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ARTICLE

Dietary enrichment with crude protein content and feed additives (*Bacillus* spp. and yeast strains) improves growth performance, survival and circulating hemocytes in juvenile white shrimp, *Litopenaeus vannamei*

Enriquecimiento de la dieta con proteína y aditivos alimentarios (cepas de *Bacillus* spp. y levaduras) mejora el crecimiento, supervivencia y hemocitos circulantes de juveniles de camarón blanco, *Litopenaeus vannamei*

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Resumen.- En este estudio se evaluó el efecto del enriquecimiento de dietas con diferentes niveles de proteína cruda (CP) y aditivos en el crecimiento, supervivencia, tasa de conversión alimenticia (FCR), consumo de alimento (FC) y conteo total de hemocitos (THC) en juveniles de camarón blanco *Litopenaeus vannamei*. El estudio consta de dos bioensayos: en el primero, los juveniles fueron alimentados diariamente por 45 días con cuatro dietas experimentales con diferente contenido de proteína: (1) Alimento comercial o control, CP 35%; (2) CP 29%; (3) CP 32%; (4) CP 35%. Después de seleccionar CP 29%, en el bioensayo II los juveniles fueron alimentados diariamente por 45 días con una dieta proteica complementada con probióticos: (1) Alimento comercial o control, CP 35%; (2) CP 29%; (3) CP 29% + mezcla de *Bacillus* a 1×10^6 UFCg^{-1} alimento; y (4) CP 29% + mezcla de levaduras a 1×10^6 UFCg^{-1} alimento. Los camarones juveniles alimentados con las dietas experimentales mostraron ganancia en peso significativamente mayor e incremento en supervivencia, FCR, FC y THC comparado con la dieta control. Sin embargo, no hubo diferencias significativas entre las dietas experimentales. En el segundo bioensayo, los juveniles alimentados con la dieta experimental + aditivos alimentarios incrementaron significativamente supervivencia, FCR, FC y THC comparado con la dieta control. El crecimiento de juveniles se incrementó significativamente con las dietas CP29% y CP29%+mezcla de levaduras, comparado con el crecimiento de la dieta control. La dieta complementada con la mezcla de levaduras mostró el valor más alto de supervivencia y THC de juveniles comparado con los demás tratamientos experimentales. Los diferentes niveles experimentales de CP incluidos en la dieta de camarón incrementaron crecimiento, supervivencia y hemocitos circulantes; la adición de una mezcla de levaduras como aditivo alimentario, indujo mejor supervivencia y respuesta inmune en juveniles de camarón.

Palabras clave: *Litopenaeus vannamei*, acuicultura, levadura, nivel de proteína, hemocitos

Abstract.- In this study the enrichment dietary effect with different crude protein levels (CP) and feed additives on growth, survival, feed conversion ratio (FCR), feed consumption (FC) and total hemocyte count (THC) in juvenile white shrimp *Litopenaeus vannamei* were evaluated. The study covered two bioassays: in the first one, juveniles were daily fed for 45 days with four experimental diets containing: (1) Control, commercial feed (35% CP); (2) 29% CP; (3) 32% CP; (4) 35% CP. After the 29% CP diet was selected, juveniles in bioassay II were daily fed for 45 days with a single CP diet complemented with probiotics: (1) Control, commercial feed (35% CP); (2) 29% CP; (3) 29% CP + *Bacillus* mix at 1×10^6 CFUg^{-1} feed; and (4) 29% CP + yeast mix at 1×10^6 CFUg^{-1} feed. Juvenile shrimp fed with experimental diets gained significantly more weight and increased survival, FCR, FC and THC compared with control diet. However, differences among experimental diets were not significant. In bioassay II, juvenile shrimp fed with experimental diet + feed additives significantly increased survival, FCR, FC and THC compared with control diet. Growth of juveniles significantly increased with 29% CP and 29% CP + yeast mix diets, compared with control group. Complementing the diet with yeast mix showed higher survival and THC of juveniles compared with other experimental treatments. Different CP levels in shrimp diet improved growth, survival and circulating hemocytes, and addition of mixed yeast as feed additive induced better survival and immune response in juvenile shrimp.

Key words: *Litopenaeus vannamei*, aquaculture, yeast, protein level, hemocytes

Introduction

Aquaculture industry is supported by cultivation of target species that survive well and grow fast in captivity (Martínez-Córdova *et al*. 2009, Ayiku *et al*. 2020). Worldwide food production by aquaculture has had an average annual increase of 6.7% in recent years (FAO 2020). Consequently, aquaculture is considered one of the most lucrative and fastest growing livestock industries (Stentiford *et al*. 2012). Among many species of commercial interest, the Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) is particularly relevant based on the analysis of nine production years (2007-2016) that positioned Thailand (588,370 t), Indonesia (489,555 t), India (461,302 t), Ecuador (422,000 t) and Vietnam (380,000 t*)* as the most prominent productive countries worldwide (FAO 2020). In Mexico, *L. vannamei* is the second most produced species among aquaculture crops, with average yields of 133,338 t that exceeds 4.5 times the volumes of natural catches from estuaries and coastal lagoons, and in 3.2 times the catches from the open sea (CONAPESCA 2017).

