et al.'s methodology (2018) in order to provide better utilization of the diets offered. The amount of feed offered followed that proposed by Jory et al. (2001) and was adjusted weekly after biometry. Pellet sizes (0.50 to 1.25 mm) were adjusted according to animal growth. The experimental period was adjusted to obtain a minimum growth of 300% for all treatments, with the duration of 35 days.

Bromatological analysis

The analysis of the centesimal composition of the diets, shrimps and biofloc were performed at the Laboratory of Nutrition of

Table 1. Formulations of the experimental diets and centesimal composition of the diets.

Ingredients	Diets (g·kg^{-1})				
	320 CP	360 CP	400 CP	440 CP	480 CP
Fishmeal[a]	170.0	235.0	300.0	365.0	430.0
Soy flour[b]	330.0	330.0	330.0	330.0	330.0
Fish oil[c]	65.0	60.0	55.0	50.0	45.0
Yeast	50.0	50.0	50.0	50.0	50.0
Gelatin	50.0	50.0	50.0	50.0	50.0
Cornstarch[d]	262.1	203.1	144.0	85.0	25.0
Cellulose[e]	30.0	30.0	30.0	30.0	30.0
Calcium carbonate	20.0	20.0	20.0	20.0	20.0
Pré-mix[f]	20.0	20.0	20.0	20.0	20.0
Methionine[g]	2.9	1.9	1.0	-	-
Centesimal composition (g·kg^{-1})					
Crude protein	316.5	364.3	401.1	423.4	468.4
Ether extract	93.0	82.3	92.7	89.5	93.9
Ash	85.5	98.9	113.2	128.9	142.7
Nitrogen free extract[h]	505.0	454.5	393.0	358.2	295.0
Dry matter (%)	95.2	97.1	97.0	96.3	97.7
Gross energy (KJ·g^{-1})[i]	19.8	19.7	19.9	19.7	19.8
Crude protein:gross energy (mg KJ^{-1})[j]	1.60	1.85	2.02	2.15	2.36

CP: crude protein; [a]Leal Santos, Rio Grande, RS, Brazil; [b]Sulino, RS, Brazil; [c]Campestre, São Paulo; [d]Maizena, Brazil; [e]Synth ®, Brazil; [f]Mineral and vitamin mix – Tectron, São Paulo, Brasil (vitamin A 1,000,000 UI·kg^{-1}, vitamin D3 500,000 UI·kg^{-1}, vitamin E 20,000 UI·kg^{-1}, vitamin K3 500 mg·kg^{-1}, vitamin B1 1,900 mg·kg^{-1}, vitamin B2 2,000 mg·kg^{-1}, vitamin B6 2,400 mg·kg^{-1}, vitamin B12 3,500 mg·kg^{-1}, vitamin C 25 g·kg^{-1}, niacin 5,000 mg·kg^{-1}, pantothenic acid 4,000 mg·kg^{-1}, folic acid 200 mg·kg^{-1}, biotin 40 mg·kg^{-1}, manganese 7,500 mg·kg^{-1}, zinc 25 g·kg^{-1}, iron 12.50 g·kg^{-1}, copper 2,000 mg·kg^{-1}, iodine 200 mg·kg^{-1}, selenium 70 mg·kg^{-1}, and BHT 300 mg·kg^{-1}); [g]Evonik; [h]nitrogen free extract = 1,000 - (crude protein + ethereal extract + ash + crude fiber); [i]gross energy = (crude protein × 23.6) + (ethereal extract × 39.5) + (NFE × 17.2); [j]crude protein:gross energy (mg CP KJ^{-1} gross energy) = mg PB KJ^{-1} gross energy.

Aquatic Organisms (FURG, Brazil). For each experimental diet, samples were taken for determination of moisture by oven drying (105°C), crude protein by the Kjeldahl method, lipids by the Bligh-Dyer methodology, ash by combustion in a muffle furnace at 550°C for five hours, and crude fiber, all methods described in the Association of Official Analytical Chemists (2007) compendium, with all results expressed on a dry matter basis. Shrimp samples were collected at the beginning and at the end of the experimental period, as well as biofloc samples (at the end of the experiment) using mesh (150 µm) for further analysis, as described before. Composition data of shrimps and bioflocs were calculated based on natural and dry matter, respectively.

