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# PROTEIN HYDROLYSATE OF POULTRY BY-PRODUCT AND SWINE LIVER IN THE DIET OF PACIFIC WHITE SHRIMP*

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## ABSTRACT

This study aimed to evaluate the use of protein hydrolysate of poultry by-product and swine liver in the diet of *Litopenaeus vannamei* and its effect on the intestinal microbiota and on the enzymatic activity of the hepatopancreas. Shrimp (10.94 ± 0.90 g) were fed with diets containing 0%, 25%, 50%, 75% and 100% of replacement of salmon by-product meal by protein hydrolysate, in triplicate. The hepatopancreas enzymatic activity and composition of intestinal microbiota was studied. It was observed that the protein hydrolysate in the diet changed the enzymatic activity of the shrimp when compared to the control group ($p$ <0.05). Amylase activity increases directly with the percent of protein replacement in the diet. Metagenomic analysis revealed change in the gut biome of the shrimps. The increasing levels of protein replacement provided greater richness and diversity in gut microbiota in the 75% and 100% treatments, which were mainly related to changes in the abundances in the families Rhodobacteraceae and Flavobacteriaceae. A reduction in the abundance of the Vibrionaceae family was observed with the inclusion of protein hydrolysate in the diet. These results indicate that the protein hydrolysate demonstrated beneficial changes when added at concentrations of 25% in the diet of *L. vannamei*.

**Keywords:** *Litopenaeus vannamei*; animal by-products; digestive enzymes; metagenomics; nutrition.

## PROTEÍNA HIDROLISADA DE SUBPRODUTOS DE FRANGO E FÍGADO SUÍNO NA DIETA DO CAMARÃO-BRANCO-DO-PACÍFICO

## RESUMO

Este estudo teve como objetivo avaliar a utilização de hidrolisado proteico de subproduto de aves e fígado de suíno na dieta do *Litopenaeus vannamei* e seu efeito na microbiota intestinal e na atividade enzimática do hepatopâncreas. Camarões (10,94 ± 0,90 g) foram alimentados com dietas contendo 0%, 25%, 50%, 75% e 100% de substituição da farinha de subproduto de salmão pela proteína hidrolisada, em triplicata. A atividade enzimática do hepatopâncreas e a composição da microbiota intestinal foram estudadas. Observou-se que a proteína hidrolisada da dieta alterou a atividade enzimática do camarão quando comparado ao grupo controle ($p$ <0,05). A atividade da amilase aumentou diretamente com a porcentagem de reposição de proteínas na dieta. A análise metagenômica revelou mudança no bioma intestinal dos camarões. Os níveis crescentes de reposição proteica proporcionaram maior riqueza e diversidade no trato digestório nos tratamentos 75% e 100%, estando principalmente relacionadas a mudanças na abundância das famílias Rhodobacteraceae e Flavobacteriaceae. Uma redução na abundância da família Vibrionaceae foi observada com a inclusão do hidrolisado proteico na dieta. Esses resultados indicam que a proteína hidrolisada demonstrou alterações benéficas quando adicionada em concentrações de 25% na dieta do *L. vannamei*.

**Palavras-chave:** *Litopenaeus vannamei*; subproduto animal; enzimas digestivas; metagenômica; nutrição.

## INTRODUCTION

In aquaculture, most production success is related to the supply of balanced feeds with high nutritional value and good digestibility. The nutrients contained in some agro-industrial by-products have high nutritional value for animal feed, which supports a good quality diet (Villamil et al., 2017; Kim et al., 2018). Protein is an essential

nutrient for the development and metabolism of the organism, but it is one of the most limiting ingredients in the formulation of diets for shrimp and fish. However, protein hydrolysates have been considered a promising biotechnological solution for the feed industry for some years now (Nasri, 2016; Kim et al., 2018). We hypothesized that the combination of hydrolysates of poultry by-products and swine liver would improve the balance of essential amino acids and, hence, the nutritional value of protein hydrolysate produced to shrimp feed. The combination of different sources of protein in the same feed, can, in addition to improving the nutritional value, increase its digestibility by the animals that consume it (Castro et al., 2016).

Protein hydrolysates are produced using proteolytic enzymes which break the peptide bonds and generate ingredients with low molecular weight peptides having high absorption and digestibility (Zheng et al., 2012; Saadi et al., 2015). Low molecular weight peptides are considered bioactive compounds, and they can be characterized as having antioxidant and antimicrobial activity, in addition to improving immune system function and enzymatic modulation (Saadi et al., 2015; Wald et al., 2016; Hou et al., 2017; Verma et al., 2017). Intestinal health is determined by host (mucosal barrier, immunity), microbial, nutritional and environmental factors (Kelly and Salinas, 2017). Moreover, intestinal microbiota is directly associated with the production of digestive enzymes (Ramirez and Dixon, 2003; Izvekova, 2006; Kar and Ghosh, 2008; Li et al., 2018). Intestinal functions, such as digestion and absorption of nutrients, are achieved through microbial metabolism, which makes intestinal microbiota an essential component for the physiological homeostasis of the host (Li et al., 2018). The gut microbiota plays a critical role in the competitive exclusion of pathogenic bacteria in addition to generating essential elements for the host's metabolism (Tzuc et al., 2014). It is relevant to know how protein hydrolysates could affect the intestinal microbial communities in farmed shrimp species.

Shrimp digestive enzymes are produced by the hepatopancreas. Their production is directly related to feeding habits and the composition of the diet (Gamboa-Delgado et al., 2003). Variation in the activity of digestive enzymes in shrimp is a consequence of physiological and nutritional responses (Moullac et al., 1996). Thus, the study of digestive enzymes is a crucial step toward understanding the mechanisms of digestion and the nutritional needs of the species, thus helping those involved in aquaculture to formulate appropriate diets (Moullac et al., 1996; Li et al., 2018).

In this context, the present study aimed to replace salmon by-product meal with protein hydrolysate of poultry by-product and swine liver in the diet of *L. vannamei* shrimp and then determine the effects of such replacement on the composition of intestinal microbiota and enzymatic activity of the hepatopancreas.

## MATERIALS AND METHODS

### Biological and Ethics Statement

The test was carried out at the Marine Shrimp Laboratory of the Federal University of Santa Catarina (Florianópolis, SC, Brazil). The experimental rearing of *L. vannamei* shrimp and all diets used in the present work were the same as those described in Soares et al. (2020). The experimental units were 800 L tanks with seawater (34.74 ± 0.21 g $L^{-1}$).

This study does not need ethical approval according to Brazilian law once it is with invertebrates.

### Experimental design

Five diets were formulated using Optimal Formula 2000 software, and they were based on nutritional recommendations for the good performance of the species. The protein of salmon by-products meal was replaced in the formulation by the protein hydrolysate of poultry by-products and swine liver (Table 1). The treatments consisted of increasing replacement levels (0%, 25%, 50%, 75% and 100%), and they were carried out in triplicate, totaling 15 tanks.