Currently, shrimp aquaculture faces a favorable growing scenario, yet some problems such as the availability of raw materials for food processing and the increase of pathogenic diseases undermine the efficiency of productive systems. Moreover, the costs associated with diet preparation can represent between 50 to 80% of total operating costs, which directly affects profitability (Cummins *et al*. 2017). Thus, formulation of well-balanced, low-cost diets, together with definition of adequate feeding strategies are key for ensuring the supply of nutrients (energy) for overall development and growth of the species, maximizing performance and production (Hernández *et al*. 2011, Shahkar *et al*. 2014). On this basis, the inclusion of an adequate protein level in the diet is essential to ensure that body tissues use them optimally, particularly to favor growth rather than baseline maintenance (Güroy *et al*. 2012, Tacon & Metian 2015).

Among penaeid shrimp, studies relative to the definition of optimal diets have mainly focused on recommended protein content between 30 and 50% of total body mass (Tacon *et al*. 2002). In *L. vannamei*, the understanding of protein metabolism in relation to amino acid composition is essential to develop a balanced diet (Portella *et al*. 2013, Méndez-Martínez *et al*. 2017). When protein content in the diet is insufficient, a lack or unbalance of essential amino acids can lead to decreased body resistance capacity and insufficient body protein formation, which in consequence affects growth, food conversion ratio, stress resistance, immune response and survival (Jin *et al*. 2013, Méndez-Martínez *et al*. 2018). Excess of dietary protein content can also be harmful, since crustaceans only use a small part of this molecule to generate new tissue, being the rest used to produce waste energy and greater amounts of ammonia (NH_3) that finally leads to decreased water quality (Zhou *et al*. 2007, Shahkar *et al*. 2014).

Under culturing conditions, research efforts of the last decades have focused on the combat and spread-out of new pathogenic diseases in shrimp, including White Spot Syndrome Virus (WSSV), Black Gill Disease (BGD), Running Mortality Syndrome (RMS), Loose Shell Syndrome (LSS), White Fecal Syndrome (WFS), White Muscle Disease (WMD) and Infectious Hypodermal and Hematopoietic Necrosis (IHHN) which cause severe economic losses to the industry in countries such as China (Ayiku *et al*. 2020), Thailand (Flegel 2012, Boonyawiwat *et al*. 2017), Taiwan (Kumar *et al*. 2020), Ecuador (Gainza & Romero 2020), Brazil (Costa *et al*. 2009) and Mexico (López-Tellez *et al*. 2020). The most recent evidence indicates that shrimp pathogens are not the main cause of these mass mortalities and should be classified as opportunistic infections. Therefore, the use of prophylactic and therapeutic (antibiotics) treatments has become necessary to maximize the health and yield of cultivated shrimp. However, antibiotics have proved to be inefficient, since they impregnate in body tissues, subtracting them quality and generating many health problems (Sapcharoen & Rengpipat 2013, Peredo *et al*. 2015). Antibiotics are also widely criticized for their impact on the environment (Cabello *et al*. 2013) and induction to resistance among many pathogenic microorganisms (van den Bogaard & Stobberingh 2000).

The current limitation of antibiotics as growth promotors to increase production volumes, demands other alternatives to improve crop technologies, especially during the juvenile stage where disease effects are critical (Walker & Winton 2010). On this basis, treating cultivated shrimp with microbial immunostimulants, such as probiotics, becomes relevant to improve the host's health based on the properties of certain bacteria and marine yeast strains. These properties include synthesis of antimicrobial compounds and digestive enzymes that enhance food conversion and nutrient assimilation by the host, and strengthen its immune system and capacity to tolerate stress (Yang *et al*. 2013, Chai *et al*. 2016). Some desirable features of probiotics include its origin, adhesion to intestinal mucosa, safety to the host, antagonism to pathogens, stimulation of the host's immune system and removal of organic matter (Verschuere *et al*. 2000). Recently, there is a special attention in searching for *Bacillus* spp. (Silva *et al*. 2013, Chien *et al*. 2020), and yeasts (Tovar-Ramírez *et al*. 2010, Phupet *et al*. 2018) to culture many species of aquaculture interest. *Bacillus* spp., for example, has the ability to sporulate, grow fast and tolerate a wide range of physiological conditions, which has been of great value in modern aquaculture to improve the quality of seawater, reduce the load of harmful bacteria and maximize the host's response without antibiotics (Nemutanzhela *et al*. 2014). Additionally, the oral administration of yeast species, particularly *Saccharomyces cerevisiae* and *Debaryomyces hansenii* has demonstrated to enhance the immune response in shrimp and other aquaculture species (Tovar-Ramírez *et al*. 2010, Babu *et al*. 2013). Despite of this advancement,

current scientific knowledge relative to the use of probiotics as feed additives and the immune response in aquaculture is scarce for early-development stages (Kesarcodi-Watson *et al*. 2012, Gyan *et al*. 2020).