Water quality

During the experiment, temperature, dissolved oxygen, and pH data were measured daily twice a day (7 a.m. and 4:30 p.m.) using an oximeter (EcoSense, DO200A, YSI) and a pH meter (FiveEasy, Metler Toledo). Nitrogen compounds, total ammonia nitrogen (TAN) and nitrite nitrogen (NO_2^--N) were measured according to United Nations Educational, Scientific and Cultural Organization (1983) and Aminot and Chaussepied's (1983) methodology, respectively. Nitrate nitrogen (NO_3^--N) and phosphate (PO_4^{-3}) were analyzed weekly according to Aminot and Chaussepied's methodology (1983); salinity with a multiparameter (Hanna, HI98194) and total suspended solids (TSS) (APHA, 2005). Alkalinity was measured twice a week (APHA, 2005), and corrections with hydrated lime were performed to maintain alkalinity concentrations at 150 mg·L^{-1} $CaCO_3$ (Furtado et al., 2015). In addition, 1 mg·L^{-1} of commercial probiotic (Pro-W, Sanolife, INVE) was added once a week to help maintain water quality. In addition, partial water renewals were performed when SST concentrations were higher than 500 mg·L^{-1}. C:N (6:1) was adjusted with molasses according to Samocha et al. (2017) protocol if necessary during the experimental period.

Zootechnical performance

Biometrics were performed once a week. Fifteen animals were randomly sampled in each experimental unit for weighing and adjustment of the amount of feed offered. At the end of the experimental period, the performance data of the shrimps were evaluated by the parameters final weight (g), weight gain (GP; %), final biomass (g), protein efficiency (PER), specific growth rate (%·day^{-1}), survival (Sr; %), Productivity (Prod; kg·m^{-3}), feed conversion ratio (FCR), and protein retention rate (PRR) (Eqs. 1–7).

$$WG = 100\ (Wf\text{-}Wi)/Wi \quad (1)$$

$$PER = (Bf\text{-}Bi)/Protein\ intake \quad (2)$$

$$FCR = Amount\ of\ feed/(Bf–Bi) \quad (3)$$

$$SGR=100\ [(\ln Wf–\ln Wi)/Time\ culture] \quad (4)$$

$$PRR=100\ [((CPf*Wf)–(CPi*Wi))/PI] \quad (5)$$

$$Sr=100\ (Final\ population/Initial\ population) \quad (6)$$

$$Y=Final\ biomass/Volume \quad (7)$$

Where: Wf: final weight (g); Wi: initial weight (g); FCR: amount of feed (g); Bf: final biomass (g); Bi: initial biomass (g), CPf: final body protein, CPi: initial body protein, PI: protein intake in dry matter;

In addition, broken line and second order regression analyses were applied to the final weights of the animals using Statistica 10, and the intercept point (between these two models) was calculated using Baker et al. (2002).

Feeding costs

During the present experiment, the total amount of feed provided was recorded. Financial analysis was performed based on the following parameters: average feeding cost (FC; USD $), feed cost ratio (RCA; %), economic feed conversion factor (eFCR; USD $/kg), rate of increase of economic FCR (IeFCR; %) (Eqs. 8–11).

$$FC=Feed\ Price.Amount\ feed \quad (8)$$

$$RCA=100\ [(Fc–Fc\ control)/Fc\ control] \quad (9)$$

$$eFCR=FC/Bf \quad (10)$$

$$IeFCR=100\ [(eFCR–eFCR\ control)/eFCR\ control] \quad (11)$$

All costs were calculated for the production of 1,000 animals, and for the purpose of comparing feed costs the 320 g·kg^{-1} diet was considered the control diet. The exchange rate used was that employed by the Central Bank of Brazil on October 29, 2021, when 1.00 USD $ = 5.64 R$.

Biochemical analyses

Biochemical analyses were performed at the Laboratory of Functional Biochemistry of Aquatic Organisms. At the end of the experimental period, three animals per tank were collected (n = 12 per treatment), and stored in ultra-freezer (-80°C) for further analysis. Shrimp tissues (muscle and hepatopancreas) were homogenized at a 1:5 weight:volume ratio, with buffer containing Tris-Base (20 mM, pH 7.6), EDTA (1 mM) and sucrose (5 mM) (Gallagher et al., 1992). The homogenate was centrifuged at 20,000 g (14,010 rpm) for 20 min at 4°C, and the supernatants were collected and used for the subsequent analyses.

The total protein content in the samples was analyzed by the biuret method (550 nm), in triplicates, using a commercial kit (Bioclin, Brazil). Subsequently, the samples were diluted to 2-mg protein·mL^{-1}, with the homogenization buffer previously used, then the antioxidant capacity against peroxide radicals (ACAP) was measured according

to Amado et al.'s methodology (2009) by determining the ROS of the samples. Lipid oxidation was measured using thiobarbituric acid reaction (TBARS) according to the methodology proposed by Oakes and Van Der Kraak (2003).

Statistical analysis

All data were assessed for compliance with the assumption of normality and homoscedasticity of samples. In the case of validation of parametric assumptions, one-way analysis of variance (one-way ANOVA) was performed, using as decision criteria the significance level of $\alpha = 0.05$, and, in case of significant differences, Tukey's post-hoc test was applied ($p < 0.05$). For percentage data, such as survival and specific growth rate, arcsin transformations were performed according to the methodology proposed by Zar (2010). In case the assumptions of ANOVA were not met, the Kruskall-Wallis non-parametric test was used with the same level of significance described above. Significant differences between treatments were determined with the aid of Duncan's post-hoc test. All analyses were performed in the free software R-Studio version 4.2.0, using the "nparcomp" packages.

