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ANIMAL PRODUCTION

Soybean protein concentrate in Pacific white shrimp reared in bioflocs: effect on health and vibrio challenge

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ABSTRACT. *Litopenaeus vannamei* shrimp were reared in a bioflocs system and fed different levels of soybean protein concentrate as a replacement for fishmeal, and both immunological parameters of this marine shrimp and its resistance to *Vibrio* sp. infection (CPQBA 378-12 DRM01) were evaluated. Four different diets were formulated with 0, 33, 66 and 100 % of soybean protein concentrate as a substitute for fishmeal. Shrimp were reared in a biofloc system in twelve 800 L tanks (250 shrimp m^{-3}) maintained at constant aeration and temperature. After 42 days, 36 animals (14.21 ± 0.89 g) per treatment were challenged with *Vibrio* sp. (1 x 10^5 CFU mL^{-1} – LD_{10}), and hemolymph was collected before and after challenge to perform immunological assays (agglutination titer, concentration of protein and phenoloxidase activity). Shrimp fed with the experimental diets showed no difference in their resistance to infection and haemato-immunological parameters. Thus, rearing *L. vannamei* in a biofloc system on diets containing either partial or total replacement of fishmeal for soybean protein concentrate did not affect either immunocompetence or susceptibility to infection.

Keywords: immune response; infection; plant protein; *Litopenaeus vannamei*.

Concentrado proteico de soja em dietas para camarão-branco-do-pacífico criado em sistema de bioflocos: efeito na saúde e desafio com vibrio

RESUMO. O presente trabalho avaliou os parâmetros imunológicos e a resistência contra o *Vibrio* sp. (CPQBA 378-12 DRM01) de camarões marinhos *Litopenaeus vannamei* alimentados com diferentes níveis de concentrado proteico de soja em substituição à farinha de peixe e cultivados em sistema de bioflocos. Foram formuladas quatro dietas com 0, 33, 66 e 100 % de concentrado proteico de soja em substituição a farinha de peixe. Os camarões foram cultivados em 12 tanques de 800 L com 250 camarões m^{-3}, sob temperatura e aeração constantes. Após 42 dias, 36 animais (14,21 ± 0,89 g) por tratamento foram desafiados com *Vibrio* sp. (1 x 10^5 UFC mL^{-1} - DL_{10}). Antes e após o desafio foi coletada hemolinfa para avaliação dos parâmetros imunológicos (título aglutinante, concentração de proteína no soro e atividade da fenoloxidase). Não houve diferença na resistência ao desafio e nos parâmetros imunológicos entre os tratamentos. Portanto, o cultivo de *L. vannamei* em sistema de bioflocos utilizando dietas com substituição total ou parcial com concentrado proteico de soja não afeta sua imunocompetência e susceptibilidade a infecção bacteriana.

Palavras-chave: resposta imunológica; infecção; proteína vegetal; *Litopenaeus vannamei*.

Introduction

Fishmeal stands out among the different sources of animal protein used to produce marine shrimp diets based on nutritional value, attractiveness and palatability (Suárez et al., 2009). However, stagnation of fishmeal production in association with increasing demand has contributed to its current high price. Thus, the replacement of fishmeal for cheaper and more available ingredients has become a subject of considerable interest for the shrimp industry, mostly by their effects on shrimp farming profitability (Sookying, Davis, & Soller, 2013; Tacon & Metian, 2008).

Soybean and its derivatives have significant potential as a fishmeal replacement. Soybean ingredients have high protein content (45 to 50%) and a good balance of amino acids, except a deficiency in methionine. Additionally, they show lower price fluctuation when compared to fishmeal, potential for long-term storage, and can be

considered a renewable source of protein (Sookying et al., 2013). In addition to its amino acid profile, soybean concentrate can be distinguished from other derivatives for its higher digestibility (energy and protein) and palatability, as well as fewer anti-nutritional factors (Suárez et al., 2009). However, studies evaluating the effect soybean protein concentrate as a replacement for fishmeal have mostly considered performance parameters (Bauer, Prentice-Hernandez, Tesser, Wasielesky Jr., & Poersch, 2012; Paripatananont, Boonyaratpalin, Pengseng, & Chotipuntu, 2001), not immune response. Moreover, diseases like vibriosis and viral diseases affect shrimp production; therefore, susceptibility to disease, which has also been poorly studied, is important as a factor in the assessment of shrimp health (Lightner et al., 2012). In fact, the assessment of haemato-immunological parameters in association with disease challenge is commonly performed to verify the health status of crustaceans (Elshopakey et al., 2017; Jia et al., 2017).

