GROWTH AND ENZYMATIC PROFILE OF THE PACIFIC WHITE SHRIMP FED WITH *Porphyridium cruentum* EXTRACT

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ABSTRACT

The study was developed to evaluate the influence of diet supplementation of a polysaccharide extract from the microalgae *Porphyridium cruentum,* on weight gain, digestive enzyme activity, and *Litopenaeus vannamei* juvenile survival. The polysaccharides crude extract from microalgae *P. cruentum* was added at different concentrations (0, 0.5, 1, 1.5, and 2.0%) in a commercial diet and provided to juvenile *L. vannamei* (6.6 ± 0.2 g). The shrimps (n = 2,000) were fed *ad libitum* for 30 days in circular tanks with 12 m^2 of bottom area (100 shrimp tank^{-1}) and environmental variables (temperature, salinity, dissolved oxygen, photoperiod and levels of total ammonia) were controlled. The supplemented diet with 1% crude extract was responsible for the biggest weight gain (7.28 g) in 30 days. The shrimp body muscle centesimal composition and survival were not affected by the polysaccharide extract supplementation. It was observed a shift in the activity of digestive enzymes from the hepatopancreas to the anterior midgut and mid midgut portions in shrimps with diet supplemented with 1% polysaccharide extract, which may have contributed to a better digestive efficiency. The results indicate that dietary supplementation with crude extract of *P. cruentum* polysaccharides in a range between 1 and 1.5% increases weight gain and enhances the activity of digestive enzymes in *L. vannamei* juveniles.

Keywords: *Litopenaeus vannamei*; microalgae; polysaccharide; digestive enzymes

CRESCIMENTO E PERFIL ENZIMÁTICO DO CAMARÃO BRANCO DO PACÍFICO ALIMENTADO COM EXTRATO DE *Porphyridium cruentum*

RESUMO

O estudo foi desenvolvido para avaliar a influência da suplementação dietética do extrato de polissacarídeo da microalga *Porphyridium cruentum* no ganho em peso, na atividade das enzimas digestivas e na sobrevivência de juvenis do camarão *Litopenaeus vannamei*. O extrato bruto de polissacarídeos, extraído da microalga *P. cruentum,* foi adicionado em diferentes concentrações (0, 0,5, 1, 1,5 e 2,0%) a uma dieta comercial fornecida a juvenis de *L. vannamei* (6,6 ± 0,2 g). Os camarões (n = 2.000) foram alimentados *ad libitum*, durante 30 dias, em tanques circulares com 12 m^2 de área de fundo (100 camarões tanque^{-1}) e as variáveis ambientais (temperatura, salinidade, oxigênio dissolvido, fotoperíodo e níveis de amônia total) foram controladas. A dieta suplementada com 1% de extrato bruto foi o responsável pelo maior ganho em peso (7,28 g) em 30 dias, porém, a composição centesimal muscular e a sobrevivência não foram afetadas pela suplementação. Entretanto, verificou-se um deslocamento da atividade das enzimas digestivas do hepatopâncreas para as porções anterior e média do intestino médio nas suplementações com até 1% do extrato, o que pode ter contribuido para uma melhor eficiência digestiva. Os resultados indicam que a suplentação da dieta com extrato bruto de polissacarídeos de *P. cruentum* numa concentração entre 1 e 1,5% aumenta o ganho em massa e melhora a atividade das enzimas digestivas de juvenis de *L. vannamei*.

Palavras chave: *Litopenaeus vannamei*; microalgas; polissacarídeos; enzimas digestivas

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INTRODUCTION

The research conducted on the use of artificially prepared diets with functional food, which have the capacity of acting on the cultivated organisms' health and disease causing agents, shows that stress resistance is of great importance. The additives or food supplements used in aquaculture with the purpose of enhancing the zootechnical performance and animal sanity can be immunonutrients, immunostimulants, probiotics, and prebiotcs (GATLIN, 2002; GATLIN *et al*., 2006).