In the present work, the combined effects of different dietary protein contents, mixed with *Bacillus* spp. and yeasts strains used as feed additives to improve nutritional and immune rearing parameters in juvenile white shrimp (*L. vannamei*) were evaluated.

Materials and methods

Origin of juvenile shrimp

Two different trials were developed with *L. vannamei* juveniles having an initial fresh weight of 0.25 ± 0.03 g (Bioassay I) and 0.14 ± 0.03 g (Bioassay II). Juveniles obtained from the cultivation facilities of Centro de Investigaciones Biológicas del Noroeste (CIBNOR, Mexico) were acclimatized for two days in 1,500 L fiberglass tanks at 29 °C and 35 of salinity before running the trials. During acclimatization, shrimp were fed *ad libitum* twice a day (10:00 AM and 4:00 PM) with a commercial diet, selected for this study according to the recommended protein content of shrimp feed up to 35% crude protein, formulated for shrimp by PIASA™ (La Paz, B.C.S., Mexico). The feed composition was: protein (35.03%), crude lipids (9.04%), fiber (2.84%), moisture (8.84%), ash (6.09%) and nitrogen-free extract (38.16%).

Formulation and preparation of pelletized food

Three experimental diets containing 29, 32 and 35% crude protein (CP) (Table 1) were formulated according to the needs of *L. vannamei* juveniles (Tacón *et al*. 2002, Gucic-Soriano *et al*. 2013). The diets were formulated using 5 Pro Nutrion software (Guadalajara, Jalisco, Mexico), following procedures described by Méndez-Martínez *et al*. (2018). The ingredients were firstly reduced in particle size with a sprayer (Molinos Pulvex, Mexico City) and then screened through a 250 µm mesh. Each diet was prepared mixing all macro-ingredients in an industrial mixer (Kitchen Aid MR, St. Joseph, MI, USA) until being homogenized. The micro-ingredients (vitamin premix, sodium dibasic phosphate, mineral premix, vitamin C, BHT, carboxymethyl cellulose, DL-methionine, Lysine-HCl, L-threonine) were then mixed in a plastic container before being added to the macro-ingredients. Fish oil and soy lecithin were included as emulsion before their addition to the mixture. Choline chloride dissolved in distilled water at 40 °C was added until reaching 35% weight of the ingredients (Table 1). The food was passed twice through a meat mill (TorRey™ Monterrey, N.L. Mexico) to form 2-mm diameter granules or pellets, which were dried for 10 h in an air flow oven at 35 °C. Dried pellets were finally packed in plastic bags and kept at 4 °C until use.

Table 1. Composition of experimental pelletized diets for *L. vannamei* juveniles / Composición de dietas experimentales peletizadas para juveniles de *L. vannamei*

Ingredients	Crude protein levels		
	29%	32%	35%
Fishmeal[1]	24.0	28	33.3
Soybean Paste[1]	26.0	30	30
Whole Wheat Flour[2]	33.2	25.2	20
Sardine Oil[3]	4.0	4.0	4.0
Sodium Alginate[3]	2.0	2.0	2.0
Soy Lecithin[3]	2.0	2.0	2.0
Vitamin premix[3*]	1.8	1.8	1.8
Sodium dibasic phosphate[3]	1.2	1.2	1.2
Mineral Premix[3**]	0.5	0.5	0.5
Choline Chloride (62% active agent)[3]	0.2	0.2	0.2
Vitamin C (35% active agent)[3*]	0.09	0.09	0.09
Butyl-hydroxy-toluene (BHT)[4]	0.004	0.004	0.004
carboxymethyl cellulose (CMC)[4]	4.9	4.9	4.9
DL-methionine[4]	0.015	0.008	-----
Lysine-HCl[4]	0.014	-----	-----
L-threonine[4]	0.054	0.045	0.033
Total	100.0	100.0	100.0

[1]Laboratory and Wearhouse of Balanced Foods from the Zootechnical Post of Universidad Autónoma de Baja California Sur (UABCS)
[2]Commercial house (Bravo Market, La Paz, B.C.S.)
[3]Shrimp balanced food plant PIASA
[4]Laboratory and Wearhouse of Balanced Foods from the Zootechnical Post of Universidad Autónoma de Baja California Sur (UABCS)
[*]VITCRU0409: Vitamin A acetate, 15000 IU: D3, 7500 IU; thiamine monohydrate, 150 mg; riboflavin, 100 mg; pyridoxine HCl, 50 mg; pantothenic acid, 100 mg; niacin, 300 mg; biotin, 1 mg; inositol, 500 mg; folic acid, 20 mg; cyanocobalamin, 0.1 mg
[**]MINCRU0409 (g/kg food): $MgSO_4$ $7H_2O$, 0.5; $ZnSO_4$ $7H_2O$, 0.09; KCl, 0.5; $MnCl_2$ $4H_2O$, 0.0234; $CuCl_2$ $2H_2O$, 0.005; KI, 0.5; $CoCl_2$ $6H_2O$, 0.0025

Chemical analysis of dietary ingredients

Before using the ingredients and diets, triplicate samples (10 g) were finely grounded, sieved, and analyzed by the Kjeldahl nitrogen method (Foss, Hillerød, DK) to determine their final CP content. Ethereal extract content was determined using the ether extraction method (Soxtec Avanti, Höganäs, Sweden) and raw fiber content was determined according to Weende and Van Soest methods (Fibertec, Foss, Hillerød, Denmark). Ashes were determined by sample incineration in flasks at 550 °C for 6 h and nitrogen-free extract was determined according to Weende system (AOAC 2006). The final composition of diets is detailed in Table 2.