RESULTS

Water quality

The results of the effect of different protein levels in the diets of *L. vannamei* juveniles reared in superintensive biofloc systems on water quality variables are presented in Table 2. Significant differences ($p < 0.05$) were found in the alkalinity concentrations of the system, in which it was possible to observe a decrease in alkalinity levels with increasing protein level in the diets.

Zootechnical performance

The zootechnical performance results are presented in Table 3. Significant differences between treatments were found for average weight, weekly weight gain, biomass, productivity, FCR, and specific growth rate, with the lowest results found in the 320 CP treatment ($p < 0.05$). Survival and protein efficiency results were not affected by the different treatments ($p > 0.05$). Furthermore, the result of the broken-line analysis indicated a requirement in protein levels of 382.35 $g \cdot kg^{-1}$ ($R^2 = 0.775$). Thus, the quadratic regression indicated that protein levels in diets during intensive rearing of *L. vannamei* juveniles should be 463.55 $g \cdot kg^{-1}$ ($R^2 = 0.783$) (Fig. 1).

Nutritional composition of bioflocs and shrimps

The bromatological data of the animals and bioflocs are present in Table 4. For *L. vannamei* juveniles, significant differences ($p < 0.05$) were recorded between protein and lipid results in the shrimps. The lowest protein contents were found in the 320 and 360 CP treatment, while the lowest lipid level was also recorded in the 320 CP treatment ($p < 0.05$). In addition, significant differences in biofloc composition (protein, ash, and dry matter levels) were observed among treatments. The lowest protein value (23.69%) of the biofloc was recorded in the 440 CP treatment ($p < 0.05$). The increase in the level of protein in the diets also resulted in an increase in the ash contents in the biofloc, evidenced in the 440 and 480 CP treatments.

Biochemical analysis

Data from ACAP and TBARS analyses, for muscle and hepatopancreas are expressed in Fig. 2. For muscle, the highest total

Table 2. Water quality variables (mean ± standard deviation) during growth out phase of *Litopenaeus vannamei* in superintensive biofloc system fed with different protein levels in diets*.

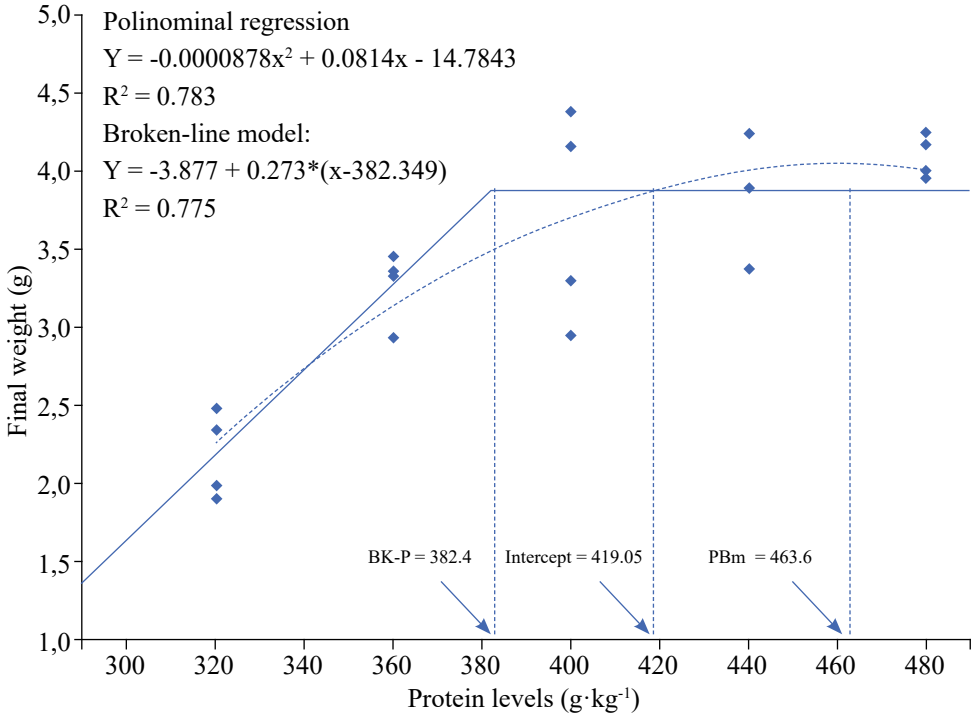
Variables	Treatments				
	320 CP	360 CP	400 CP	440 CP	480 CP
Temperature (°C)	27.25 ± 0.58	28.25 ± 0.64	28.37 ± 0.54	28.19 ± 0.53	28.10 ± 0.58
Dissolved oxygen (mg/L)	5.61 ± 0.57	5.56 ± 0.61	5.53 ± 0.63	5.56 ± 0.54	5.45 ± 0.63
pH	8.03 ± 0.24	8.06 ± 0.23	8.07 ± 0.23	8.05 ± 0.23	8.03 ± 0.24
Total ammonia nitrogen ($mg \cdot L^{-1}$)	0.14 ± 0.09	0.13 ± 0.06	0.14 ± 0.08	0.13 ± 0.06	0.14 ± 0.08
NO_2-N ($mg \cdot L^{-1}$)	0.47 ± 0.60	0.56 ± 0.75	0.57 ± 0.77	0.58 ± 0.82	0.70 ± 1.08
NO_3-N ($mg \cdot L^{-1}$)	45.55 ± 24.99	49.31 ± 28.16	51.96 ± 30.54	56.24 ± 30.78	58.15 ± 34.61
Phosphate ($mg \cdot L^{-1}$)	3.00 ± 1.38	2.70 ± 1.24	2.69 ± 1.20	2.62 ± 1.00	2.96 ± 1.54
Alkalinity ($mg \cdot L^{-1}$)	155.67 ± 20.94^{a}	151.50 ± 22.38^{ab}	150.67 ± 23.13^{ab}	143.50 ± 23.64^{bc}	141.00 ± 21.92^{c}
Salinity ($g \cdot L^{-1}$)	30.90 ± 0.89	31.00 ± 0.70	30.73 ± 1.05	30.84 ± 1.04	31.07 ± 1.14
Total suspend solids ($mg \cdot L^{-1}$)	260.58 ± 149.3	264.75 ± 133.53	269.12 ± 151.89	289.00 ± 137.72	302.00 ± 207.13