According to BRICKNELL and DALMO (2005), the immunostimulants are important tools with the potential to be used in aquaculture to minimize loss due to disease. The immunostimulant compound administration, as dietetic supplementation, presents advantages since it can be given simultaneously for a great number of individuals, preventing the resulting stress from animal handling (SMITH, 2003). Stimulating the non-specific defense system improves growth and disease resistance, in addition to being a potential method to improve microbial control in juvenile marine fish, crustacean, and mollusk production (SKJERMO *et al.* 2006).

In recent years, scientific interest has grown regarding the investigation and identification of microalgae biologically active compounds, among these the polysaccharides. These molecules have been increasingly used as immunostimulants in aquaculture, due to their great parasite variety of fungi, bacteria, and viruses that affects the aquatic organisms' production, causing great economic losses (BOISSON-VIDAL *et al*., 1995).

Despite those advantages, a new ingredient addition, even in small quantities, can cause enzymatic inhibition and diminished weight gain (LEMOS *et al*., 2004). Therefore, the digestive enzymes behavior is a relevant point to understand the digestion of any nutrient included or supplemented in the diet (LE MOULLAC *et al*., 1997; ALMEIDA NETO, 2011), being an alternative to indicate the animal nutritional state (MELO, 2004).

Generally, the penaeid shrimps are very well adapted to diet composition changes by the induction of digestive enzymes synthetized and secreted by the hepatopancreas (LE MOULLAC *et al*., 1997). This enzymatic activity adaption to the diet nutritional content was also described by DELGADO *et al*. (2003) and DEVAKARAN *et al*. (2004) in experiments involving groups of *Litopenaeus vannamei* shrimp submitted to different composition diets.

This study aim was to evaluate the effect of dietetic supplementation with different sulfated polysaccharides crude extract concentrations of the microalgae *Porphyridium cruentum* on the survival, weight gain, and digestive enzymatic activity of *L. vannamei* shrimp juveniles.

MATERIAL AND METHODS

Polysaccharide crude extract

The polysaccharide (PS) crude extract of the microalgae *P. cruentum* was extracted with a crude papain solution (30 mg mL^{-1}) in 250 mL 0.1 M sodium acetate buffer (pH 5.0) plus 5 mM EDTA and 5 mM of cysteine from 5 g biomass. The material was filtered and centrifuged (7.965 × g; 20 min.; 10 °C), the PS in the supernatant was concentrated by precipitation with 16 mL of 10% Cetilpiridinium chloride (CPC), washed (200 mL; 0.05% CCP) and after dissolved in 174 mL of 2M NaCl: ethanol (100:15; v/v). Shortly after another precipitation with absolute ethanol (24 h; 4 °C), the material was washed with 80% ethanol (200 mL, 2x), absolute ethanol (200 mL, 1x) and oven dried (24 h, 60ºC) (FARIAS *et al*., 2000).

Diet preparations

Five different *P. cruentum* PS crude extract concentrations (0.5, 1.0, 1.5, and 2.0%) were added to a commercial marine shrimp feed (Guabi®, 40% crude protein and 7.5% crude fat). The feed was separately grinded in 5 kg lots, and each lot received a different supplementation of PS crude extract according to the concentrations specified above. These supplemented lots were mixed one by one for 15 minutes in a model Y mixer. The dried mixture was placed in a mixer and warm water was added in sufficient amounts until consistent dough was formed. After, the dough was pelleted in an electrical meat grinder and the formed pellets were oven dried with air circulation (24 h, 60 ºC), bagged and stored at 4 ºC.

The control lot, without PS supplementation, was submitted to the same procedures.