Table 2. Proximal chemical composition of experimental diets used in bioassay I for *L. vannamei* juveniles / Composición química proximal de dietas experimentales utilizadas en el bioensayo I para juveniles de *L. vannamei*

Protein content (%)	Ethereal extract (%)	Crude fiber (%)	Ash (%)
29	9.15	8.93	10.09
32	1.02	1.20	1.21
35	7.48	7.25	8.83

Preparation of probiotic mixtures

Two marine yeast strains obtained from CIBNOR collection were used: *Candida insectorum* (DHHBCS005) and *Debaryomyces hansenii* (DHHBCS006). A third yeast strain (*Debaryomyces hansenii* L1) was isolated from the pericarp of Mexican lime, *Citrus aurantifolia* (Hernández-Montiel *et al*. 2010). In addition, three *Bacillus* spp. strains were isolated from the gut of wild *L. vannamei* shrimp: *Bacillus tequilensis* (YC5-2), *Bacillus endophyticus* (YC3-B) and *Bacillus endophyticus* (C2-2) (Luis-Villaseñor *et al*. 2011).

Preserved yeast and *Bacillus* strains at –80 °C were thawed and individually reactivated on Petri dishes with Potato Dextrose Agar (PDA) at 30 °C for 24 h (for yeasts) and Trypticase Soy Agar (TSA) with 2.5% NaCl at 37 °C for 24 h (for *Bacillus* spp.). The colonies were extracted from agar and suspended in test tubes with 10 mL NaCl solution (3%). Bacterial suspension was concentrated until reaching 1×10^9 CFU mL^{-1}, which occurred at 540 nm and an absorbance of 1.0; a concentration of 3×10^7 CFU mL^{-1} occurred at 600 nm for yeasts. Once the desired concentrations were obtained, 33.3% of each microbial suspension was added to a 5 mL plastic sprinkler with saline solution, which was added to the food using large sterile Petri dishes to obtain a final concentration of 1×10^6 CFU g^{-1} feed. The procedure was carried out under aseptic conditions in a laminar flow hood for both marine *Bacillus* and yeasts strains.

Shrimp rearing conditions

The culture system consisted of twelve 60 L fiberglass aquaria (50x55x38 cm) holding ten shrimp each one (166.67 shrimp m^{-3}). The system was supplied with seawater previously passed through 70 µm sand filters (Cristal-Flo, Santa Rite Industries Inc., Delavan, WI, USA), 10 and 5 µm activated carbon cartridges, and UV light. Physical-chemical parameters of seawater were controlled with 200 W submersible heaters for temperature (28 ± 0.2 ºC), external exhausters and 5 HP blowers for oxygen content (5.44 ± 0.3 mg mL^{-1}; oximeter YSI 550A, OH, USA), and an optical refractometer for salinity (40 ± 0.26). Photoperiod was controlled for 12 h light and 12 h darkness with a 200 W neon light system. All aquaria were siphoned daily before the first feeding and 60% of total water volume was replaced.

Bioassays

For bioassay I, juvenile shrimp were daily fed for 45 days with three levels of experimental CP: (1) Control, commercial feed (the same used for acclimatization); (2) 29% CP; (3) 32% CP; (4) 35% CP. Based on these results, bioassay II was conducted for 45 days and shrimp were daily fed base experimental diet at 29% CP, complemented with microbial feed additives: (1) mix of *B. tequilensis* + *B. endophyticus* + *B. endophyticus* (Bmix at a 1:1:1 ratio and 1×10^6 CFU g^{-1} feed); (2) mix of *C. insectorum* + *D. hansenii* + *D. hansenii* (Ymix at 1:1:1 ratio and 1×10^6 CFU g^{-1} feed). Again, a control group of juvenile shrimp fed the same commercial diet was included. A completely randomized design with three replicates was used for each bioassay. Food was supplied at 10% of total juvenile biomass in three daily rations (9:00, 13:00 and 17:00 h). Food intake was determined by feeding to apparent satiation. Food remains, which could be readily identified by its swollen pellet shape, were removed the next day in the morning and quantified by concentrating on Whatman No.1 filter paper with a vacuum pump (Gast Manufacturing, Benton Harbor, MI). This was done before drying at 50 °C for 18 h in an air flux oven (Hafo Series 1600, Sheldon Manufacturing, Cornelius, OR) (Méndez-Martinez *et al*. 2018). Ration was adjusted to minimize the amount of uneaten feed. Growth gain, survival and other productive indicators were determined at the beginning of both bioassays and every 15 days until finishing them at each sampling, six randomly-collected shrimps per treatment were processed as described below.