*Different letters on the same line indicate significant differences ($p < 0.05$) between treatments.

Table 3. Zootechnical performance (mean ± standard deviation) during fattening of *Litopenaeus vannamei* cultured in superintensive biofloc system fed with different protein levels in diets*.

Variables	Treatments				
	320 CP	360 CP	400 CP	440 CP	480 CP
Initial weight (g)	0.59 ± 0.003	0.59 ± 0.003	0.59 ± 0.003	0.59 ± 0.003	0.59 ± 0.003
Final weight (g)	2.27 ± 0.25^{c}	3.27 ± 0.23^{b}	3.69 ± 0.68^{ab}	3.85 ± 0.35^{ab}	4.09 ± 0.14^{a}
Weight gain (%)	284.75 ± 31.63^{c}	453.87 ± 28.32^{b}	526.09 ± 97.61^{ab}	552.24 ± 38.83^{a}	593.05 ± 19.15^{a}
Final biomass (g)	53.34 ± 12.12^{b}	73.92 ± 9.02^{a}	83.77 ± 3.63^{a}	91.96 ± 4.03^{a}	91.53 ± 17.45^{a}
Survival (%)	77.78 ± 11.70	75.83 ± 11.01	77.50 ± 14.50	80.00 ± 6.08	75.00 ± 16.44
Protein efficiency rate	1.00 ± 0.31	1.34 ± 0.18	1.37 ± 0.07	1.43 ± 0.08	1.25 ± 0.25
Productivity (kg·m^{-3})	1.07 ± 0.24^{b}	1.48 ± 0.18^{ab}	1.68 ± 0.07^{a}	1.84 ± 0.08^{a}	1.83 ± 0.35^{a}
Specific growth rate (% dia^{-1})	3.99 ± 0.33^{b}	5.03 ± 0.21^{a}	5.35 ± 0.54^{a}	5.50 ± 0.27^{a}	5.68 ± 0.09^{a}
Feed conversion ratio	3.39 ± 1.22^{b}	2.08 ± 0.30^{a}	1.82 ± 0.10^{a}	1.66 ± 0.09^{a}	1.75 ± 0.37^{a}
Protein retention rate (%)	12.74 ± 5.25	21.20 ± 3.14	20.06 ± 0.50	20.54 ± 1.32	19.08 ± 3.73

*Different letters on the same line indicate significant differences ($p < 0.05$) between treatments.



BK-P: breakpoint of the Broken-line model; PBm: point of maximum average weight by the regression model; Intercept: point of maximum efficiency suggested by Baker et al. (2002).

Figure 1. Influence of different protein levels on the average weight of *Litopenaeus vannamei* juveniles cultured in intensive biofloc systems.

antioxidant activity (lowest relative area) was observed in the 400 CP treatment ($p < 0.05$), while the lowest lipid peroxidation indices (TBARS) were observed in the 360 and 480 CP treatments. For the hepatopancreas of the animals, a reduction in ACAP was observed in the 320 CP treatment ($p < 0.05$), and the lowest concentrations of TBARS were found in the 360 and 440 CP treatments.