The molecular weight of the protein fraction of the salmon by-product meal and the protein hydrolysates of poultry by-product and swine liver was determined by Nuclear Magnetic Resonance (NMR). The salmon by-product meal showed more than 97% of protein fraction with a molecular weight greater than 100 kDa (whole proteins), while the protein hydrolysate presented 91% of protein fraction with molecular weight lower than 1.2 kDa (peptides).

After six weeks of feeding with experimental diets, shrimp with an average weight of 10.94 ± 0.90 g were collected to perform the analyses described below. Analyses of enzymatic activity and protein electrophoresis were carried out at the University Marist of Mérida (Mérida, Yucatán, Mexico).

### Determination of enzymatic activity

To perform enzymatic analyses, the hepatopancreas of six shrimp (after 12 h of fasting) from each treatment were extracted, frozen in Falcon tubes at -80°C and subsequently lyophilized. The enzymatic extracts were obtained from 25 mg of hepatopancreas (pool of two shrimp per tank) dissolved in 1.5 mL of Tris-HCl (10 mM, pH 8.0). The mixture was centrifuged at 10,621 x g for 15 min at 4°C, and the supernatant was stored in microtubes at -20°C for the determination of enzymatic activity. The total protein concentration was determined according to the method of Lowry et al. (1951), using a visible light spectrophotometer (Multiskan, Thermo Scientific, Rockford, IL).

Amylase activity was determined by the method of Rick and Stegbauer (1974) and Villasante et al. (1999). Briefly, 250 µL of Tris-HCl (50 mM, pH 8.0) were mixed with 50 µL of enzymatic extract and 250 µL of 1% soluble starch (solubilized in Tris-HCl, 50 mM) and incubated for 10 min at 25°C. Then 250 µL of DNS

**Table 1.** Composition of the experimental diets and percentage of replacement of the protein by-product of salmon by protein hydrolysate of poultry by-product and swine liver.

| Ingredients (g kg$^{-1}$) | Protein Replacement (%) | | | | |
|---|---|---|---|---|---|
| | 0 | 25 | 50 | 75 | 100 |
| Wheat flour[1] | 150.0 | 150.0 | 150.0 | 150.0 | 150.0 |
| Soybean meal[2] | 380.0 | 380.0 | 380.0 | 380.0 | 380.0 |
| Cod liver oil[3] | 33.5 | 33.5 | 33.5 | 33.5 | 33.5 |
| Carboxymethyl cellulose[4] | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Soybean lecithin[5] | 21.0 | 21.0 | 21.0 | 21.0 | 21.0 |
| Monocalcium phosphate[4] | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 |
| Soybean oil[6] | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Vitamin C[4] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Vitamin premix[7] | 3.6 | 3.6 | 3.6 | 3.6 | 3.6 |
| Mineral premix[8] | 17.0 | 17.0 | 17.0 | 17.0 | 17.0 |
| Magnesium sulfate[4] | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Kaolin[9] | 114.4 | 114.4 | 114.3 | 114.0 | 113.8 |
| Sodium chloride[4] | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| PHPPL[10] | 0.0 | 50.0 | 100.0 | 150.0 | 200.0 |
| Fishmeal[11] | 200.0 | 150.0 | 100.0 | 50.0 | 0.0 |
| Potassium chloride[4] | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| DL-methionine[12] | 0.0 | 0.0 | 0.1 | 0.4 | 0.6 |
| **Centesimal composition (dry matter g kg$^{-1}$)** | | | | | |
| Dry matter | 873.5 | 890.1 | 913.6 | 923.8 | 944.8 |
| Crude protein | 365.0 | 364.9 | 362.3 | 364.9 | 361.5 |
| Digestible protein | 322.4 | 324.0 | 325.6 | 327.2 | 328.8 |
| Gross energy (kcal kg$^{-1}$) | 3,948 | 4,029 | 4,059 | 4,118 | 4,117 |
| Digestible energy (kcal kg$^{-1}$) | 3,278 | 3,291 | 3,304 | 3,317 | 3,330 |
| Ether extract | 106.9 | 106.6 | 107.8 | 114.2 | 116.6 |
| Ash | 238.5 | 232.7 | 226.6 | 218.1 | 212.6 |
| NFE[13] | 133.3 | 162.0 | 193.1 | 201.8 | 232.5 |
| Crude fiber | 29.8 | 23.9 | 23.8 | 24.8 | 21.6 |
| Phosphorus | 13.3 | 13.1 | 13.6 | 13.5 | 13.7 |

[1] Moinhos Cruzeiro do Sul S/A (Canoas, Brazil). [2] Nicoluzzi Rações Ltda. (Penha, Brazil). [3] Distributed by Química Delaware Ltda. (Porto Alegre, Brazil) and manufactured by Berg Lipid Tech (Aalesund, Noruega). [4] Labsynth Produtos para Laboratórios Ltda (Diademas, Brazil). [5] Quimidrol Com. Ind. Imp. Ltda (Joinville, Brazil). [6] Bunge Alimentos (São Paulo, Brazil). [7] InVivo mix (Paulínia, Brazil) – Vitamin premix: vitamin A: 3,000,000 IU; vitamin $D_3$: 1,000,000 IU; vitamin E: 70,000 IU; vitamin $K_3$: 14 g; vitamin $B_1$: 30 g; vitamin $B_2$: 20 g; vitamin $B_6$: 33 g; vitamin $B_{12}$: 50,000 µg; pantothenic acid: 40 g; biotin: 750 mg; nicotinic acid: 70 g; folic acid: 3,000 mg; excipient q.s.p.: 1,000 g. [8] InVivo mix (Paulínia, SP, Brazil) – Mineral Premix: Potassium: 6,100 mg; copper: 23,330 mg; zinc: 10,000 mg; manganese: 20,000 mg; selenium: 125 mg; iodine: 1,000 mg; cobalto: 50 mg; excipient q.s.p.: 1,000 g. [9] Rhoster Indústria e Comércio Ltda. (Araçoiaba da Serra, SP, Brazil). [10] Protein hydrolysates from poultry by-product and swine liver manufactured by BRF S.A. (Curitiba, PR, Brazil). [11] Salmon by-product meal protein - Tectron Imp. e Exp. de Prod. Veterinários Ltda. (Toledo, PR, Brazil). [12] Evonik Degussa Brasil Ltda. (Guarulhos, SP, Brazil). [13] Nitrogen-Free Extract = Dry matter - (crude protein + mineral matter + ether extract + fiber).

acid were added, and the mixture was placed in water for 5 minutes at 100°C. Maltose (1 mg mL$^{-1}$) was used as a positive control and 50 mM Tris-HCl as a negative control. After the reaction, the absorbance was measured at 541 nm (Multiskan, Thermo Scientific, Rockford, IL). The units of amylase activity were calculated using the molar extinction coefficient ε = 4950 cm$^{-1}$ M$^{-1}$.