Evaluation of productive indicators

After removing excess of water with absorbent paper, collected juvenile shrimp were individually counted to estimate survival (%) and weighed on a digital balance (± 0.01 g; PE 3600 Mettler-Toledo, Columbus, OH, USA) to estimate gain in total fresh weight. With this data, the following parameters were determined:

Percentage of survival (%):

$$\text{Survival} = \frac{\text{initial numbers of organisms}}{\text{final number of organisms}} \times 100$$

Apparent feed consumption (FC, g):

$$\text{FC} = \text{food supplied (g)} - \text{residual food (g)}$$

Feed conversion ratio (FCR, g day^{-1}):

$$\text{FCR} = \frac{\text{consumed apparent food}}{\text{corrected weight gain}}$$

Total hemocyte count (THC)

Hemolymph samples (40 µL) were extracted from pleopod base of the first abdominal segment near the genital pore of each collected shrimp, using a 3 mL syringe with 160 µL precoagulant solution precooled at 4 °C (450 mM NaCl, 10 mM KCl, 10 mM EDTA-Na_2, 10 mM HEPES, pH 7.3, 850 mOsm kg^{-1}) and 4% formalin (Fermont, Monterrey, Mexico) (Vargas-Albores *et al*. 1996). Extracted hemolymph was placed in sterile 1.5 mL Eppendorf tubes and kept in an ice bed for immediate counting of circulating hemocytes. Subsequently, the samples (100 µL) were placed in a hematocytometer (Marienfeld, Germany) for counting the total number of hemocytes under an optical microscope (Optika, Italy). Total hemocyte count was expressed as 1x10^6 hemocytes mL^{-1}.

Statistical analysis

Data homoscedasticity and homogeneity were determined with Kolmogorov-Smirnov and Bartlett tests, respectively. To identify differences in total weight gain, FC, CR, and THC between treatments, one-way ANOVA, followed by *post-hoc* Duncan test for mean comparisons when necessary, was used at 95% confidence level (Zar 1984). All tests were determined with STATISTICA software (v.12.0, StatSoft, Tulsa, OK, USA)

Results

Productive variables and THC: Bioassay I

After 45 days of culture, mean weight (Fig. 1), FC, FCR, and survival of juvenile shrimp fed on experimental diets showed significant differences ($P \leq 0.05$) compared to the control diet (Table 3). Weight gain significantly ($P \leq 0.05$) increased in shrimp fed on 32% CP diet at 30 and 45 days, compared to control diet. Similarly, shrimp fed on 29 and 35% CP diets significantly increased weight gain after 45 days of cultivation, compared to commercial diet (Fig. 1). THC of juveniles fed on experimental diets, particularly 32% CP diet, was significantly ($P < 0.05$) higher than counts of juveniles fed on control diet (Fig. 2). No significant ($P \geq 0.05$) differences in productive indicators occurred among experimental diets (29, 32, and 35% CP). Based on these results, 29% CP experimental diet was selected as optimal for the second bioassay that included probiotics as feed additives.

Productive variables and THC: Bioassay II

Final weight, FC, and FCR of juvenile shrimp fed on the base experimental diet (29% CP) complemented with probiotics after 45 days culture is shown in Table 2. Juveniles fed on 29% CP and 29% CP + Ymix showed a significant ($P \leq 0.05$) increase in all productive variables, compared to control group. In contrast, juvenile shrimp fed on 29% CP diet + Bmix did not significantly ($P \geq 0.05$) increase final weight and FC compared to control group (Table 4).

Table 3. Productive variables for *L. vannamei* juveniles fed with different levels of crude protein for 45 days / Variables productivas en juveniles de *L. vannamei* alimentados por 45 días con diferentes niveles de proteína cruda

Treatment	Apparent feed consumption (g)	Feed conversion ratio (g day^{-1})	Survival (%)
Commercial food (Control)	14.49 ± 0.13^a	1.5 ± 0.05^a	73 ± 25.2^a
29% CP	16.6 ± 0.95^b	3.6 ± 0.15^b	100^b
32% CP	17.7 ± 1.67^b	3.6 ± 0.38^b	100^b
35% CP	16.9 ± 0.49^b	3.8 ± 0.46^b	96 ± 5.7^b
Significance	0.00032	0.00004	0.00001

Different superscript letters denote significant differences between treatments according to Duncan (1951)