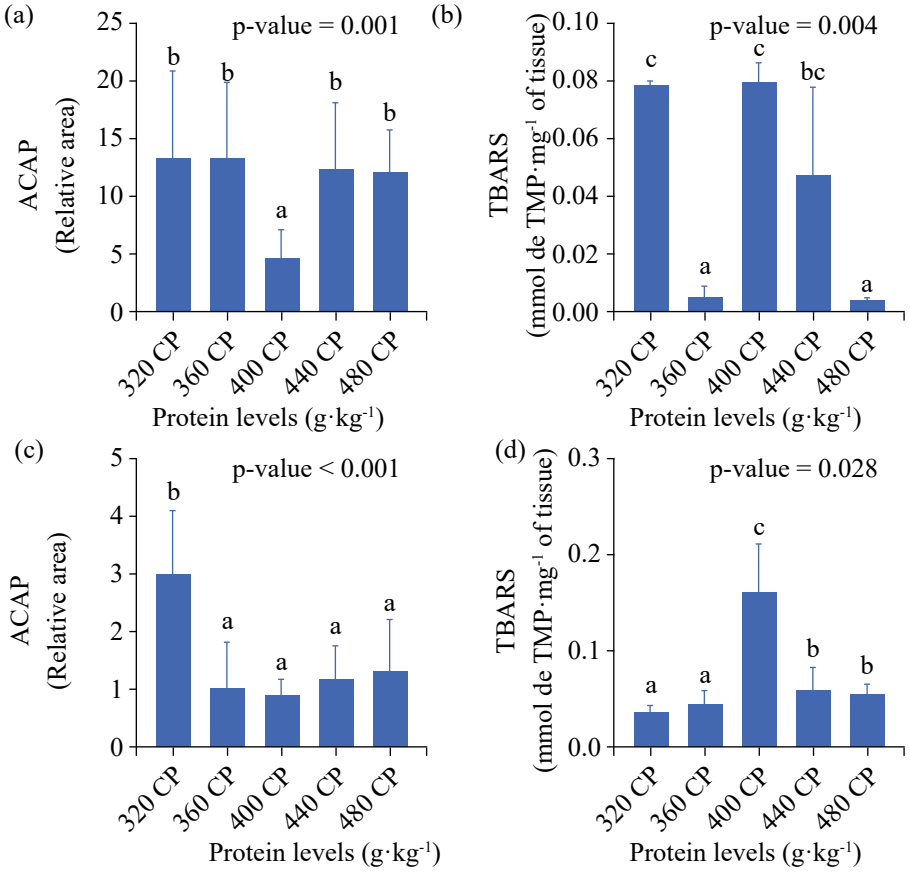
Feeding costs

The evaluation of feed costs is presented in Table 5. The increase in protein content in the diets resulted in an increase of up to 10% in total feed cost. Feed costs were proportional to the increase in the price of diets, with the highest costs observed in the 440 and 480 CP treatments ($p < 0.05$), totaling an increase of up to 30%

Table 4. Effect of different protein levels in *Litopenaeus vannamei* feed during superintensive cultures in biofloc system on the centesimal composition (mean ± standard deviation) of biofloc and shrimps*.

Variables	Initial population	Treatment				
		320 CP	360 CP	400 CP	440 CP	480 CP
Shrimp						
Moisture (%)	78.46	82.66 ± 1.50	81.02 ± 0.80	80.51 ± 0.82	80.41 ± 1.18	80.60 ± 0.54
Crude protein (%)	9.49	11.68 ± 1.34^{b}	13.45 ± 0.77^{b}	14.00 ± 0.32^{a}	13.73 ± 0.40^{ab}	14.12 ± 0.79^{a}
Lipid (%)	1.16	0.98 ± 0.41^{b}	1.38 ± 0.17^{ab}	1.30 ± 0.17^{a}	1.42 ± 0.14^{a}	1.09 ± 0.26^{ab}
Ash (%)	2.99	3.25 ± 0.32	3.17 ± 0.28	3.13 ± 0.26	3.20 ± 0.28	3.01 ± 0.28
Biofloc						
Crude protein (%)	-	27.95 ± 2.34^{a}	26.59 ± 1.58^{a}	25.76 ± 2.91^{ab}	23.69 ± 1.44^{b}	25.19 ± 1.26^{ab}
Lipid (%)	-	3.17 ± 0.83	2.82 ± 0.61	2.41 ± 0.26	2.75 ± 0.60	2.54 ± 0.41
Ash (%)	-	47.49 ± 4.97^{a}	49.28 ± 3.27^{ab}	48.62 ± 2.12^{ab}	53.22 ± 2.58^{b}	53.46 ± 2.39^{b}
Dry matter (%)	-	91.30 ± 0.99^{a}	92.42 ± 0.30^{ab}	94.56 ± 2.21^{b}	92.37 ± 0.86^{ab}	92.50 ± 0.86^{ab}

*Different letters on the same line indicate significant differences ($p < 0.05$) between treatments.