In an even more granular sense, few studies have reported on the effect of diets containing soybean protein concentrate on shrimp reared in bioflocs technology (BFT) systems (Sá, Sabry Neto, Cordeiro Júnior, & Nunes, 2013). The BFT system is based on the presence of microbial aggregates. The manipulation of the C:N ratio favors the conversion of inorganic nitrogen in microbial biomass, maintaining water quality and, consequently, reducing the need for water exchange and increasing biosecurity (McIntosh et al., 2000). This manipulation takes place through the addition of organic carbon based on the nitrogen concentration present in the feed and water (Ahmad, Rani, Verma, & Maqsood, 2017). The microbial aggregates present in BFT systems can also be used as a feed source for the animals, and the consumption of these microbial aggregates seems to improve the immune system, increasing both antioxidant defense (Cardona et al., 2016; Liu, Zhu, Liu, Guo, & Ye, 2017; Xu & Pan, 2013) and resistance against Infectious myonecrosis virus (IMNV) challenge (Ekasari et al., 2014).

This study aimed to evaluate the haemato-immunological parameters of the marine shrimp *Litopenaeus vannamei* reared in a bioflocs system, as well as its resistance to *Vibrio* sp. infection, when fed different levels of soybean protein concentrate as a replacement for fishmeal.

Material and methods

Biological material

The Pacific white shrimp (*Litopenaeus vannamei*) used in this study were purchased from AQUATEC (Rio Grande do Norte, Brazil) and represent a lineage of specific–pathogen-free (SPF) breed under the mandatory notification (WSSV, IHHNV, TSV, IMNV, and YHV) by the OIE-World Organization for Animal Health.

Lethal dose

For bacterial challenge, a strain of *Vibrio* sp. (CPQBA 378-12 DRM01) was isolated from *L. vannamei* broodstock maintained at the Laboratório de Camarões Marinhos of the Universidade Federal de Santa Catarina (LCM-UFSC). The strain was identified using 16S rRNA sequencing and showed higher similarity with *Vibrio natriegens* and *Vibrio alginolyticus* on phylogenetic analysis (Figure 1). A lethal dose (LD) assay was performed, using 10 experimental units (40 L) with 10 L. *vannamei* individuals (11.5 ± 1.2 g). These units were filled with sterilized seawater under constant temperature (28.0 ± 1.0ºC) and aeration. Nine different concentrations were evaluated, ranging from 5×10^4 to 1×10^7 colony forming units (CFU) per shrimp, one for each experimental unit. One unit was inoculated with 3 % sterile saline solution (SSE) as control (Buglione et al., 2010).

The *inoculum* was cultivated in Brain and Heart Infusion Broth (BHI) at 28°C for 18h and then centrifuged at 1,800 *g* for 30 min. The supernatant was discharged, and the precipitate was resuspended in 3% SSE at 1×10^8 CFU mL^{-1} based on a previously established relationship between absorbance at 600 nm (OD600) and CFU. Afterwards, the bacterial suspension was diluted to the desired concentration for the challenge (ranging from 5×10^4 to 1×10^7) and then inoculated (100 μl) at the first abdominal dorsal segment. Mortality was monitored every 6 h over the course of 96h (data not shown).

Rearing conditions

Four isocaloric diets were formulated with different levels of soybean protein concentrate as a replacement for fishmeal (0, 33, 66 and 100 %). Diets were formulated with 271-274 g kg^{-1} of estimated digestible protein (Jatobá et al., 2017) and similar amounts of marine-origin fat (fish oil + fat contained in the fishmeal), ensuring a similar profile of fatty acids. The protein content, crude lipids, crude fibre, ash and moisture of all diets were determined (Table 1).