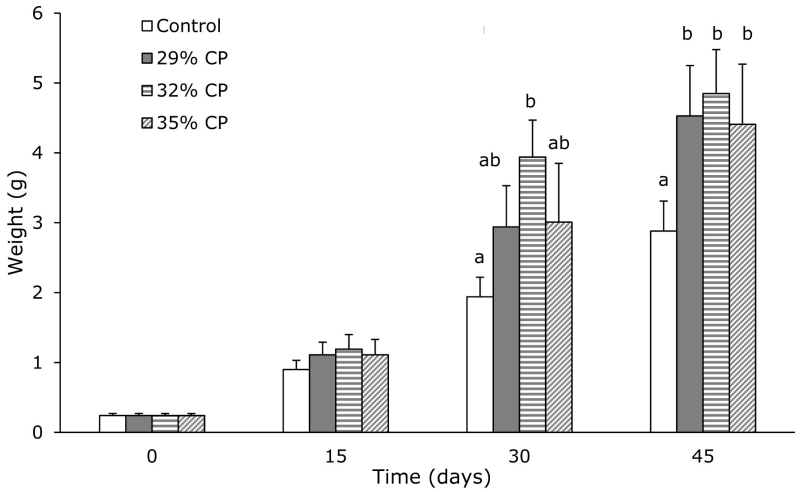

Figure 1. Growth of juvenile *Litopenaeus vannamei* fed with different levels of crude protein (CP) for 45 days. Different superscript letters indicate significant ($P \leq 0.05$) differences between treatments / Crecimiento de juveniles de *Litopenaeus vannamei* alimentados con diferentes niveles de proteína cruda (CP) por 45 días. Literales diferentes indican diferencias significativas ($P \leq 0,05$) entre tratamientos

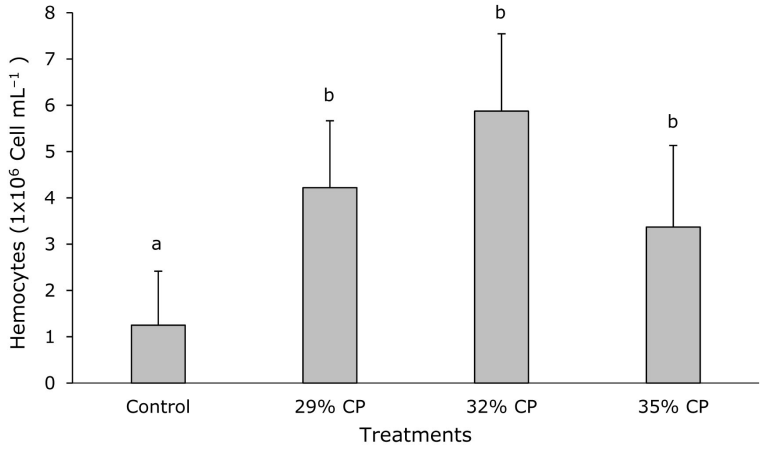

Figure 2. Total hemocyte count in juvenile *Litopenaeus vannamei* fed with different levels of crude protein for 45 days. Different superscript letters indicate significant ($P \leq 0.05$) differences between treatments / Conteo total de hemocitos en juveniles de *Litopenaeus vannamei* alimentados con diferentes niveles de proteína cruda por 45 días. Literales diferentes indican diferencias significativas ($P \leq 0,05$) entre tratamientos

Table 4. Productive variables for *L. vannamei* juveniles fed with isoproteic and probiotic diets / Variables productivas en juveniles de *L. vannamei* alimentados con dietas isoprotéicas y probióticas

Treatment	Apparent feed consumption (g)	Feed conversion ratio (g day^{-1})	Survival (%)
Commercial food (Control)	2.21 ± 0.44^{a}	0.81 ± 0.06^{a}	63 ± 11.5^{a}
29% de CP	6.98 ± 0.43^{b}	1.56 ± 0.18^{b}	80 ± 7.3^{b}
29% CP +B mix (1x10^{6} CFU g^{-1} feed)	5.87 ± 0.16^{ab}	1.52 ± 0.26^{b}	83 ± 7.7^{b}
29% CP+ Ymix (1x10^{6} CFU g^{-1} feed)	6.09 ± 0.62^{b}	1.45 ± 0.05^{b}	93 ± 5.7^{b}
Significance	0.025	0.020	0.00001

Different superscript letters denote significant differences between treatments according to Duncan (1951)

Survival of juvenile shrimp fed with probiotic treatments for 45 days shown in Figure 3. Juveniles fed on 29% CP diet complemented with additives significantly ($P \leq 0.05$) increased survival, compared with control group. In particular, complementing the base diet with a yeast mix significantly ($P \leq 0.05$) increased survival compared with experimental groups. Counts of circulating hemocytes were significantly ($P \leq 0.05$) higher in juvenile shrimp fed on experimental diets than in those only fed on a control diet (Fig. 4). Significantly ($P \leq 0.05$) higher THC values occurred in juveniles treated on the base 29% CP diet +Ymix.