ACAP: antioxidant capacity against peroxyl radicals; TBAR: thiobarbituric acid reactive substances.

Figure 2. Values of total ACAP (expressed in relative area) in (a) muscle and (c) hepatopancreas and concentration of TBARs in (b) muscle and (d) hepatopancreas of *Litopenaeus vannamei* juveniles reared in intensive biofloc systems fed with different protein levels.

Table 5. Economic variables (mean ± standard deviation) during superintensive biofloc culture of *Litopenaeus vannamei* fed with different protein levels in the diets*.

Variables	Treatments				
	320 CP	360 CP	400 CP	440 CP	480 CP
Feed prices (US$)	1.92	1.97	2.02	2.07	2.12
Increase in feed prices (%)	-	2.65	5.29	7.94	10.56
Average feed cost (US$)	0.21 ± 0.01^{a}	0.23 ± 0.01^{a}	0.25 ± 0.01^{ab}	0.25 ± 0.00^{b}	0.27 ± 0.01^{b}
Feed cost ratio (%)	-	10.86 ± 3.49^{c}	$17,76 \pm 2.76^{bc}$	21.34 ± 1.12^{ab}	30.67 ± 6.12^{a}
Economic feed conversion rate (US$·kg^{-1})	4.37 ± 0.82^{b}	2.83 ± 0.46^{a}	2.90 ± 0.13^{a}	2.83 ± 0.19^{a}	2.75 ± 0.56^{a}
Ratio of economic feed conversion factors (%)	-	-34.85 ± 10.54	-33.38 ± 3.09	-35.00 ± 4.35	-36.70 ± 13.04

*Different letters on the same line indicate significant differences ($p < 0.05$) between treatments.

when compared to the 320 BW treatment. However, the highest eFCR was observed in the 320 CP treatment ($p < 0.05$).

DISCUSSION

Studies to determine and optimize the protein content of aquatic feeds in biofloc systems are widespread, especially for fish (Green et al., 2019; Gullian Klanian et al., 2020) and shrimp (Panigrahi et al., 2020; Pinho and Emerenciano, 2021; Chu and Brown, 2022). The protein requirement for *L. vannamei* varies widely from 303 to 480 g·kg^{-1}, depending on the life stage and environmental conditions of the culture, mainly temperature and salinity (Henriques et al., 2021; Strebel et al., 2023). However, Henriques et al. (2021) found that, in the initial rearing of *L. vannamei* in a BFT system (450 Pl·m^{-3}), the crude protein content of the feed can be reduced to 362.9 g·kg^{-1}. The same authors indicate that, in order to maximize growth and feed efficiency of the culture, the crude protein of the diets should remain between 442.6 and 471.2 g·kg^{-1}.

In the present study, the final weight and weekly weight gain are indicative of the possible sparing effect of proteins (between 400 and 480 g·kg^{-1} protein) performed by bioflocs, however more severe reductions in the protein levels of the diets (mainly to 320 g·kg^{-1} protein) limited the growth of *L. vannamei* juveniles. Corroborating that, Mansour et al. (2022a, 2022b) and Yun et al. (2016) observed reductions in growth of juveniles, of 0.23 and 1.30 g, respectively, when the protein levels of the diets were lower than 350 g·kg^{-1}. In addition, the broken-line (non-linear model) and quadratic regression results for final weight showed that the protein levels of the diets should be between 382.35 and 463.55 g·kg^{-1} protein. The optimal level (419.05 g·kg^{-1}) was established at the first intercept of the polynomial regression to the plateau of the broken-line model, according to Baker et al.'s methodology (2002), corroborating Henriques et al. (2021), who found the same rearing phase. In addition, survival in the present study was around 80%, with not significant difference among treatments. Usually the survival rates to this phase are above 85% (Henriques et al., 2021; Silveira et al. 2022a), however, in some cases, when the density are higher than 300 shrimps·m^{-3}, the survival rates can be affected (Irani et al., 2023).

In the presence of bioflocs, an improvement in FCR is a recurrent topic, due to the high availability of natural food in the water (Lara et al., 2017; Silveira et al., 2022b). However, in the present study, the better results in terms of FCR were found among 400 to 480 CP treatments (around 1.7). These values were similar to other studies of white shrimp superintensive culture (Silveira et al., 2020). In addition, the highest FCR values were observed in the treatments fed with the lowest protein contents (320 and 360 g·kg^{-1}), which may be an attempt of the animals to compensate for the low protein content in the diets by increasing the consumption of the feed provided. On the other hand, the increase in FCR may be directly related to the protein:gross energy (CP:GE) ratio of the diets. Yun et al. (2016) reported the lower utilization of diets with CP:GE lower than 2 g protein kJ^{-1}, corroborating with the findings of the present experiment.