Biological material and experimental conditions

Two thousand white shrimp *L. vannamei* juveniles with an initial weight of 6.6 ± 0.2 g, SPF (specific pathogen free), from Marine Shrimp Laboratory (LCM), were randomly distributed in 20 glass fiber tanks (12 m^2 bottom, 4 tanks $treatment^{-1}$, 100 shrimp $tank^{-1}$). The five experimental diets were randomly designated to four tanks. During 30 days, the shrimp were fed *ad libitum* twice a day, 10:00 h and 17:00 h. Tank water was renewed 50% daily at 8:00 h and 14:00 h.

The environmental variables were monitored during the experiment, as dissolved oxygen; water temperature and salinity were measured twice a day. Total ammonia was analyzed every 10 days immediately after sampling, and after previously having been filtered in cellulose acetate micro filters (0.45 µm). These analyses were conducted according to STRICKLAND and PARSONS (1972).

Shrimp samples were obtained every 10 days to follow the animal weight gain (WG). The collected animals were not returned to the tank, but they had their intestinal tracts dissected for enzymatic activity determination. By the end of 30 days, the following variables were calculated:

$$\mathrm{WG} = 100 * \left(\frac{\text{final weight} - \text{initial weight}}{\text{initial body weight}}\right)$$

$$\text{Survival} = 100 * \left(\frac{\text{final shrimp number}}{\text{initial shrimp number}}\right)$$

Tissue harvest for enzymatic assays

For midgut and hepatopancreas removal, live shrimps were put on ice and then dissected in a cold saline solution (250 mM NaCl). The intestinal tract portions used in this study were the hepatopancreas, the anterior third of the midgut, the middle third of the midgut ant the posterior third of the midgut. The hepatopancreas and the midgut sections were homogenized with double distilled cold water, using a Potter-Elvehjem homogenizer. The homogenized tissue was centrifuged (10,000 g, 30 minutes, at 4 °C), and the supernatant and pellets were used in the enzymatic assays.

Protein determination and enzymatic assay conditions

The activities over cellobiose (20 mM), maltose (10 mM) and sucrose (20 mM) were determined by the glucose liberation assay according to the Dahlqvist method (1968). The activities over raffinose (20 mM) and jellified starch (0.5%, w v^{-1}) were measured by the determination of the medium reducing power, according to NOELTING and BERNFELD (1948). All carbohydrases assays were performed in a 50 mM citrate-phosphate buffer, pH 5.2, except for α-amylase, when it was used a 50 mM sodium acetate buffer, pH 5.2, plus 20 mM NaCl and 2 mM $CaCl_2$.

The total protease activity was determined using azoalbumin as a substrate. The assays were adapted from a method described by LEMOS *et al*. (1990). Extracts (100 µL each assay) were incubated with 100 µL mixture of 1% substrate (w v^{-1}) in 100 mM phosphate buffer, pH 7.5. The proteolytic reaction was terminated by the addition of 50 µL 30% trichloroacetic acid (w v^{-1}) and the reaction was incubated during 15 min in ice. The precipitated substrate was removed by centrifugation (7.000 g, 5 min, at room temperature). A hundred µL supernatant samples were mixed with 100 µL 2N NaOH solution and the absorbance was read at 440 nm. The hydrolysis of p-nitroanilide succinyl-alanyl–alanyl-prolyl-phenylalanine-p-nitroanilide (SAAPF-pNA) and benzoyl-L-arginyl-p-nitroanilide (BAPNA) were assayed by the liberation of p-nitroaniline, with the absorbance maximum at 410 nm (ERLANGER *et al*., 1961).

The assays used 50 µL of four 2 mM substrates, 50 µL enzyme sources and 100 µL 100 mM phosphate buffer, pH 7.5, with or without inhibitors. The reaction was terminated by the addition of 100 µL 30% trichloroacetic acid (w v^{-1}), and the absorbance was read at 410 nm.