Trypsin and chymotrypsin activities were based on the method of Geiger and Fritz (1988), using the respective substrates BAPNA (96 mM) and SAPNA (19.2 mM) solubilized in DMSO and diluted 1:17 in Tris-HCl (Erlanger et al., 1961). The enzymatic extract (10 µL) was mixed with 170 µL of Tris-HCl (60 mM, pH 8.0), 10 µL of $CaCl_2$ (192 mM, solubilized in 60 mM Tris-HCl, pH 8.0)

and 10 µL of substrate. After the reaction, the absorbance was measured at 405 nm (Multiskan, Thermo Scientific, Rockford, IL) until the equilibrium point. Trypsin and chymotrypsin units were calculated using $\varepsilon = 8800\ cm^{-1}\ M^{-1}$.

Aminopeptidase activity was based on Marouax et al. (1973). Fifty µL of enzymatic extract were mixed with 950 µL of 10 mM p-nitroanilide leucine (solubilized in 80 mM sodium phosphate, pH 7.2) and left to react at 37°C for 10 min. Then, 200 µL were transferred to a flat-bottomed 96-well microplate, and the absorbance was measured at 405 nm, until the equilibrium point. The aminopeptidase units were calculated using $\varepsilon = 8200\ cm^{-1}\ M^{-1}$.

To determine acid protease, 50 µL of the enzyme extract were mixed with 1 mL of 0.5% hemoglobin in glycine-HCl (100 mM, pH 3.1) and maintained at 25°C for 10 min. Subsequently, 500 µL of trichloroacetic acid (TCA, 20%) were added and kept at 25°C for an additional 10 min to stop the reaction. The samples were then incubated at 4°C for 10 min and then centrifuged at 10, 621 x g for 5 min at 4°C. The absorbance reading was performed at 405 nm. One unit (U) of activity corresponded to the amount of enzyme that catalyzes the formation of 1 µg of tyrosine per minute per milligram of protein, using $\varepsilon = 8200\ cm^{-1}\ M^{-1}$.

To determine alkaline protease activity, 240 µL of the enzymatic extract were mixed with 200 µL of 5% casein solution (solubilized in 100 mM Tris-HCl, pH 8.0) and incubated at 37°C for 1 h. Then, 240 µL of TCA solution (10%) were added and kept at 25°C for 15 min. Subsequently, the solution was centrifuged at 10, 621 x g for 20 min at 4 °C, and 100 µL of the supernatant were mixed with 93 µL of NaOH (100 mM). Then, 180 µL were transferred to a flat-bottomed 96-well microplate, and the absorbance was measured at 405 nm. The units of activity of alkaline protease were calculated using $\varepsilon = 8200\ cm^{-1}\ M^{-1}$.

Units of enzymatic activity of all enzymes were expressed in units per milligram of soluble protein (U min $mg^{-1}$) based on the methodology described by Alvarez-González (2003).

## Characterization of enzymes using SDS-PAGE

Polyacrylamide gels were prepared with Sodium Dodecyl Sulfate (SDS-PAGE) containing 4% polyacrylamide in storage gel and 12% in separation gel (Laemmli, 1970; García-Carreño et al., 1993). The samples were prepared in microtubes by adding 25 µL of enzymatic extract (0%, 25%, 50%, 75% and 100%) in 25 µL of Laemmli buffer (0.25 M Tris-HCl, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue (4% SDS, pH 6.8). Two µL of albumin were used as a positive control. The molecular marker Precision Plus Protein™ WesternC™ (Bio-Rad, USA) was used to assist in identifying the molecular weights of the samples. After denaturation (5 minutes at 100°C), samples were loaded onto the gel using 15 µL of sample for each treatment, 15 µL of positive control and 10 µL of molecular marker. Electrophoresis was carried out in the Bio-Rad PowerPac 300 chamber (Hercules, California, USA) at 100 V for 420 minutes at 4°C, and Tris base (25 mM, glycine; 192 mM, SDS 0.1%) was used as a run buffer.

Alkaline proteases were identified by immersing the gel at 4°C for 30 minutes in a 1% casein solution containing 50 mM Tris-HCl, pH 9.0. After washing with distilled water, a second bath was performed in the same solution for 1:30 h at 37°C and rinsed with TCA (12%). Staining was carried out in Coomassie's solution (0.25% Coomassie Brilliant Blue R-250, 50% methanol, 10% glacial acetic acid and 40% water) with constant stirring for 30 min. A mixture of distilled water (67.5%), acetic acid (7.5%), and methanol (25%) was used as a bleaching solution.

To detect acid proteolytic activity, the gel was immersed in a 0.1 M HCl solution until it reached pH 2 and incubated at 4°C in a 25% hemoglobin solution in 0.1 M glycine-HCl pH 2 buffer for 30 minutes and for more 1: 30 h at 37°C in a similar solution. Finally, the gel was washed successively with distilled water and TCA solution (12%). Band staining was performed in the same way as that described for the alkaline proteases.

## Gut Collection and Genomic DNA Extraction

The midgut of 15 animals per treatment was sampled (after 12 h of fattening), pooled and analyzed (one combined single pool per treatment). The intestinal content (feces) of each sample was collected, stored individually in free DNase and RNase microtubes and immediately frozen at -20°C for subsequent DNA extraction.

Sequencing analysis was performed by Neoprospecta Microbiome Technologies (Florianópolis, SC, Brazil), using the Illumina sequencer MiSeq. The technique was based on sequencing the 16S rRNA gene by amplifying the V3-V4 regions using 314F / 806R primers (Wang and Qian, 2009; Caporaso et al., 2011). The DNA concentration of the amplicon pool was estimated with Picogreen dsDNA assays (Invitrogen, USA), and then the pooled libraries were diluted for accurate quantitation of qPCR using the KAPA Library Quantification Kit by Illumina platforms (KAPA Biosystems, Woburn, MA). The library bank was adjusted to a final concentration of 11 pM and sequenced in the MiSeq system, using the Illumina primers provided in the kit. A 300 nt single-ended run was performed using a V2x300 sequencing kit.

## Data and statistics analysis

Protein concentration in the hepatopancreas and enzymatic activity was analyzed by ANOVA (after the verification of homoscedastic and homogeneity of variance assumptions) supplemented by Tukey test at significant level of 0.05.

Canonical variant analysis (CVA) was used to identify differences among treatments based on the interaction of all enzymatic data for each treatment. The significant effect for interactions was represented by Wilks' multivariate statistical test ($p < 0.05$). Statistical analyses were performed using the XLStatistcs for CVA.

The sequences representing the abundance of Operational Taxonomic Units (OTU) in the digestive tract of the animals were analyzed using USEARCH (version 10.0.240) at 97% similarity with the UPARSE algorithm, and for the taxonomic attribution, we used the SILVA database (version 132) at 91% identity. The rarefaction

curve and the diversity indices (Richness, Chao, Inverse Simpson e Shannon) were calculated using the vegan R package. The "good coverage" index was calculated using the QsRutils R package.