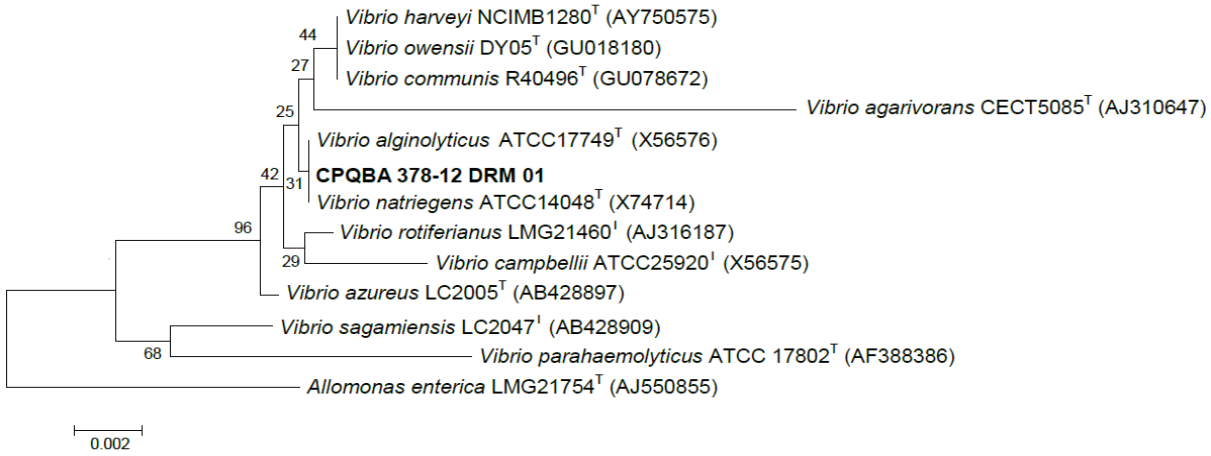

Figure 1. Phylogenetic tree derived from partial 16S rDNA sequence data for bacteria related to the CPQBA 378-12-DRM 01 strain, which was isolated from *L. vannamei* broodstock from the maturation sector of LCM-UFSC and used for bacterial challenge. Sequence data was from The Ribosomal Database Project (RDP) and GenBank.

Table 1. Ingredients and proximal composition of the experimental diets with different replacement levels of fishmeal by soybean protein concentrate.

Ingredients (g kg^{-1})	Replacement levels (%)			
	0	33	66	100
Fish meal (590 g kg^{-1} crude protein)	208.7	131.9	60.0	0.0
[1]Soybean protein concentrate	0.0	65.0	120.0	171.6
Soybean meal (450 g kg^{-1} crude protein)	350.0	350.0	350.0	350.0
Rice grits	80.0	80.0	80.0	80.0
Wheat flour	250.0	250.0	250.0	250.0
Soy lecithin	15.0	15.0	15.0	15.0
Fish oil	6.2	13.4	20.0	25.1
Soybean oil	20.0	20.0	20.0	20.4
Potassium chloride	15.0	14.0	10.0	9.1
Sodium chloride	15.0	15.0	15.0	15.0
Magnesium Sulfate	8.0	8.0	8.0	8.0
Vitamin-C	0.3	0.3	0.3	0.3
Kaolin	8.8	13.4	23.7	26.6
Monocalcium phosphate	20.0	0.00	0.00	0.00
[2]Premix	15.0	15.0	15.0	15.0
Proximal composition (g kg^{-1})				
[3]Digestible protein	272.0	274.0	272.0	271.0
Crude protein	333.9	326.7	321.6	318.8
Crude lipid	63.1	66.4	60.6	66.3
Nitrogenan-free extract	350.6	369.3	380.0	389.9
Ash	126.7	121.0	114.3	104.3
Crude fiber	21.9	23.9	21.9	26.9
Moisture	103.9	92.8	101.6	98.8
[4]Energy	3909.0	3973.0	3.903.0	4002.0
[5]Energy.Crude protein^{-1}	11.7	12.1	12.1	12.5

[1]Proximate composition: 63.07 % crude protein, 1.38 % ether extract, 4.66 % crude fiber, 6.79 % moisture and volatiles, 6.32 % ash, 17.78 % nitrogen free extract, 1.38 % acid hydrolysis extract; 4,426.0 cal.g^{-1}; amino acid profile: aspartic acid 6.67 %, glutamic acid 10.03 %, serine 2.65 %, glycine 1.90 %, histidine 1.68 %, arginine 3.69 %, threonine 1.74 %, proline 2.73 %, tyrosine 1.69 %, valine 2.73 %, methionine 0.71 %, methionine + cystine 1.37 %, isoleucine 2.82 %, leucine 4.99 %, phenylalanine 3.04 % and lysine 3.92 %. [2]DSM Produtos Nutricionais Brasil Ltda. (São Paulo, SP, Brazil): vit. A – 1,250,000 UI; vit. D3 – 350,000 UI; vit. E – 25,000 UI; vit. K3 – 500.0 mg; vit. B1 – 5,000.0 mg; vit B2 4,000.0 mg; vit. B6 – 5,000.0 mg; B12 – 10.0 mg; nicotinic acid – 15,000.0 mg; pantothenic acid – 10,000.0 mg; biotin – 150.0 mg; folic acid - 1.25 mg; vit. C – 25,000.0 mg; choline – 50,000.0 mg; inositol 30,000.0 mg; iron – 2,000.0 mg; copper – 3,500.0 mg; copper chelate – 1,500.0 mg; zinc – 10,500.0 mg; zinc chelate – 4,500.0 mg; manganese – 4,000.0 mg; selenium – 15.0 mg; selenium chelate – 15.0 mg; iodine – 150.0 mg; cobalt 30.0 mg; chromium – 80.0 mg. [3]Estimation according to the ingredients used (Jatobá et al., 2017); [4]Cal.g^{-1}. [5]kcal.g crude protein^{-1}. Amino acids were measured using AOAC (2005) official method 994.12, except methionine and cystine measured using AOAC (2005) official method 985.28. Protein contents were also measured according to AOAC (2005) using the Dumas nitrogen combustion method. Crude lipids were quantified using the ether extraction method, and energy was determined with a bomb calorimeter. Crude fibre, ash, and moisture were determined using AOAC (2005) official methods 978.10, 942.05, and 930.15, respectively.