All assays were performed at 30 °C. The buffers (50 mM) used in the pH optimum determination were sodium acetate, sodium citrate-phosphate, Tris-HCl buffer, with pH values ranging from 3 to 9, with 0.2 pH units intervals. The incubations were made at least at four different time periods and the initial

hydrolysis speeds were calculated. One enzymatic unity was defined as the enzyme quantity which catalyzes the cleavage of 1 µmol of substrate min^{-1}. Small enzymatic quantities were expressed in mU.

The protein concentration was determined according to the method described by SMITH *et al*. (1985), modified by MORTON and EVANS (1992), using bovine serum albumin as a protein standard.

Statistical analysis

The experimental design used was entirely randomized, with 5 levels of polysaccharide extract supplementation (0, 0.5, 1.0, 1.5, and 2.0%) and four repetitions for each treatment. The weight gain, body composition, and survival data were submitted to Analysis of Variance ANOVA and, when necessary, the Duncan test was applied at 5% significance level.

RESULTS

During the experimental period, the water temperature, salinity, dissolved oxygen, ammonia total levels (NH_3 + NH_4^+) and photoperiod were, respectively, 25.9 ± 0.15 °C; 33 ± 0.1; 6.0 mg L^{-1}; 0.06 to 0.3 mg L^{-1} and 12 h.

The polysaccharide concentration that provide the best weight gain, at approximately 7.28 g in 30 days, was the 1% diet. The survival rates were high (90 to 97%), without significant difference among treatments (Table 1). The polysaccharide supplementation diet did not cause any important alterations to the shrimp muscle centesimal composition, without significant differences (P>0.05) among treatments in the body protein, moisture, and ash content (Table 2).

The sulfated polysaccharide crude extract supplementation did not affect the shrimp survival rates; however, it significantly influenced the juveniles' weight gain, when compared to the control, without supplementation. This supplementation increases the weight gain in a 0.5 to 1.5% interval, but from this point on, it decreases. With this concentration, the weight gain is optimized and estimated in 7.28 g by the end of the experiment.

Table1. Weight gain and survival rates of the *Litopenaeus vannamei* juveniles fed for 30 days with diets containing different concentrations of polysaccharides (PS) crude extract from microalgae *Porphyridium cruentum.*

Diet	Final weight (g)	Weight gain (g)	Survival (%)
1 (0% PS)	12.43 ± 0.3^{b}	5.73 ± 0.16^{b}	90.7 ± 7.4^{a}
2 (0.5% PS)	13.50 ± 0.8^{a}	6.90 ± 0.39^{a}	97.4 ± 1.4^{a}
3 (1.0% PS)	13.80 ± 0.5^{a}	7.28 ± 0.62^{a}	94.1 ± 7.9^{a}
4 (1.5% PS)	13.55 ± 0.6^{a}	6.73 ± 0.29^{a}	95.0 ± 8.2^{a}
5 (2.0% PS)	13.18 ± 0.4^{ab}	6.68 ± 0.62^{ab}	94.5 ± 7.1^{a}

Values are mean ± standard deviation. Different superscript letters on same column show significant difference as determined by Duncan´s Test (P<0.05).

Table 2. White shrimp body muscle centesimal composition after 30 days fed with different concentrations of the sulfated polysaccharides (PS) crude extract from microalgae *Porphyridium cruentum* (g kg^{-1} wet weight, P>0.05).

Fractions	% PS Supplementation				
	0.0	0.5	1.0	1.5	2.0
Crude Protein	21.24	22.01	21.88	21.50	21.78
Moisture	76.67	75.35	75.29	75.95	75.29
Crude fat	0.30	0.40	0.10	0.10	0.10
Ash	1.34	1.46	1.42	1.36	1.00

Enzymatic determinations suggest that digestive enzymes are produced and secreted in the hepatopancreas and they are distributed in concentration gradient along the midgut (Figure 1). The proteolytic activities were found in higher levels in the hepatopancreas and decrease along the mid and posterior midgut (Figures 1A, 1C, and 1D). Comparing the activity with the growing polysaccharide concentration in the diets, there was no alteration in the hepatopancreas, but there was an increase in the proteolytic activity in the anterior intestine to the following distal segments, which suggests the existence of digestive enzymes produced in the hepatopancreas and displaced to the midgut.