## RESULTS

### Enzymatic activity

Total replacement of the salmon by-products meal by the protein hydrolysate of poultry by-product and swine liver decreased the total protein concentration of hepatopancreas, when compared to treatments with 0%, 25%, 50% and 75% of replacement ($p$ <0.05). The activity of amylase, proteases, trypsin, chymotrypsin, and aminopeptidase had no significant effect ($p \geq 0.05$) on the differences among treatments (Table 2).

The CVA showed that the enzymatic activity of different treatments was completely different from that of the control group (Figure 1). The greatest similarity was observed in the 75% and 100% replacement treatments. The enzymatic activity of the 25% treatment was completely different from that of both the 75% and 100% treatments. The differences among treatments could be attributed to amylase activity, which explained 62.69% of the variation, followed by the activity of acid proteases (23.15%; Table 3).

**Table 2.** Enzymatic activity of the shrimp hepatopancreas, *Litopenaeus vannamei*, with diets at different substitution levels (0%, 25%, 50%, 75% and 100%) of salmon by-product meal by protein hydrolysates from poultry by-product and swine liver.

| Parameters | Protein Replacement (%) | | | | |
|---|---|---|---|---|---|
| | 0 | 25 | 50 | 75 | 100 |
| Protein (mg mg$^{-1}$ of tissue) | 73.92±4.39$^{a}$* | 76.32±4.32$^{a}$ | 75.32±0.61$^{a}$ | 68.76±2.32$^{a}$ | 64.64±2.83$^{b}$ |
| Trypsin (U mg$^{-1}$ of protein) | 0.0102±0.0048 | 0.0155±0.0063 | 0.0163±0.0052 | 0.0329±0.0016 | 0.0285±0.0193 |
| Chymotrypsin (U mg$^{-1}$ of protein) | 0.0213±0.0048 | 0.0250±0.0191 | 0.0097±0.0039 | 0.0060±0.0024 | 0.0113±0.0054 |
| Aminopeptidase (U mg$^{-1}$ of protein) | 0.0483±0.0141 | 0.0567±0.0062 | 0.0566±0.0233 | 0.0418±0.0071 | 0.0291±0.0087 |
| Amylase (U mg$^{-1}$ of protein) | 0.0683±0.0057 | 0.0604±0.0010 | 0.0637±0.0005 | 0.0677±0.0026 | 0.0694±0.0021 |
| Acid proteases (U mg$^{-1}$ of protein) | 1.5337±0.2898 | 1.0800±0.0574 | 1.1605±0.0924 | 1.1786±0.1277 | 1.0465±0.3064 |
| Alkaline proteases (U mg$^{-1}$ of protein) | 0.0504±0.0066 | 0.0537±0.0119 | 0.0567±0.0130 | 0.0706±0.0230 | 0.0633±0.0113 |

*Different letters indicate difference by Tukey test ($p$ <0.05).

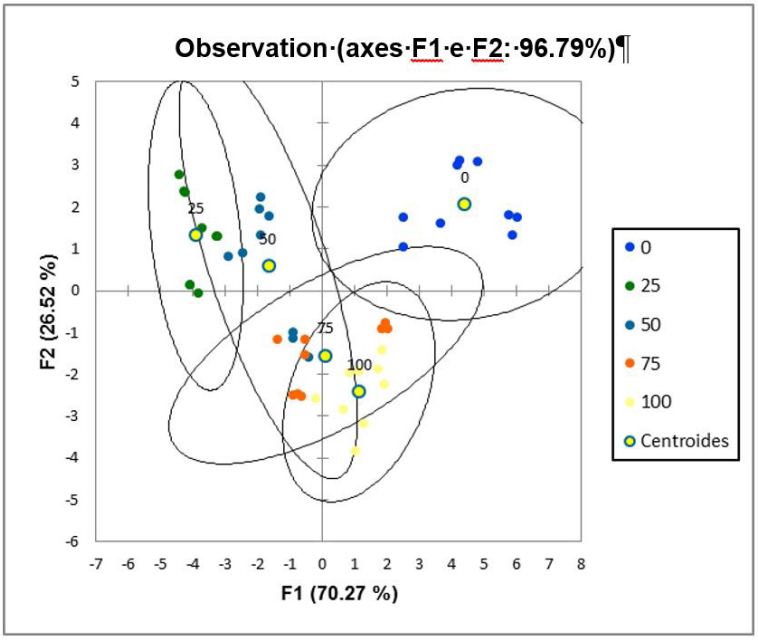


**Figure 1.** Canonical variant analysis (CVA) of the hepatopancreas enzymatic activity of the shrimp, *Litopenaeus vannamei*, fed different levels of protein substitution (0%, 25%, 50%, 75% and 100%). *Points represent the result of the CVA regression for each treatment. Eigen values are shown in parentheses.

**Table 3.** Discriminant variables of the Canonical variant analysis (CVA) based on the activity of five enzymes of the shrimp hepatopancreas, *Litopenaeus vannamei* as a function of diets with protein replacement.

| Enzymatic activity | Variance explained (%) | F | Pr > F | Wilk's lambda | *Pr < Lambda |
|---|---|---|---|---|---|
| Amylase | 62.69 | 12.41 | <0.0001 | 0.446 | <0.0001 |
| Acid proteases | 23.15 | 16.65 | <0.0001 | 0.165 | <0.0001 |
| Trypsin | 8.30 | 16.99 | <0.0001 | 0.059 | <0.0001 |
| Aminopeptidase | 3.48 | 12.79 | <0.0001 | 0.025 | <0.0001 |
| Chymotrypsin | 2.37 | 4.22 | 0.007 | 0.017 | <0.0001 |

SDS-PAGE electrophoresis showed three main protein bands with molecular weight of 150, 100 and 50-37 kDa (Figure 2-A). The protein bands of the hepatopancreas enzyme extracts were similar in all treatments (Figure 2-A). The alkaline proteases showed a pattern similar to that of all treatments, appearing as smooth bands between 100 and 150 kDa (Figure 2-B). The protein profile revealed the absence of the 50 -37 kDa band in the treatment with 100% protein substitution (Figure 2-C).

## Diversity of the intestinal microbiota

After quality filtering, trimming and chimera removal, a total of 902,360 sequences (74% of the original dataset; median of 225,590 reads per sample) were grouped into 333 Operational Taxonomic Units (OTUs). The rarefaction curve (Figure 3) and Goods coverage index (min = 99.96 and max = 100.00) reflected an almost complete coverage of the sampled microbial community.

