Growth Enhancing Effects of Squid By-Product Hydrolysate in Plant Protein-Based Diet Fed to Black Tiger Shrimp, *Penaeus monodon* Fabricius, 1798

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Abstract

The present study evaluated the potential of squid by-product hydrolysate as fishmeal replacement in the plant-based diet of juvenile black tiger shrimp, *Penaeus monodon* Fabricius, 1798. Five experimental plant protein-based diets were formulated containing squid by-product hydrolysate to replace fishmeal at 0, 25, 50, 75 and 100 %. These experimental diets were fed to triplicate groups of *P. monodon* for 8 weeks. Results revealed that 25 % fishmeal replacement with squid by-product hydrolysate resulted in growth enhancement, attributable to increased feed intake and gut lactic acid bacteria proliferation. The shrimp fed with 100 % replacement level exhibited a similar growth response with the control group. Protein retention was not affected by the fishmeal replacement levels, but lipid retention was found higher in all treatments with squid by-product hydrolysate than the control. Digestive protease activity decreased with increasing levels of hydrolysate while α-amylase and lipase activities were not affected. Hepatopancreas histology showed that B-cells dominated the control group while R-cells proliferated with increasing inclusion of dietary squid by-product hydrolysate. These results collectively indicate that fishmeal could be totally replaced with squid by-product hydrolysate and 25 % fishmeal replacement could promote the growth of juvenile *P. monodon*.

Keywords: fishmeal replacement, growth promotion, digestive enzymes, lactic acid bacteria

Introduction

Fishmeal is considered an ideal protein source in fish feed due to its high protein content, balanced essential fatty and amino acids profile, good nutrient digestibility, and highly available in mass quantity (Daniel, 2018). However, fishmeal supply has become erratic in recent years due to dwindling wild fish stocks and increasing demands, leading to price increases and supply shortages (Tantikitti, 2014). This situation leads to intensive efforts to develop alternative feed ingredients such as plant proteins for aquaculture feeds. Several authors have reported the potential of various plant ingredients to partially- or fully replace fishmeal in the diet of cultured organisms (Brinker and Reiter, 2011; Al-Thobaiti et al., 2018; Lazzarotto et al., 2018). However, the presence of anti-nutritional factors, deficiency in certain essential amino acids, low nutrient digestibility and poor palatability are issues that limit the use of plant ingredients in aquaculture feeds (Daniel, 2018).

Several strategies to overcome the problems associated with plant-based diets have been developed. Dietary and feeding interventions, including supplementation of essential amino acids, the combination of different plant proteins, application of exogenous enzymes, and mixed feeding schedules have been evaluated (Nandeesha et al., 2002; Aksnes et al., 2006; Liti et al., 2006; Sarker et al., 2007; Jiang et al., 2014). The inclusion of fish industry by-product hydrolysate has also been shown to enhance the feed value, palatability, and acceptability of plant protein-based diets for aquatic organisms.

Moreover, earlier works on protein hydrolysates produced from fish industry by-products have indicated that this material could significantly replace

fishmeal in aquaculture feeds (Hardy and Tacon, 2002; Naylor et al., 2009). These by-product hydrolysates have undergone specific enzymatic hydrolysis that improved their nutritional value and biological functionality (Chalamaiah et al., 2012; Kim and Venkatesan, 2014; Xu et al., 2017). Fish hydrolysates have been shown to influence growth, diet digestibility, plasma biochemical composition, intestinal morphology, innate immune responses and antioxidant enzyme activities, gene expression and disease resistance of the cultured species (Zheng et al., 2012; Khosravi et al., 2015; Li et al., 2018). These were also reported to support the growth and colonisation of beneficial lactic acid bacteria in the gut of cultured aquatic animals (Vazquez et al., 2008; Ringo et al., 2018). Furthermore, earlier works have provided evidence on the potential use of fish protein hydrolysate as a growth-promoting dietary supplement for cultured shrimp (Mendoza et al., 2001; Niu et al., 2014; Valle et al., 2015; Quinto et al., 2018).

At present, as the world oceans are experiencing climatic changes and as the global fish stocks decline due to overfishing, the rise of squid stocks has been found as an ecological consequence. The squid processing industry has recently become a lucrative investment and has expanded in terms of production volume and the number of new processors in a global context (Doubleday et al., 2016). Similar to the fish processing industry, the squid processing plants generate a significant amount of protein-rich waste by-products with potential for development as a feed ingredient for aquaculture.