Similar to the proteases, the enzymatic activity distribution over starch substrate showed a different pattern in the hepatopancreas, and along the midgut (Figure 1B). It is worth to mention that the higher α-amylase distribution shows a higher activity displacement along the midgut, mainly to the middle and posterior thirds of the midgut, when the polysaccharide concentration was higher than 1.0%.

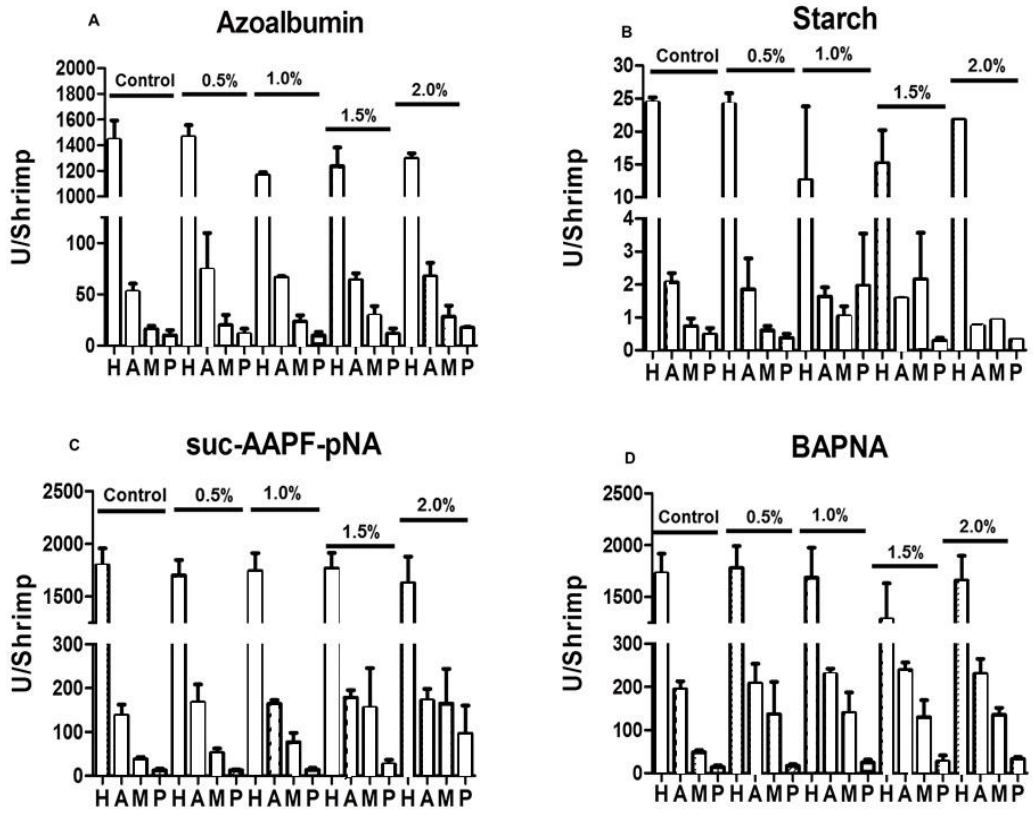

Figure 1. Digestive enzymatic activity displacement, proteases (A, C D) and α-amylase (B) in the hepatopancreas (H), anterior third of the midgut (A), middle third of the midgut (M) and posterior third of the midgut (P) in the Pacific white shrimp, fed with different concentrations of the polysaccharides crude extract from microalgae *Porphyridium cruentum.*

The activity over maltose (Figure 2) has the same pattern as the starch, forming an enzymatic gradient along the midgut, in response to crescent doses of polysaccharides. The other activities over other substrates such as melibiose (Figure 2B), sucrose (Figure 2C) and cellobiose (Figure 2D), showed diffuse patterns, where it was not possible to relate any activity difference to the crescent polysaccharides concentration in the diets.