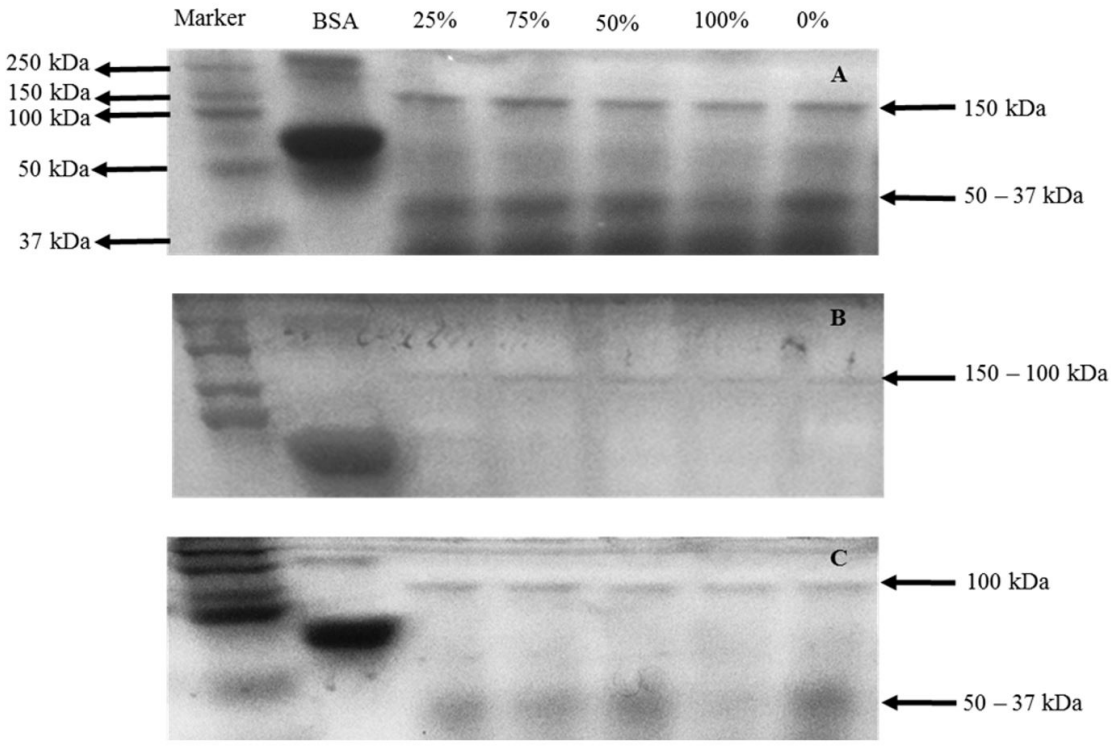


**Figure 2.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of the shrimp hepatopancreas enzymes *Litopenaeus vannamei*, fed different replacement levels (0%, 25%, 50%, 75% and 100%) of the salmon by-product meal protein by the protein hydrolysates from poultry by-product and swine liver. (A) total protein size profile; (B) alkaline proteases activity; (C) acid proteases activity. Bovine Serum Albumin (BSA) was used as positive control.

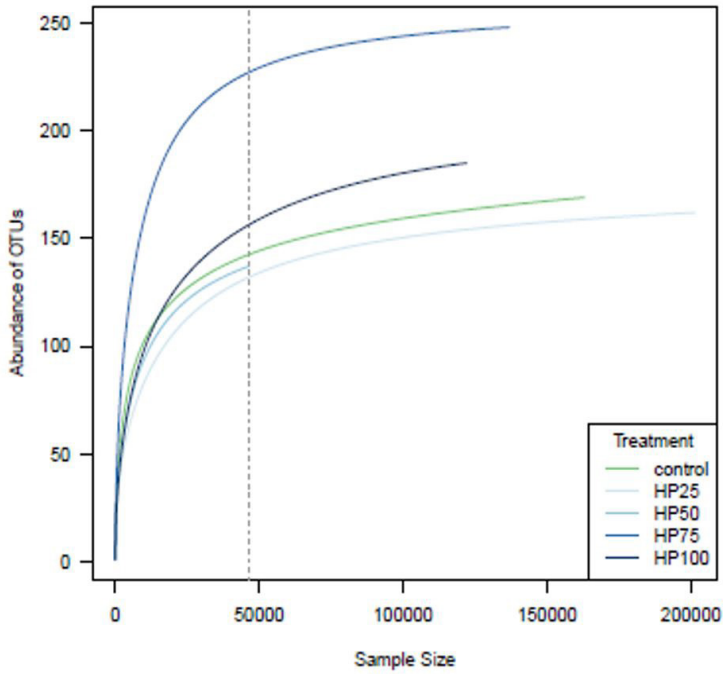


**Figure 3.** Rarefaction curves of the gut microbiome of the shrimp, *Litopenaeus vannamei*, fed different replacement levels (0% - control, 25%, 50%, 75% and 100%) of the protein of salmon by-products meal by the protein hydrolysates from poultry by-product and swine liver (HP). OTU - Operational Taxonomic Units.

The richness, as calculated by the Chao and Ace indexes, was higher in the gut bacterial communities of shrimp fed a diet with 75% of protein substitution, followed by 100% (Table 4). According to the Shannon and Simpson indices, the diversity of the bacterial communities was similar among the treatments. However, it was possible to observe less diversity in animals fed 100% protein substitution.

Multidimensional scaling (MDS) showed that the gut biome of shrimp feed with a diet of 75% replacement differed substantially from that of the other treatments. In fact, the 25%, 50%, and 100% treatments had a microbial profile similar to each other, but the control (0%) showed a profile different from all replacement levels (Figure 4).

The phylum Proteobacteria showed the highest relative abundance of all treatments, being more representative in the 75% replacement treatment (65%) and less representative in the 25% replacement treatment (49%). In descending order of relative abundance, the other dominant bacterial phyla were Bacteroidetes, Verrucomicrobia, and Actinobacteria. The phyla Bacteroidetes and Actinobacteria showed behavior opposite from that of Proteobacteria, with more abundance in the 25% treatment (41% and 4%, respectively) and less in the 75% treatment (30% and 2%, respectively) (Figure 5-A).

Considering family abundance, the five most abundant bacterial families were Cellulomonadaceae (Phylum Actinobacteria), Rubritaleaceae (Phylum Verrucomicrobia), Thiotrichaceae (Phylum

**Table 4.** Richness and diversity indices of the microbiota of the shrimp *Litopenaeus vannamei* fed with different levels of protein substitution (0%, 25%, 50%, 75% and 100%).

| Treatment | Richness | Shannon | Simpson | Chao | ACE | Good's coverage |
|---|---|---|---|---|---|---|
| 0% | 175 | 2.25 | 5.94 | 174.06 | 175.78 | 100 |
| 25% | 175 | 2.11 | 5.73 | 165.10 | 173.85 | 100 |
| 50% | 145 | 2.05 | 5.15 | 163.07 | 159.27 | 99.96 |
| 75% | 259 | 2.36 | 5.65 | 267.83 | 266.38 | 100 |
| 100% | 193 | 1.95 | 4.67 | 232.25 | 220.92 | 100 |