Squid by-product hydrolysate as a feed additive has been previously documented to improve the performance of larval and juvenile fish (Lee et al., 2018). Several studies were also conducted using squid by-product hydrolysate as a supplement in experimental diets of the omnivorous shrimp, *Penaeus vannamei* Boone, 1931 (Gonzalez-Felix et al., 2014; Zhou et al., 2016). However, to date, there has been a scarcity of information regarding the feed value of squid by-product hydrolysate in cultured aquatic species, specifically, the carnivorous shrimp species that requires a higher amount of high-quality feed protein to achieve optimum growth.

Therefore, the present study evaluates the feed value and potential of squid by-product hydrolysate to replace fishmeal in plant-based diet of the juvenile black tiger shrimp, *Penaeus monodon* Fabricius, 1798.

Materials and Methods

Ethical statement

All the applicable guidelines (international, national, and/or institutional) on the care and use of animals during the conduct of the study were meticulously followed.

Chemicals and reagents

The enzyme bromelain (3000 gelatine-digesting units.g^{-1}) was procured from Doctor's Best, Inc. (California, USA). Most of the chemicals and reagents used for the enzyme assays and other analyses were analytical grades obtained from Sigma-Aldrich (Missouri, USA).

Squid by-products and hydrolysate

Squid by-products were procured from the local squid processing industry at Zamboanga, Philippines. The by-products that include heads, tentacles, fins, viscera, and skin, were cleaned of any debris, washed with distilled water, minced, weighed, and stored in a freezer until used.

To generate the hydrolysate, the squid by-products were enzymatically hydrolysed with bromelain following the methods described by Haslaniza et al. (2010) and Soufi-Kechaou et al. (2012). The enzymatic hydrolysis was initiated by adding bromelain to the homogenised squid material and the reaction was allowed to proceed for 4 h at 60 °C, pH 6.0 until the desired hydrolysis degree of 57.85 % was achieved. The protocol of Hoyle and Meritt (1994) was used to analyse the degree of hydrolysis. The hydrolysed squid biomass was dried to powder and stored at -30 °C until used.

Experimental feed preparation

A plant-based diet containing low levels of fishmeal at 15 % (40 % crude protein), serving as the basal diet was formulated following Alvarez et al. (2007) and Suarez et al. (2009). The basal diet was formulated to contain all essential fatty and amino acids to support all the dietary requirements of *P. monodon* for optimal growth. Additional four experimental diets were formulated with the replacement of fishmeal with squid by-product hydrolysate at increasing levels of 25 %, 50 %, 75 % and 100 %. The diet ingredients and proximate composition are presented in Table 1.

All dry ingredients were ground, sieved in 100 µm, weighed and mixed. Feed oil was added to the dry ingredients, mixed, and an appropriate amount of water was added. The moistened dough was pelletised in a low-pressure benchtop electric extrusion machine. The resulting pellet-strands were dried at 60 °C until the desired moisture content was reached. The dried pellets were pulverised and sieved to appropriate sizes. Diets were stored at 4 °C until used.

Experimental animals and acclimation

Black tiger shrimp were purchased from a private hatchery in Tigbauan, Iloilo and transported to the Institute of Aquaculture Multi-Species Hatchery, University of the Philippines Visayas, Miag-ao, Iloilo.

Table 1. Composition of the control and experimental diets (g.100 g^{-1} diet) for black tiger shrimp *Penaeus monodon*.