It is important to note that the activity in these substrates on the hepatopancreas was not higher when compared to the measured activities in the midgut.

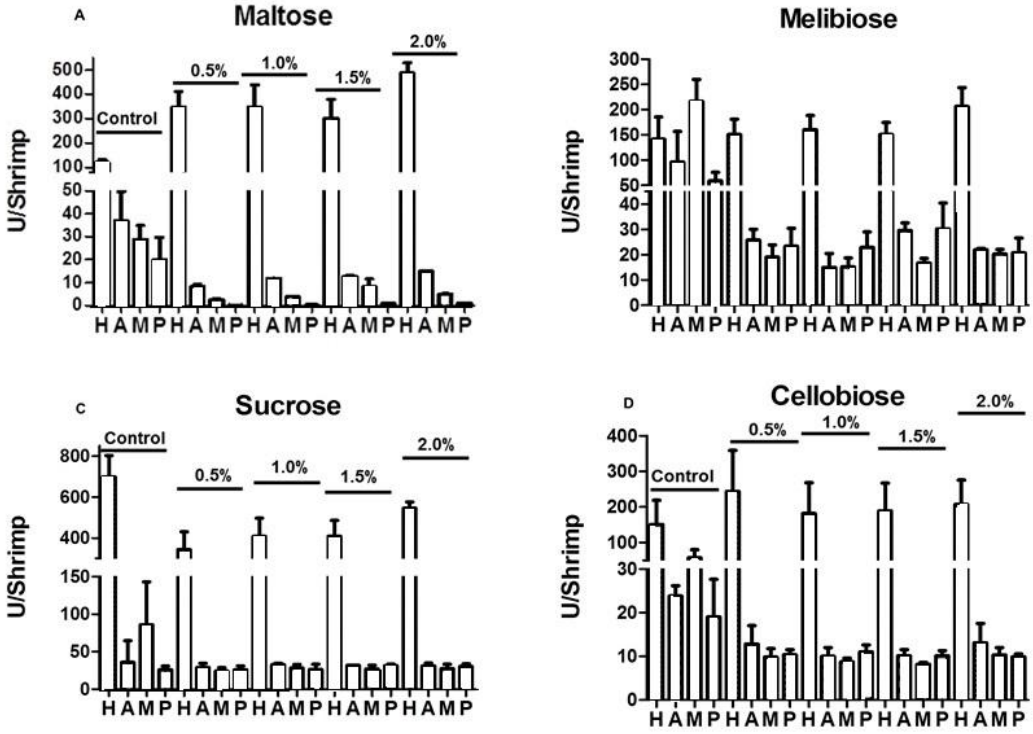

Figure 2. Digestive enzymatic activity displacement, carbohydrases (A, B, C, and D) in hepatopancreas (H), anterior third of the midgut (A), middle third of the midgut (M) and posterior third of the midgut (P) in the Pacific white shrimp, fed with different concentrations of the polysaccharides crude extract from microalgae *Porphyridium cruentum.*

The enzymatic activity displacement in the face of the crescent polysaccharide crude extract doses on the tested substrates has a direct influence on the nutrient digestive efficiency. The gradient formation where the complex enzyme-substrate fades into smaller units to be absorbed is the ideal pattern.

In the present work, it was verified that the polysaccharide extract supplementation can maintain the enzymatic gradient in the majority of the tested substrates until 1% supplementation, considering the tested dosage. From this concentration forward, the gradient became undone and the enzymatic occurrence on the posterior intestine increased. This is not a desirable feature, once there is no nutrient absorption in this region and these enzymes may possibly be lost in the feces. The importance in enzymatic gradient formation along the midgut can be better understood when the peritrophic membrane is taken into account (ALEXANDRE *et al.* 2014).