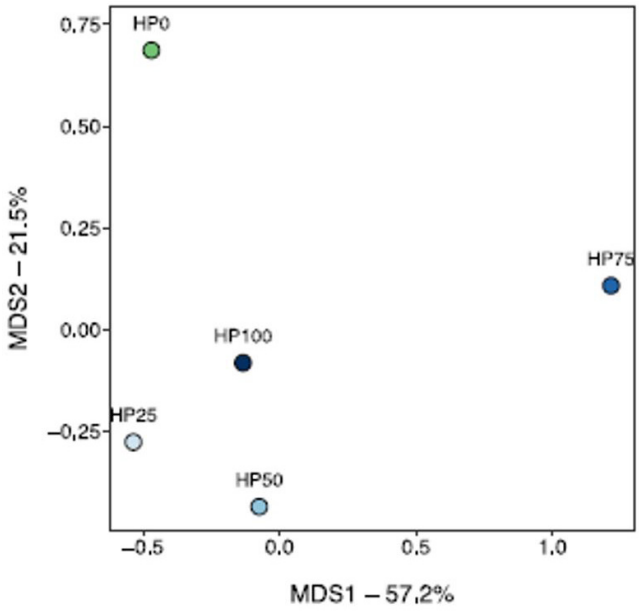


**Figure 4.** Multidimensional scaling plot of the intestinal microbiota of the shrimp, *Litopenaeus vannamei*, fed different replacement levels of salmon by-product meal by protein hydrolysates from poultry by-product and swine liver (0%, 25%, 50%, 75% and 100%), assigned as HP0, HP25, HP50, HP75 and HP100, respectively.

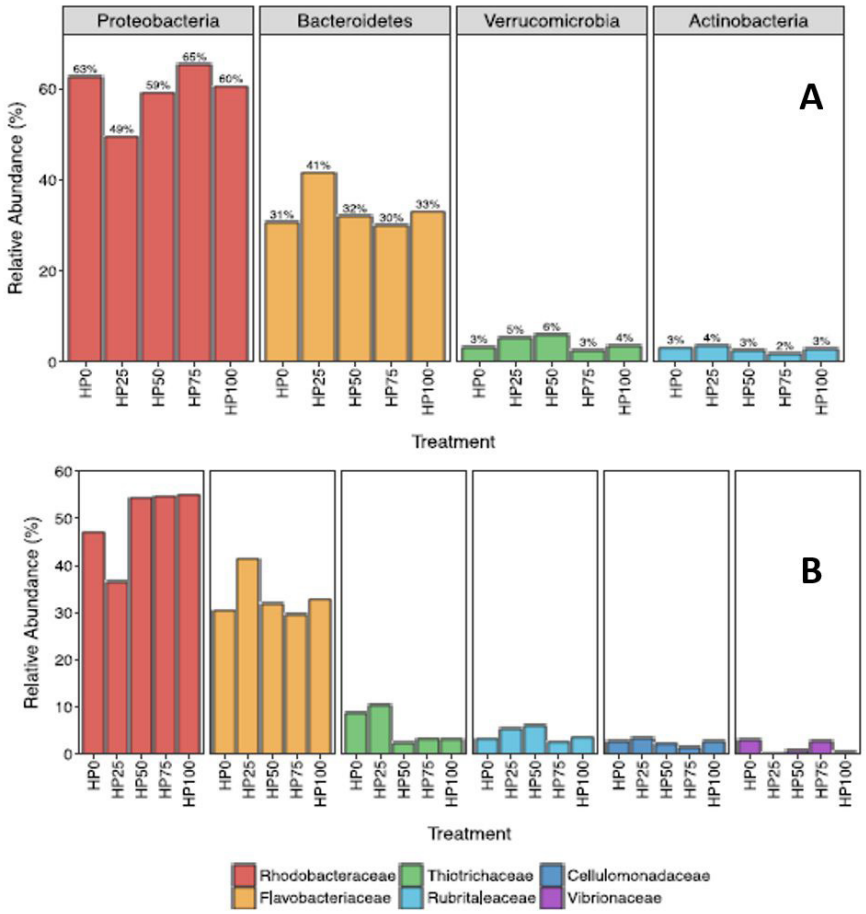


**Figure 5.** Relative abundance of bacterial phyla (A) and families (B) identified in the intestinal microbiota of shrimp, *Litopenaeus vannamei*, fed diets different replacement levels of salmon by-product meal by the protein hydrolysates from poultry by-product and swine liver (0%, 25%, 50%, 75% and 100%), assigned as HP0, HP25, HP50, HP75 and HP100, respectively.

Proteobacteria), Flavobacteriaceae (Phylum Bacteroidetes), and Rhodobacteraceae (Phylum Proteobacteria) in all treatments (Figure 5-B). The Thiotricaceae family was more abundant in treatments with lower replacement levels (25% and 0%), while the Rhodobacteraceae family was more abundant in the treatments with higher replacement levels (50%, 75%, and 100%). The Vibrionaceae family was not identified in treatments with 25% and 100% of protein substitution and had low abundance in the 50% treatment.

The most abundant genera were *Pseudoruegeria* and *Ruegeria* (family Rhodobacteriaceae) and *Formosa* and *Actibacter* (family Flavobacteriaceae) (Figure 6). Among these, the genera *Actibacter* and *Pseudoruegeria* decreased in abundance as dietary replacement increased. The opposite behavior was observed in all treatments for the genus *Formosa*. The genus *Ruegeria*, on the other hand, suffered a reduction in its abundance in the 25% treatment, while in treatments with higher levels of protein substitution (50%, 75%, and 100%), it presented a substantial increase. Additionally, a small increase in the concentration of *Donghicola* (family Rhodobacteraceae) was observed in the 75% and 100% treatments. The Legionellaceae family was observed only in the control treatment (0%).

## DISCUSSION

The results of our study showed that the composition of protein hydrolysate influenced the enzymatic activity of shrimp. It is likely that the composition of low molecular weight peptides and free amino acids present in the hydrolysates is the cause variation in the enzyme profiles. A similar result was observed in *L. vannamei* fed diets containing different sources of protein hydrolysates (fish and krill) and replacement levels of 3%, 9%, and 15%. The total proteolytic activity showed significant differences between control (commercial diet) and some of the inclusion levels of protein hydrolysates (Cordova-Murueta and García-Carreño, 2002). A later result confirmed the influence of the dietary composition on the enzymatic activity of *L. vannamei* (Cordova-Murueta et al., 2003).