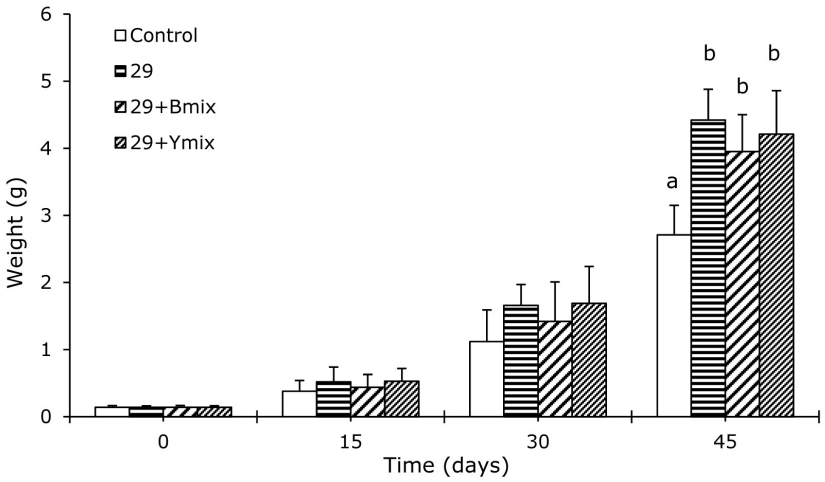

Figure 3. Survival of juvenile *Litopenaeus vannamei* fed with 29% CP and feed additives for 45 days. Different superscript letters indicate significant (*P* ≤ 0.05) differences between treatments / Supervivencia de juveniles de *Litopenaeus vannamei* alimentados con CP 29% y aditivos alimentarios por 45 días. Literales diferentes indican diferencias significativas (*P* ≤ 0,05) entre tratamientos

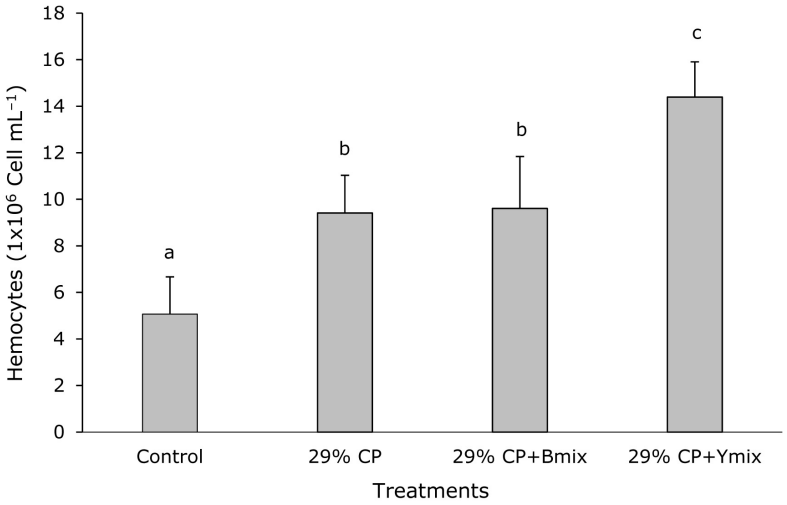

Figure 4. Total hemocyte count in juvenile *Litopenaeus vannamei* fed with 29% CP and feed additives for 45 days. Different superscript letters indicate significant (*P* ≤ 0.05) differences between treatments / Conteo total de hemocitos en juveniles de *Litopenaeus vannamei* alimentados con CP 29% y aditivos alimentarios por 45 días. Literales diferentes indican diferencias significativas (*P* ≤ 0,05) entre tratamientos

Discussion

In this study, the formulation of experimental diets was not only based on the requirements of CP recommended for penaeid shrimp (Tacon *et al*. 2002, Terrazas *et al*. 2010, Gucic-Soriano *et al*. 2013, Ayisi *et al*. 2017), but on the need of increasing profits and decreasing operational costs in the Mexican shrimp aquaculture. The evidence from bioassay I showed that the inclusion of different levels of CP to base commercial diet significantly increased FC, FCR, and improved growth, survival, and THC compared to control group. However, as none of these variables yielded significant differences among the three experimental diets (29, 32, and 35% CP), the diet containing 29% CP was selected as optimal for bioassay II to reduce operational costs.

Several studies have analyzed the inclusion of different CP levels to the diet of cultivated *L. vannamei* juveniles, but results of growth and FCR between commercial and experimental diets have been inconclusive so far. Some authors recommend high CP levels (40 to 45%) to maximize these traits (Tacon *et al*. 2002), lower CP contents between 30-35% have been proposed for adults and <30% for juveniles (Colvin & Brand 1977), or even <25% CP in commercial ponds (Martínez-Córdova *et al*. 2002). Similar to our study, inconclusive patterns are reported in other shrimp species treated with different CP contents added to the diet. For example, no significant differences in weight gain of juvenile white shrimp *Penaeus schmitti* (*L. schmitti*) occurred when using diets containing 25, 30, and 35% CP (Parra 1992), 28

and 33% CP (Galindo *et al*. 2002), or 25, 30, 35, and 40% CP (Pérez-Velázquez *et al*. 2008). In contrast, García *et al*. (1998) supplied four different CP levels (40, 50, 60 and 65%) to the diet of *Penaeus setiferus* and *Farfantepenaeus duorarum* juveniles and reported maximal growth and survival at 50% CP. Usually, when dietary protein is offered in excess, catabolism produces high concentrations of NH_2 and NH_3 compounds that are mobilized in the hemolymph and interfere with most metabolic functions. In turn, these compounds may cause different problems that include unbalanced osmotic pressure and oxygen transport (Schmitt & Santos 1998), slow growth due to increasing metabolic cost of nitrogen excretion (Rosas *et al*. 2001), and toxicity from gradual accumulation in body fluids of free amino acids (Méndez-Martínez *et al*. 2018).