Shrimp juveniles (initial weight: 3.96 ± 0.04 g) were grown under a super-intensive biofloc system (BFT) in polyethylene units (800 L) supplied with water pumped from a bioflocs matrix tank. The units had an area of 4 m² (bottom and sides), and an additional 2 m² were provided as artificial substrate. Constant aeration was provided through diffusion hoses connected to a 7.5 hp blower, while 1,000 W titanium heaters with a thermostat were used to maintain the temperature at 29.0 ± 0.5ºC.

The experimental units were distributed in a completely random design among the four treatments (0, 33, 66 and 100 % of soybean protein concentrate) in triplicate. Each tank received 200 shrimp, maintaining an initial density of 250 shrimp m^{-3}. The rearing trial lasted 42 days, until the animals reached 14.21 ± 0.89 g (Jatobá et al., 2017).

Bacterial challenge and haemato-immunological analysis

After the rearing period, 12 shrimp from each experimental unit were transferred to 40 L tanks filled with sterilized seawater under constant temperature (28.0 ± 1.0ºC) and aeration. Then, 100 μl of bacterial suspension in SSE at the concentration of 1 x 10^5 CFU mL^{-1} (LD_{10}, calculated using the lethal dose assay), produced as described above, were inoculated in the dorsal region of the first abdominal segment of each shrimp. As in the lethal dose assay, mortality was monitored every 6 h over 96h.

Hemolymph from five shrimp per experimental unit was collected to form three pools for each treatment, before and 96h after challenge, for haemato-immunological analysis. Samples were collected using 1-mL sterile syringes with a 21 G needle and left to clot at 4°C for 2h. The coagulated hemolymph was frozen at -20°C, thawed, and then

centrifuged at 10,000 *g* for 10 min to obtain the serum, which was then aliquoted and stored at -20°C for later use. Haemato-immunological assays, including serum agglutination titer, concentration of protein and phenoloxidase activity (PO) were performed as described by Silva et al. (2016).

Data analysis

A bacterial lethal dose curve was performed by using a linear regression model. Data adjustment to the model was based on the significance level ($p < 0.05$), the regression coefficients by t test, the determination coefficient (R2 = S.Q.Reg./ S.Q.Treatment), the sum of squared deviations, and the phenomenon under study. Mortality data from the experimental challenge and data from haemato-immunological assays were first subjected to Cochran analysis to verify the homogeneity of variance, and then the data were subjected to repeated-measures ANOVA and ANOVA factorial (replacement level x challenge), respectively. If differences were found between the means, the Tukey test for comparison of means was used at 5 % probability.

Results and discussion

In the bacterial challenge assay, shrimp fed with 33 and 66 % of soybean protein concentrate replacement showed lower mortality rate in the first 70 h, but not different ($p \geq 0.05$). However, after 96 h, all treatments reached similar ($p \geq 0.05$) mortality rate (Figure 2).

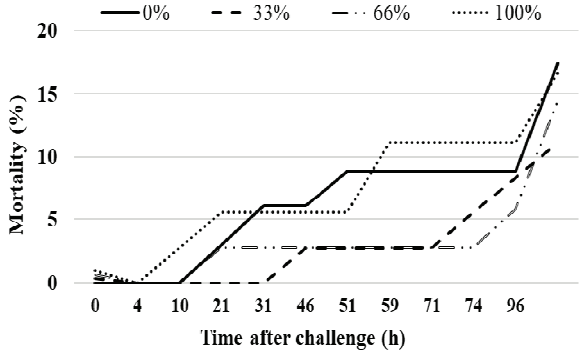

Figure 2. Cumulative mortality of BFT-reared *Litopenaeus vannamei* fed with diets containing different replacement levels of fishmeal by soybean protein concentrate and challenged with *Vibrio* sp. strain (CPQBA 378-12 DRM01) for 96h.