In the present work, the activity of amylase was affected by the replacement levels of protein hydrolysate in the diet (Table 3). It is likely that the increase in activity was a consequence of the availability of amino acids and the increase in the concentration of carbohydrates (133.3 to 232.5 g $kg^{-1}$) in the experimental diets. Studies carried out in *Litopenaeus stylirostris* showed that the increase in carbohydrates in the diet (1%, 10%, 21%, and 33%) stimulated the activity of α-amylase and α-glucosidase

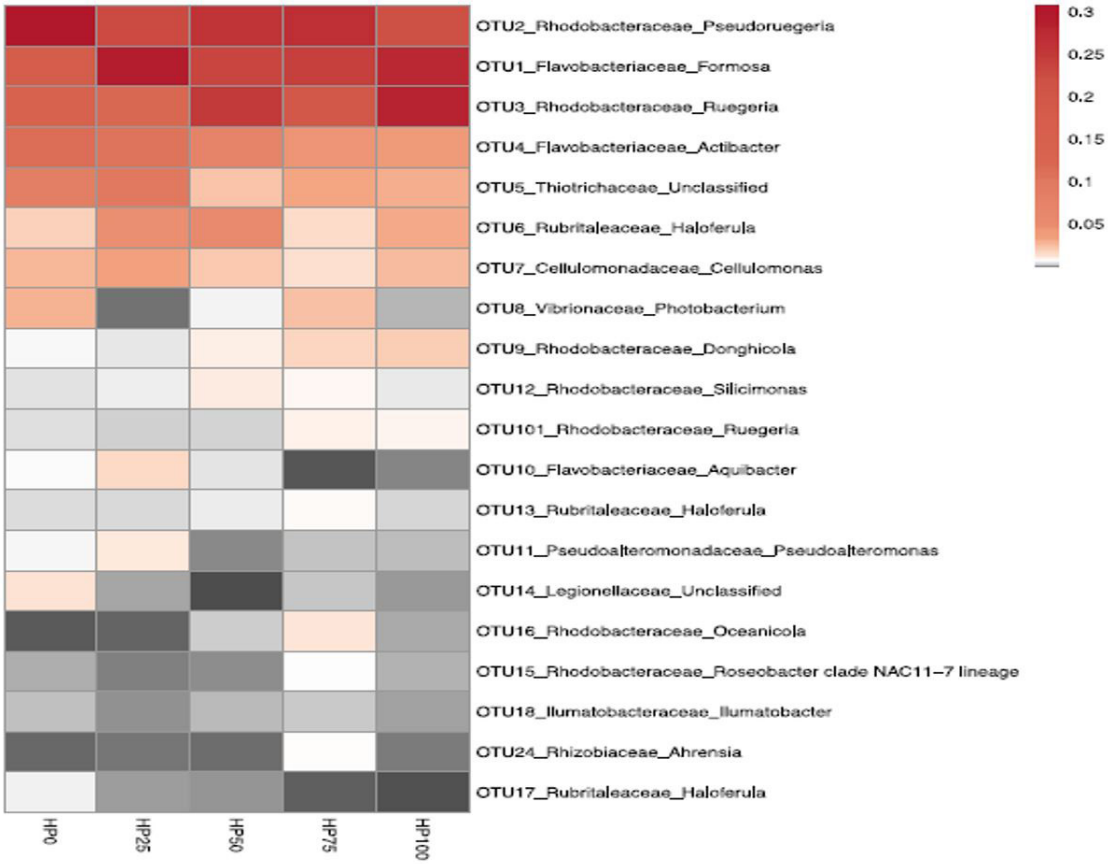


**Figure 6.** Heatmap of the 20 most abundant operational taxonomic units (OTUs) identified in the intestinal microbiota of shrimp, *Litopenaeus vannamei*, fed different replacement levels (0%, 25%, 50%, 75% and 100%), assigned as HP0, HP25, HP50, HP75 and HP100, respectively.

in the hepatopancreas, observing their maximum activity with 21% inclusion (Rosas et al., 2000). Another possibility is that the difference of dry matter in the diets affected the amylase activity once the dry matter increased with the substitution of the protein source.

The results of enzymatic physiological behavior, as determined in the present work, may be related to the degree of hydrolysis of the evaluated protein sources which conferred distinct functional characteristics on the proteins. NMR revealed that the protein hydrolysate had peptides of lower molecular weight (<1.2 kDa) compared to from salmon by-products meal (>100 kDa). The presence of free amino acids or small polypeptides in food can reduce the substrate for enzymes, mainly endopeptidases, especially if the specific enzyme binding sites along the protein chain have been previously hydrolyzed (Cordova-Murueta and García-Carreño, 2002). In another study, when *L. vannamei* shrimp were fed different sources of protein hydrolysates (krill and fish) with different molecular weights and at different levels of inclusion in the diet (3%, 9% and 15%), less activity was observed for trypsin, chymotrypsin, and total protease (Cordova-Murueta et al., 2003).

In addition, the low molecular weight peptides present in the protein hydrolysate may have acted as functional ingredients, acting in a manner similar to that of hormones and, hence, regulating enzymatic activity of the shrimp. Effects of specific hormone-like peptides derived from hydrolyzed proteins have been associated with the regulation of gastrointestinal motility, endocrine metabolism, and ingestion, positively affecting animal performance (Martínez-Alvarez, 2013). However, more studies need to be carried out to understand the effect of protein hydrolysates on the physiology of shrimp.

Shrimp fed diets with 25% and 50% of protein substitution displayed a similar enzymatic behavior (Figure 1) that correlated with improved growth performance of these animals, as reported in our previous study (Soares et al., 2020). The growth of shrimp increased with 25% protein substitution, even while growth with the 50% treatment remained similar to that of control. Both treatments, however, showed good results for growth. Shrimp can regulate the enzymatic digestive system according to their diet, and digestive enzymes can be induced in response to growth or in an attempt to provide more nutrients in a deficient diet (Moullac et al., 1996; Gamboa-Delgado et al., 2003). Hernández et al. (2011) reported that the inclusion of protein hydrolysate of tuna by-products improved the digestibility of diets containing pork meat flour as the main protein source, and, consequently, caused better growth performance of *L. vannamei*.

Electrophoresis analysis confirmed the enzymatic activity in the evaluated treatments through the molecular weights observed in the enzymatic extracts. Molecular weights are similar to those previously reported for enzymes from *L. vannamei* and *Farfantepenaeus paulensis* (Hernandez-Cortés et al., 1997; Lemos et al., 2000). However, owing to the diversified pattern of proteinases among the peneid shrimp species (*Farfantepenaeus californiensis*, *L. vannamei*, *F. paulensis* and *Litopenaeus schmitti*), different properties related to the digestion of the protein fraction can be found for each species (Lemos et al., 2000). Studies show that bands between 24 and 30 kDa can be associated with chymotrypsin in *L. vannamei* (Lemos et al., 2000; Muhlia-Almazán and García-Carreño, 2002; Cordova-Murueta et al., 2003), as also verified in our study.

In this study, differences in the protein profile between protein sources may have influenced the diversity of intestinal microbiota among treatments. Protein hydrolysis generates a product containing free amino acids and low molecular weight peptides (Nguyen et al., 2012), both important to produce microbial peptone (Kristinsson and Rasco, 2000). Therefore, protein hydrolysates are essentially used in culture media to maintain the growth of different microorganisms (Chalamaiah et al., 2012; Safari et al., 2012; Villamil et al., 2017).