Ingredients	Control	Squid by-product hydrolysate inclusion levels			
		25 %	50 %	75 %	100 %
Fish meal	15.00	11.25	7.5	3.75	0.00
Squid by-product hydrolysate[a]	0.00	3.75	7.5	11.25	15.00
Wheat gluten	5.00	5.00	5.00	5.00	5.00
Soybean meal	25.00	23.12	21.24	19.36	17.48
Brewer's yeast	5.00	5.00	5.00	5.00	5.00
PECM®[b]	10.00	10.00	10.00	10.00	10.00
ProEn-K™[c]	25.00	25.00	25.00	25.00	25.00
Rice bran	6.00	7.88	9.76	11.64	13.52
Cholesterol	0.50	0.50	0.50	0.50	0.50
Soybean lecithin	0.50	0.50	0.50	0.50	0.50
Fish oil	6.00	6.00	6.00	6.00	6.00
Mineral mix[d]	1.00	1.00	1.00	1.00	1.00
Vitamin mix[e]	1.00	1.00	1.00	1.00	1.00
Proximate composition (n = 3)					
Moisture (g.100 g^{-1} diet)	10.79 ± 0.23	9.07 ± 0.20	10.33 ± 1.27	11.44 ± 0.01	11.31 ± 0.01
Dry matter (DM; g.100 g^{-1} diet)	21 ± 0.23	90.93 ± 0.20	89.67 ± 1.27	88.56 ± 0.01	88.69 ± 0.01
Crude protein (g.100 g^{-1} DM)	50.82 ± 0.40	50.70 ± 0.12	50.10 ± 0.69	50.90 ± 0.01	49.15 ± 0.16
Crude lipid (g.100 g^{-1} DM)	13.14 ± 0.20	12.89 ± 0.24	13.03 ± 0.03	13.41 ± 0.11	13.39 ± 0.37
Crude fibre (g.100 g^{-1} DM)	3.47 ± 0.06	3.72 ± 0.04	3.83 ± 0.32	3.64 ± 0.10	3.66 ± 0.07
Ash (g.100 g^{-1} DM)	15.05 ± 0.01	14.09 ± 0.29	15.04 ± 0.20	15.51 ± 0.28	16.80 ± 0.46
NFE[f] (g.100 g^{-1} DM)	17.52	18.6	18.00	16.54	17.00

[a]Composition of the squid by-product hydrolysate: crude protein, 79.85 ± 0.22 %; crude lipid, 5.25 ± 0.06 %; ash, 3.26 ± 0.05 %; moisture, 11.64 ± 0.06 %.

[b]Composition of PECM® (fermented copra meal): crude protein, 44.40 %; crude lipid, 3.90 %; crude fibre, 13.30 %; ash, 8.80 %; NFE, 29.60 %. Commercialised by National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, Philippines.

[c]Composition of ProEn-K™ (fermented sweet potato): crude protein, 40 %; crude lipid, 0.40 %; crude fibre, 4.20 %; ash, 9 %; carbohydrate, 46.40 % (Traifalgar et al., 2019). Commercialised by Plentex Philippines, Incorporated.

[d]Mineral mix contribution.100 g^{-1} of feed: Phosphorus, 480 mg; Calcium, 480 mg; Magnesium, 60 mg; Iron, 6 mg; Zinc, 16.8 mg; Copper, 8.4 mg; Potassium, 300 mg; Cobalt, 4.4 mg; Manganese, 6.4 mg; Selenium, 0.004 mg; Molybdenum, 0.002 mg; Aluminium, 0.1 mg; Iodine, 1.6 mg.

[e]Vitamin mix contribution.100 g^{-1} of feed: β-carotene, 1.8 mg; Cholecalciferol, 0.15 mg; Thiamine, 3.6 mg; Riboflavin, 7.2 mg; Pyridoxine, 6.6 mg; Cyanocobalamin, 0.02 mg; α-tocopherol, 16.5 mg; Menadione, 2.4 mg; Niacin, 14.4 mg; Pantothenic acid, 4 mg; Biotin, 0.02 mg; Folic acid, 1.2 mg; Inositol, 30 mg, Stay C, 100 mg.

[f]Nitrogen-free extract (NFE) = 100 - (crude protein + crude lipid + crude fibre + ash).

The experimental shrimp were tested for the presence of disease-causing agents and were found negative for white spot syndrome virus, infectious hypodermal and hematopoietic necrosis virus, monodon baculovirus and other pathogenic Vibrios.

The shrimp were acclimated in culture tanks and were maintained with the commercial diet for a week prior to the start of the experiment. The physico-chemical parameters of the water were regularly monitored using a digital pH pen and dissolved oxygen meter (Yieryi, China), salinity meter (Smart Sensor, China) and a water test kit (API, USA). All parameters were found at optimum levels, viz., temperature (22–27 °C), pH (8.2), dissolved oxygen (>5 ppm), salinity (20–25 ppt), ammonia (0–0.25 ppm), nitrite (0–0.25 ppm).