DISCUSSION

In the literature, the use of polysaccharides in order to optimize marine shrimp growth, immunological parameters, and survival rates is shown as a promising alternative and is viable at the experimental level.

The works that describe the use of polysaccharides, by immersion baths, relate that this administration pathway increases the shrimp survival rates and the resistance against nature's various challenges (environmental stress, bacterial infections, viral infections), but it does not present the most positive results for weight gain, mainly because the animal handling causes stress and the immunostimulant contact is virtually external and by short periods of time (LIMA *et al.*, 2009).

Although an immunostimulant main goal is to increase individual resistance, the body weight increment is considered a quite relevant effect in shrimp cultivation. BRICKNELL and DALMO (2005) have already verified immunostimulants

supplementation in diets that were optimized with these compounds' absorption and this aspect was reflected in a body weight increase.

The sulfated polysaccharide use in marine shrimp diets is dated since 1994, when SUNG *et al.* (1994) used β-glucans as a diet supplement for *Penaeus monodon,* aiming at the vibriosis increased resistance in these animals. In this study, crustaceans, besides presenting higher resistance, also showed a higher weight gain when fed with these polysaccharides.

CRUZ-SUAREZ *et al*. (2000) verified that a diet supplementation with the sulfated polysaccharide extracted from the macroalgae *Macrocytis pyrifera* improved the juvenile *L. vannamei* growth.

The same growth positive effect was also observed in *L. vannamei* juveniles fed with Ergosan® (Merck Animal Health, EUA) supplemented diets, which is a commercial product prepared from alginic acid extracted from the macroalgae *Laminaria digitata* (MONTERO-ROCHA *et al*. 2006).

In 2012, ZHAO *et al.* (2012) reported the beneficial effect in *L. vannamei* growth fed β-glucans supplemented diets. Similar effects in growth improvement and feeding efficiency was registered for *Marsupenaeus japonicus,* when fed with a fucoidan supplemented diet, a polysaccharide extracted from the macroalgae *Undaria pinnatifida* (TRAIFALGAR *et al*., 2010).

The peritrophic membrane is an acellular layer, semipermeable, comprising chitin and protein that envelop the bolus and occur exclusively in the midgut. This membrane delimits the endoperitrophic space, where the bolus resides, and the ectoperitrophic space, between the peritrophic membrane and intestinal epithelium (TERRA, 2001).

In insects, the importance of this compartmentalization is shown by the endo-ectoperitrophic circulation of digestive enzymes and nutrients model proposed by FERREIRA *et al*. (1990). In this model, the enzyme-substrate complex is taken to the bolus until the substrate decreases its size, and then the enzymes and oligomers can pass through the peritrophic membrane pores and reach the ectoperitrophic space. There, a fluid counter flow pushes those compounds to the anterior region where it is absorbed and can again enter the endoperitrophic space, recycling the enzymes.

According to TERRA (2001) and BOLOGNESI *et al*. (2008), this endo-ectoperitrophic circulation model increases the digestion efficiency because it allows the enzymes reutilization and nutrient acquisition optimization. The weight gain data corroborate to what was found in the enzymatic gradient pattern.

In the polysaccharide extract supplementation concentrations where the enzymatic gradient was maintained, the animal weight gain was higher. In shrimp, this can be an insight into the fact that the endo-ectoperitrophic circulation assists in the diet nutrient digestion optimization (ALEXANDRE *et al*., 2014).

CONCLUSIONS

Dietary supplementation with crude extract of polysaccharides from microalgae *P. cruentum* seems to be a promising alternative option to increase the weight gain of the Pacific white shrimp *L. vannamei*. A supplementary concentration between 1 and 1.5% may be considered to maximize the weight gain and to maintain the enzymatic activity gradient along the midgut. The relationship between the endo-ectoperitrophic circulation existence and the digestion optimization in marine shrimp is a recent subject and, therefore, underestimated, which demands additional research for a better understanding on the matter.

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