Actinobacteria and Bacteroidetes phyla increased in the treatment with 25% of protein substitution, while the Proteobacteria phylum decreased in this treatment. The opposite was observed for the 75% protein substitution treatment, suggesting that a certain level of inclusion of the protein hydrolysate influenced the abundance of the main bacterial phyla identified. Similar behavior was observed in *L. vannamei* when fed diets containing different seaweed species. It was observed that specific seaweeds influenced the increase in Bacteroidetes and the reduction of Proteobacteria in the shrimp intestine (Niu et al., 2019). Some bacterial families, namely Enterobacteriaceae and Aeromonadaceae, both belonging to Gammaproteobacteria class of Proteobacteria phylum, are generally associated with intestinal dysbiosis by reducing the absorption capacity of nutrients (Rigottier-Gois, 2013; Schippa and Conte, 2014).

A few glycolytic genes from bacteria like *Bacteroides eggerthii*, *Bacteroides cellulosilyticus*, *Bacteroides intestinalis*, *Bacteroides ovatus*, and *Bacteroides xylanisolvens* of the phylum Bacteroidetes can degrade dietary fiber in humans. However, it is known that carbohydrate degradation is less efficient in shrimp and fish (Wu et al., 2012; Zhang et al., 2014). According to Li et al. (2018), the lower abundance of Bacteroidetes in the shrimp intestine may be related to the low degradation of carbohydrates. However, in our study, Bacteroidetes was the second most abundant phylum in the shrimp gut, possibly attributed to the level of carbohydrates present in the replacement diets.

Rhodobacteraceae was the most abundant family in almost all treatments, followed by Flavobacteriaceae. The Rhodobacteraceae family consists of photosynthetic bacteria containing a variety of nutrients and functional substances that can work as bioremediatiors in aquaculture systems (Mata et al., 2017). They have also been reported to produce Polyhydroxybutyrate (PHB), which is associated with greater growth in aquatic animals, in addition to having a high capacity for tolerance to gastrointestinal transit, suggesting its potential as a probiotic in aquaculture (Zhou et al., 2007; Yamazaki et al., 2016). Also, strains of this family may have antagonistic activity which would limit the survival of such pathogenic bacteria as the *Vibrio* genus (Hjelm et al., 2004; Sharifah and Eguchi, 2011).

However, the 25% protein replacement treatment, together with the 50% and 100% treatments, showed the lowest abundance level of Vibrionaceae (0%, 1% and 0%, respectively). This may be related to the presence of antimicrobial peptides in protein hydrolysates (Verma et al., 2017). The Vibrionaceae family is

naturally present in marine aquatic environments and includes gram-negative bacteria considered opportunistic pathogens for shrimp in stressful situations, such as poor water quality, immune suppression, and malnutrition (Thompson et al., 2004; Xiong et al., 2016). In shrimp farming worldwide, this bacterial group has caused many diseases, leading to massive mortality and great economic losses (Restrepo et al., 2018). Thus, the use of protein hydrolysates in the shrimp diet could help to reduce the emergence of diseases caused by the Vibrionaceae family.

Bacteria of the family Flavobacteriaceae, the largest family of the phylum Bacteroidetes, seem to play an important role in the degradation and absorption of dissolved organic material as they can degrade biopolymers like cellulose and chitin (Kirchman, 2002; Abell and Bowman, 2005). Different strains of Flavobacteriaceae are also known for their ability to produce large amounts of carotenoids (Sowmya and Sachindra, 2015), as an important source of vitamins and antioxidants, providing health benefits (Nelson and Cox, 2011). The Thiotrechaceae family was identified in all treatments, albeit in higher abundance in the 25% and 0% treatments, respectively.

Although considered the most prevalent bacterial genera, *Pseudoruegeria* (Phylum Proteobacteria, Family Rhodobacteraceae) and *Actibacter* (Phylum Actinobcteria, Family Flavobacteriaceae) showed a reduction in their relative abundance with increasing levels of protein hydrolysate in the diet. Conversely, the genus *Formosa* (Phylum Bacteroidetes, Family Flavobacteriaceae) showed an increase in abundance. Bacteria of the genus *Formosa* have enzymatic activity involving the depolymerization of polysaccharides from marine algae species, thus serving as a carbon source (Silchenko et al., 2016; 2018). In addition, the *Ruegeria* genus increased in the 50%, 75% and 100% replacement treatments.

The protein hydrolysate evaluated in the present work may have some antimicrobial effect, possibly from the presence of antimicrobial peptides in the intestinal microbiota of the shrimps, altering it according to the level of protein hydrolysate included in the diet. Swine liver protein hydrolysates showed antimicrobial activity against gram-positive and gram-negative bacteria belonging to the phylum Proteobacteria and Firmicutes (Verma et al., 2017). Chakka et al. (2015) reported antimicrobial activity against *Micrococcus luteus*, but only by poultry liver hydrolysate, after enzymatic and fermentative hydrolysis of crude matter.

Antimicrobial peptides damage the cell membrane of bacteria, interfering with the functions of their intracellular proteins and affecting their metabolism (Osman et al., 2016; Hou et al., 2017). It is assumed that the mechanism of action may be related to cationic properties and the hydrophobic surface of the peptides, which interact easily with the anionic surface and cytoplasmic membrane of microorganisms, leading to a change in membrane permeability and lysis of microorganisms (Izadpanah and Gallo, 2005; Verma et al., 2017). Lower molecular weight peptides may have a higher rate of interaction with the cytoplasmic membrane than long-chain peptides or higher molecular weight proteins (Verma et al., 2017).

The intestinal microbiota has a metabolic function directly associated with the process of digestion and absorption of nutrients (Gao et al., 2019). In some studies, the genera *Pseudoalteromonas* and *Vibrio*, isolated from *L. vannamei*, were able to synthesize extracellular enzymes (amylase, lipase, and chitinase) important for digestive processes, demonstrating that the intestinal microbiota has the potential degrade components of the diet (Tzuc et al., 2014; Gao et al., 2019). In our study, the genus *Pseudoalteromonas* was found only in the 25% protein replacement treatment, which, together with the particular changes in the gut microbiome and enzymatic activity described above, may have improved the digestive process and, consequently, the growth performance of the shrimp, as reported in our previous work (Soares et al., 2020).

Treatments with 0%, 25%, and 50% protein substitution showed similar enzymatic activity and bacterial diversity. These reported findings suggest that the behavior of gut bacterial community and enzymatic activity are influenced by the protein source offered and, particularly, the level of inclusion in the diet, supporting the hypothesis driving this work.

## CONCLUSION

To conclude, our results provided a better understanding of the use of protein hydrolysate as a new ingredient in the diet of Pacific white shrimp. The protein hydrolysate of poultry by-product and swine liver induced beneficial changes in the enzymatic activity of the hepatopancreas and the intestinal microbiota of *L. vannamei*, such as increasing the activity of the enzyme amylase and reducing the population of the family Vibrionaceae, respectively. Based on our overall findings, we recommend a 25% replacement in the diet since that replacement level produced the best dynamics in the activity enzymatic and intestinal microbiota of the shrimps. Our findings also provide guidelines for those engaged in aquaculture looking to formulate nutritionally balanced diets with less dependence on fish meal for the Pacific white shrimp.

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