The variable results of biofloc participation in reducing exogenous feed supply (Kaya et al., 2019; Tong et al., 2020; Prates et al., 2023) or even protein levels may be related to the wide nutritional composition variability of biofloc (Rajkumar et al., 2016; Ekasari et al., 2019; Binalshikh-Abubkr et al., 2021). Usually the biofloc is characterized as a high protein feed content (164.8 to 536.5 g·kg^{-1}) and low lipid content (5.7 to 33.5 g·kg^{-1}). Several factors can influence the nutritional value of biofloc, such as biotic conditions (species of cultured organisms, stocking density) and abiotic conditions (organic carbon source, carbon:nitrogen ratio, light, availability, and quality of provided

food) (Hamidoghli et al., 2019; Reis et al., 2019; Khoa et al., 2020; Huang et al., 2022; Khanjani and Sharifinia, 2022), in addition to the predominance/dominance of bacteria present in the BFT system (Ferreira et al., 2020, 2021).

In the present study, the amount of crude protein present in the diets of *L. vannamei* juveniles altered the composition of biofloc, mainly at protein levels, corroborating the previous finding by Mansour et al. (2022b). However, unlike the observed by Henriques et al. (2021), the higher protein contents in bioflocs were not directly related to the protein content in the diets. This may be associated with the difference in the predominance of bacteria present in biofloc during the study. The higher alkalinity consumption observed in the 440 and 480 CP treatments are indicative of a higher intensity of the nitrification process in these treatments (Ebeling et al., 2006), therefore with a higher predominance of chemoautotrophic bacteria when compared to the treatments fed with lower protein contents (320 CP). Thus, the lower protein contents in biofloc observed in the 440 CP treatment (236.9 $g \cdot kg^{-1}$ protein) corroborate with that found by Ferreira et al. (2020), who observed a decrease in the protein content of biofloc in systems dominated by chemoautotrophic bacteria when compared to those dominated by heterotrophic bacteria.

On the other hand, the biofloc, in addition to participating in the feeding of the animals, are the main responsible for maintaining the water quality throughout the crops. In general, water quality parameters were not affected by the different protein levels in the diets and were within the recommended range for shrimp culture in biofloc system (Emerenciano et al., 2017). However, a reduction in alkalinity levels was observed in the 480 $g \cdot kg^{-1}$ CP treatment. This highlights the excess of nitrogen in these diets, since protein-rich diets favor protein catabolism (Emerenciano et al., 2022), thus releasing more nitrogen compounds into the water. Ebeling et al. (2006) established that the nitrification process consumes 7.05 g of alkalinity for conversion of 1 g of ammonia nitrogen to nitrate nitrogen. Therefore, even if there are no significant differences between treatments for nitrogen compound contents, the reduction in alkalinity may be indicative of lower protein utilization in this treatment.

As the water parameters in the different treatments were within the suitable levels for *L. vannamei*, the physiological responses of shrimp could be attributed to the crowding that influence the growth, survival and production of ROS. High stocking density can generate a chronic stress, declining the immune and antioxidant system of the animals (Lin et al., 2015; Liu et al., 2017). In addition, ROS presence is capable of oxidizing and damaging biomolecules such as proteins, lipids, DNA and RNA, with consequent deleterious effects on some biological functions (Sies, 2015; Liang et al., 2016). In general, protein is directly correlated with increased activity of enzymes that act in the antioxidant system of animals, such as SOD (Panigrahi et al., 2019, 2020), besides sources of essential amino acids that act in the activation of the antioxidant system and innate immune response of animals (Maiti et al., 2022).

In the present study, ACAP was different according to the tissues analyzed, following the patterns found in previous studies in biofloc systems (hepatopancreas > muscle) (Silva Martins et al., 2015; Colombo et al., 2023). This is due to the hepatopancreas being the main organ of shrimps, responsible for storing and producing enzymes that participate in the immune and antioxidant system of the animals. Feeding *L. vannamei* juveniles with 320 $g \cdot kg^{-1}$ protein diets decreases ACAP in the hepatopancreas, indicating a depletion of the antioxidant status of the animals, a response similar to that found by Panigrahi et al. (2019), who observed reductions in the activity of the SOD enzyme (another antioxidant mechanism) in individuals fed 320 $g \cdot kg^{-1}$ protein diets when compared to 400 $g \cdot kg^{-1}$ diets. One possible explanation is that essential amino acid sources are important in activating the antioxidant response of organisms (Maiti et al., 2022). However, the same response profile was not observed in the muscles, in which the best ACAP (lowest relative area) was observed in the 400 $g \cdot kg^{-1}$ treatment. These variations may be related to the consumption of bioflocs, which can act as a natural source of bioactive compounds that increase the antioxidant status of animals, especially in the hepatopancreas (Silva Martins et al., 2015; Colombo et al., 2023). Colombo et al. (2023) observed higher concentrations of flavonoids and polyphenols in this organ when compared to muscle. These compounds act as ROS interceptors, thus preventing lipid damage in animals (Zamora and Hidalgo, 2016).