Defining an optimal protein intake in the diet is difficult, as most shrimp species are slow feeders that generate some leaching and nutrient loss during chewing. Protein digestion is highly efficient in crustaceans and higher protein content usually represents improved digestive efficiency. Despite this, the pattern is species-dependent and greatly varies in relation to the quality and quantity of non-protein energy available in the diet, such as lipids and carbohydrates (Méndez-Martínez *et al*. 2017). Consequently, some authors suggest including vegetal protein sources to the diet of juvenile shrimp as a strategy to enhance survival and feed conversion ratio (FCR) (Venkataramiah *et al*. 1975), maximize the assimilation of low-cost nutrients (Chen *et al*. 1985), and improve growth with an animal/vegetal protein source >25% (Cruz-Suárez *et al*. 2000). Méndez-Martínez *et al*. (2017) reported that an adequate combination of animal and vegetable protein enhances weight gain of juvenile shrimp due to the inclusion of a balanced combination of amino acids and vitamins in the mixture. For most shrimp species, the formulation of a digestible amino acids profile is important to compensate the quality level of ingredients defined in the diet (Terrazas *et al*. 2005). In *L. vannamei*, for example, Huai *et al*. (2010) compared four levels of dietary CP (35.5-41.3%), or their digestible protein equivalent (29.8-35.3%), and suggest that reducing the protein level in the diet is feasible without affecting growth and overall production, as long as essential amino acids are supplemented. In our study, all diets were combined with animal (fishmeal) and vegetable protein (soybean paste, whole wheat flour), and supplemented with essential amino acids, such as DL-methionine, lysine, and L-threonine (Table 1).

Recently, some novel protein sources that include dried microbial biomass (called microbial protein) beneficial strains (Alloul *et al*. 2021), and microbial immunostimulants have been used as a strategy to control the spread-out of infectious diseases in shrimp commercial cultivation (Gómez-Gil *et al*. 2000, Phupet *et al*. 2018). Many advantages of beneficial bacteria and yeasts are reported in relation to the synthesis of enzymes and bioactive compounds that bring balance to the host intestinal flora, improve the absorption of nutrients, strengthen its immune system, and displace harmful bacteria (Gullian *et al*. 2003). Moreover, some authors report that the mixtures of probiotics are far more effective for the control of pathogens than single strains, due to the synergic effect of the mix (Douillet 2000, Sotomayor & Balcazar 2003). In this study, the mix of *Bacillus* strains evaluated as feed additives did not increase shrimp growth, but improved survival and THC compared to control juveniles. These results, however, are not consistent with those reported by Rengpipat *et al*. (1998), where significant differences in growth between probiotics and control groups occurred for *Penaeus monodon* juveniles treated with *Bacillus* sp. (strain S11) in three different presentations: fresh cells, fresh cells with saline solution, and lyophilized cells. Similarly, Alloul *et al*. (2021) reported more weight gain and improved FCR in whiteleg shrimp (*L. vannamei*) treated with *Rhodopseudomonas palustris* and *Rhodobacter capsulatus* for 28 days.

Marine yeasts have been used in the last decades as protein source, despite of their lack of essential aminoacids and high nucleic acid contents (Palacios *et al*. 2007, Alamillo *et al*. 2017). Consequently, many yeast strains represent an alternative to partially substitute fish flour in different shrimp species, contributing thus to a more sustainable aquaculture industry (Alloul *et al*. 2021). Additionally, yeasts usually produce polyamines that accelerate gut maturation (Peulen *et al*. 2000) and increase the number of circulating hemocytes (Song & Hsieh 1994, Chaosomboon *et al*. 2017). Increasing the number of hemocytes is associated with greater resistance against pathogens and decreased susceptibility to diseases (Rodríguez & Le Moullac 2000, Gyan *et al*. 2020). Consistently, enhancing the immune system of reared juveniles was possible when the yeasts *S. cerevisiae* and *D. hansenii* were added as immunostimulants to the diet (Gatesoupe 2007, Pacheco *et al*. 2012). Accordingly, in the present study, juvenile shrimp fed on diets complemented with mixed yeasts enhanced survival and THC compared to control diet, including the mix of bacilli strains.

From the evidence collected in this study, formulating the diet for *L. vannamei* juveniles with 29% CP complemented with a mix of live yeasts is recommended to increase its productive response under culturing conditions. Analyses of the nutritional requirements of shrimp in relation to the inclusion of dietary immunostimulants, essential amino acids, and alternative sources of protein (such as microbial biomass) is necessary to broaden the understanding of overall physiological response of the species under pilot-to-commercial cultivation scale, and to reduce the environmental pressure. Future studies that focuse on reaching a functional balance between feed production costs and immune-nutritional diets in juvenile shrimp should be conducted.

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