Growth trial

An 8-week feeding trial was conducted using juvenile *P. monodon* (5.12 ± 0.38 mg) that were randomly stocked in a 60 L plastic tank at a density of 25 individuals.$tank^{-1}$. The experimental tanks were run under a recirculating culture system. This experiment followed a completely randomised design with five treatments, 25 % (SH25), 50 % (SH50), 75 % (SH75), 100 % (SH100) squid by-product hydrolysate replacement of fishmeal and control (CTRL). The experimental treatments were run in three replicates.

The experimental animals were fed to satiation with the test diets at three feeding times (0800, 1200 and 1600 h). To quantify the actual feed intake, excess and uneaten feed materials were collected 1 h after each feeding. Periodic sampling was conducted every 2 weeks for survival monitoring, the conduct of tank clean-up and adjustment of the feeding ration. Upon the termination of the feeding trial, growth of shrimps in terms of weight gain (WG) and specific growth rate (SGR), protein efficiency ratio (PER), feed conversion efficiency (FCE) and survival were computed.

$$\text{Weight gain (\%)} = \frac{\text{Final weight (mg)} - \text{Initial weight (mg)}}{\text{Initial weight (mg)}} \times 100$$

$$\text{Specific growth rate (\%)} = \frac{\ln \text{final weight} - \ln \text{initial weight}}{\text{Days of experiment}} \times 100$$

$$\text{Protein efficiency ratio} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g; dry weight basis)}}$$

$$\text{Feed conversion efficiency (\%)} = \frac{\text{Live weight gain (g)}}{\text{Feed consumed (g)}} \times 100$$

$$\text{Survival (\%)} = \frac{\text{Total number of shrimp at the end of culture period}}{\text{Total number of shrimp stocked}} \times 100$$

Proximate composition and nutrient retention

Initial and final carcass composition (moisture, crude protein, crude fibre, ash) of the experimental animals, squid by-product hydrolysate and the diets were analysed following the standard AOAC method (AOAC, 1990), while crude lipid was determined following the methods described by Bligh and Dyer (1959). Protein and lipid retentions of shrimp were computed using the following formula (Hardy and Barrows, 2002):

$$\text{Nutrient retention (\%)} = \frac{\text{CNC}_{\text{final}} - \text{CNC}_{\text{initial}}}{\text{NI}} \times 100$$

where:

$\text{CNC}_{\text{initial}}$ = carcass nutrient content at the start of experiment

$\text{CNC}_{\text{final}}$ = carcass nutrient content at the end of experiment

NI = nutrient intake during the experiment

Digestive enzyme activity analyses

Penaeus monodon digestive enzyme activities including total protease, amylase and lipase were also analysed. Hepatopancreas of shrimp from all treatments were excised, thawed, and homogenised in ice-cold 50 mM sodium phosphate buffer, pH 8.0 at a ratio of 1:25 (wet tissue to volume) using a Teflon-glass tissue homogeniser. The homogenates were centrifuged at 4,000 rpm at 4 °C for 15 min, and the supernatant collected and used in subsequent enzyme assays. All enzymes were assayed at 25 °C and were done in triplicates per treatment. The protein content of the crude enzyme extracts was determined following the method of Bradford (1976) using bovine serum albumin as standard.

The protease activity was quantified according to the method described by Buroker-Kilgore and Wang (1993). The enzyme reaction was initiated by adding 25 µL hepatopancreas homogenate extract to the substrate cocktail consisting of 0.65 % casein in 10 mM sodium acetate buffer at pH 7.5. The reaction was allowed to proceed at 25 °C for 10 min and was stopped by adding Bradford reagent to react with the amino acids formed during the hydrolysis reaction. The formation of the enzymatic hydrolysis products were read at an absorbance of 595 nm in a spectrophotometer (Shimadzu, Japan). A unit of specific protease enzyme activity was expressed as the 0.001 change in absorbance of the sample.min^{-1} mg^{-1} protein. Furthermore, α-amylase activity was measured at 546 nm using 1 % starch in 200 mM phosphate buffer, pH 7.0 (Bernfeld, 1951 as modified by Areekijseree et al., 2004). Specific enzyme activity was defined as µg glucose formed.min^{-1} mg^{-1} protein. Lipase activity was determined at 715 nm following the protocols of Pinsirodom and Parkin (2001) using olive oil/Triton X-100 emulsion as substrate. Methyl undecanoate was used as the standard and lipase specific unit activity was defined as nanomole fatty acid formed.min^{-1} mg^{-1} protein.