Stressors such as high stocking densities can cause lipid damage in animals (Xie et al., 2018). During lipid peroxidation processes, one of the bio-products formed is peroxyl radicals, which can be intercepted by ACAP, thus reducing the damage with lipid peroxidation (Okpala et al., 2016). In the present study, greater lipid damage is observed in the hepatopancreas compared to the muscle of the animal; this is mainly due to this organ having a greater presence of lipids, which makes it more susceptible to these reactions. Despite the reduction of ACAP in the treatment fed with 320 $g \cdot kg^{-1}$ of protein, TBARS would not differ from the other treatments. This may be also related to the lower availability of lipids present in the animals, since the amount of TBARS is directly correlated with the amount of lipids present in the tissues (Ouraji et al., 2011).

The lower PRR associated with the lower protein level in shrimp fed, 320 g·kg^{-1} protein diet, is an indicative of the nutritional imbalance of this diet, and consequently in the reduction of the zootechnical performance variables presented in this treatment. In addition, previous studies report that the whole-body composition can be altered by several factors, such as the quantity and quality of proteins provided in the diets, culture phase and feeding frequency (Anand et al., 2021; Lee and Lee, 2018). The present result corroborates Lee and Lee (2018), who reported that severe reductions in the protein levels of diets reduce the protein content in the proximal composition of the animals. In addition, in the present study, *L. vannamei* juveniles fed with levels higher than 400 g·kg^{-1} crude protein did not result in an increase in protein deposition in the tissues of the animals, similar to what was found by Anand et al. (2021) for *Penaeus indicus*. This may indicate that this crude protein content exceeds the nutritional requirements of *L. vannamei*. In this scenario, it is possible that amino acids are being catabolized in other metabolic functions not related to the growth of the animals (Emerenciano et al., 2022).

It is widely recognized that feed costs are the major effective cost in aquatic organism production and can represent up to 70% of the total cost operational (Rego et al., 2017; Almeida et al., 2021). Nunes et al. (2022) state that 50–65% of total dietary costs are associated with the use of protein ingredients and essential amino acids. This is mainly due to the use of ingredients such as fishmeal, which has a high market value and limited availability (Nunes et al., 2022). In the present study, we observed this effect with an increase of up to 10.56% in the cost of highest protein diet (480 g·kg^{-1}) compared to lowest protein diet (320 g·kg^{-1}). One attempt to reduce the cost of producing formulated diets is to use diets with the most precise nutrient levels for each growing condition (Xu et al., 2018; Nunes et al., 2022). There was no difference between the final weight of animals fed diets between 400 and 480 g·kg^{-1} protein. In addition, diets with lower protein contents are more advantageous in relation to cost-benefit and environmental friendliness, by reducing the use of fishmeal, the main protein source for aquatic organisms (Ashour et al., 2021).

Focusing only on the feeding cost may not reflect the most cost-effective scenario in shrimp production, mainly because these variables do not take into account the survival and biomass produced by the animals. In the present study, the highest eFCR values were observed in the 320 g·kg^{-1} protein treatment, due to the lower conversion of this diet into shrimp biomass, and consequently a lower cost-benefit ratio of using this diet, despite the lower feed cost observed in this treatment. In addition, further studies should be carried out to obtain shrimp of commercial weight.

CONCLUSION

The amount of protein provided in the diets of *L. vannamei* juveniles affects the growth of the animals, the broken-line results indicate that the animals should be fed diets containing at least 382.35 g·kg^{-1} crude protein, while the average weight regression model indicates that feeding the shrimp with 463.55 g·kg^{-1} crude protein optimizes the growth of the juveniles under superintensive conditions. Severe reductions in dietary protein levels negatively affect the antioxidant system of the animals, as well as reduce zootechnical performance and increase the feed cost/biomass produced ratio of the farms.

CONFLICT OF INTERESTS

Nothing to declare.

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AUTHORS' CONTRIBUTIONS

Conceptualization: Braga IFM, Rosas VT; **Methodology:** Rosas VT; **Investigation:** Braga IFM, Araujo ACS; **Data curation:** Braga IFM, Araujo ACS, Rosas VT; **Formal analysis:** Braga IFM, Araujo ACS; **Resources:** Monserrat JM, Tesser MB, Wasielesky Junior W, Fóes GK; **Funding acquisition:** Wasielesky Junior W, Fóes GK; **Supervision:** Monserrat JM, Tesser MB, Wasielesky Junior W, Fóes GK; **Writing – original draft:** Braga IFM; **Writing – review & edition:** Braga IFM, Rosas VT, Monserrat JM, Tesser MB, Wasielesky Junior W, Fóes GK; **Final approval:** Fóes GK.

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