Based on the haemato-immunological assays, shrimp fed with all four experimental diets showed no difference, either before or after bacterial challenge ($p > 0.05$). After challenge with *Vibrio* sp., agglutination titer and serum protein concentration showed a reduction ($p < 0.05$) in all treatments. However, PO activity remained at the same level before and after challenge (Table 2).

Table 2. Haemato-immunological parameters of BFT-reared *Litopenaeus vannamei* (n = 15) fed diets with different levels of protein concentrate to replace fishmeal before (BC) and after (AC) challenge with *Vibrio* sp. strain (CPQBA 378-12 DRM01) for 96h.

Treatments		0%	33%	66%	100%
Agglutination titer	BC*	12.33±0.58	13.00±0.0	12.67±0.58	12.25±0.0
($\log_2 x+1$)	AC	10.33±0.58	11.00±0.0	10.67±0.58	10.50±0.58
PO activity	BC	46.58±14.28	35.08±4.75	40.19±2.43	36.54±1.63
(unit min^{-1} mg^{-1})	AC	41.06±2.98	38.59±5.35	43.29±9.69	37.44±1.77
Protein concentration	BC*	167.09±25.66	161.96±10.84	135.23±13.91	153.01±14.74
(mg mL^{-1})	AC	87.14±4.76	93.65±11.97	85.99±10.50	95.16±2.99

*Indicates statistical differences in the Tukey test ($p < 0.05$) between groups before and after challenge. Mean ± standard deviation.

The replacement of fishmeal by soybean protein concentrate at levels of 66 and 100% decreased shrimp growth and yield (Jatobá et al., 2017); however, none of the different replacement levels affected the susceptibility of shrimp to *Vibrio* sp. challenge, nor did such replacement levels result in significant differences in haemato-immunological parameters evaluated before or after challenge.

The effect of plant-based ingredients, especially soybean, as the main source of feed protein, on resistance to infection and innate immune response of aquatic organisms seems to be controversial. Bulbul et al. (2016) demonstrated that the use of canola and soybean meal as the primary protein source did not affect the viability or hemocyte count of *Marsupenaeus japonicus* grown in clear water. Meanwhile, *Macrobrachium nipponense* grown in clear water and fed with fermented soybean meal greater than 25 %, as a replacement for fishmeal, showed a reduction in resistance to *Aeromonas hydrophila* infection, as well as a reduction in immunocompetence (Ding, Zhang, Ye, Du, & Kong, 2015).

In fish, soy protein concentrate at 60%, as a replacement for fishmeal, did not affect resistance against *Vibrio harveyi* in *Paralichthys dentatus* reared in clear water. However, replacement by a combination of 48% soy protein concentrate and 12% soybean meal did increase survival by 25% after challenge with *V. harveyi* (Ward, Bengtson, Lee, & Gomez-Chiarri, 2016). Kokou, Rigos, Henry, Kentouri, and Alexis (2012) found that different percentages of fishmeal replacement by bioprocessed soybean meal variously modulated innate immune response of *Sparus aurata* grown in clear water.

On the other hand, rearing marine shrimp in a bioflocs system seems to positively influence resistance to infection and immunocompetence of the animals. Bioflocs is an aggregate formed by bacteria (gram-negative and gram-positive), algae,

fungi, protozoans and nematodes (Ahmad et al., 2017). The cell walls of these microorganisms, like lipopolysaccharides and peptidoglycans, is known to be immunostimulant for shrimp (Wang, Sun, Liu, & Xue, 2017). Ekasari et al. (2014) found that *L. vannamei* reared in a biofloc system with addition of different carbon sources improved immune response and resistance against IMNV. Xu and Pan (2013) also reported improvement in immune response and antioxidant production in shrimp reared in a biofloc system. Thus, in the present study, rearing marine shrimp in a biofloc system may have overcome the effects of protein source replacement, homogeneously enhancing immunocompetence and response to bacterial infection of *L. vannamei* from the different treatments.

Nevertheless, activation of the innate immune system of the shrimp reared in biofloc system is not necessarily a positive situation, essentially because it could lead to higher energy cost and physiological impairment in these animals. Therefore, further studies should be performed to shed more light on the apparent immunostimulatory effect of biofloc system on shrimp health.

Conclusion

Rearing *L. vannamei* in a biofloc system with diets replacing fishmeal with soybean protein concentrate, either partially or completely, does not affect the susceptibility or immunocompetence (agglutination titer, concentration of protein and phenoloxidase activity) of these animals upon challenge with *Vibrio* sp.

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