Histological examination

Shrimp hepatopancreas were fixed in Davidson's solution and histological slides were prepared. Photomicrographs of the samples were taken under light microscopy (Motic, China), wherein shrimp hepatopancreatic epithelial cells were identified and described based on the normal structure as documented by Bell and Lightner (1988).

Lactic acid bacterial count

For the evaluation of lactic acid bacteria, shrimp were sanitised with 70 % alcohol and rinsed with sterile distilled water. The digestive tract was removed aseptically, weighed, and macerated in sterile saline solution. Ten-fold serial dilutions of the homogenate were prepared, 0.1 mL aliquot was spread plated on De Man-Rogosa-Sharpe agar (TM Media, India) with 2 %

NaCl, and incubated at room temperature for 48 h (Vieira et al., 2007). Colonies of lactic acid bacteria were counted and quantified as log colony forming unit (CFU).g^{-1} tissue.

Statistical analysis

Results were reported as mean ± standard error of the mean (SEM). All data were subjected to One-way analysis of variance and the differences between means were determined using Tukey Test at 0.05 significance level. Pearson correlation coefficient was also determined to measure the correlation among parameters. As needed, data were subjected to logarithmic or arcsine transformation prior to analysis. All data analyses were performed using Sigma Plot 11.0 (Systat Software Inc., USA).

Results

Growth

After the 8 weeks of feeding trial, SH25 group exhibited a higher per cent WG and SGR as compared to the CTRL and SH100 groups (Table 2). The per cent WG and SGR values in the SH25 group were not significantly different from those obtained in treatments SH50 and SH75. The experimental group at SH50 showed similar per cent WG and SGR values to all the other treatment groups. Additionally, the per cent WG and SGR values in SH75 were higher than that of SH100 but were statistically similar to those obtained in the other treatment groups. Total feed intake (TFI) was significantly higher in SH25 compared to the CTRL and SH100, which were the lowest among the treatments. However, this TFI value was not different from those obtained in SH50 and SH75. Also, PER, FCE and survival were not significantly different among the treatments.

Furthermore, correlation analysis indicated a strong positive association of per cent WG and SGR with TFI, PER and FCE (Table 3).

Carcass nutrient composition

The crude protein content of shrimp carcass was lower in SH75 and SH100 than in the other treatments (Table 4). Moreover, carcass protein contents of the CTRL, SH25 and SH50 were found to be similar and the highest among the experimental treatment groups.

In contrast to the shrimp carcass protein content, the carcass lipid content was found to increase with increasing substitution levels of fishmeal in the diet. The SH100 group had the highest lipid content but was not different from those in the SH75 group. The carcass lipid content of the SH75 group was also

Table 2. Growth performance of black tiger shrimp *Penaeus monodon* fed with squid by-product hydrolysate as fishmeal replacement for 8 weeks.

Treatments	WG (%)	SGR (%)	TFI (mg)	PER	FCE (%)	Survival (%)
CTRL	1618.13 ± 113.52^{ab}	4.73 ± 0.11^{ab}	201.65 ± 5.16^{a}	0.18 ± 0.01	42.69 ± 2.10	87.83 ± 4.43
SH25	2118.14 ± 89.01^{c}	5.16 ± 0.07^{c}	253.19 ± 12.79^{b}	0.19 ± 0.01	42.68 ± 1.15	90.60 ± 4.47
SH50	1874.63 ± 143.07^{abc}	4.96 ± 0.12^{abc}	227.24 ± 12.74^{ab}	0.18 ± 0.01	40.40 ± 1.94	87.78 ± 4.84
SH75	1989.12 ± 1.73^{bc}	5.07 ± 0.04^{bc}	225.35 ± 7.05^{ab}	0.19 ± 0.00	44.33 ± 1.08	82.57 ± 4.10
SH100	1521.59 ± 63.22^{a}	4.64 ± 0.06^{a}	190.66 ± 8.38^{a}	0.17 ± 0.00	40.94 ± 0.91	90.99 ± 2.36

Values are mean ± SEM (n = 3). Different superscript letters indicate significant differences among treatment ($P < 0.05$).
WG -weight gain, SGR – specific growth rate, TFI – total feed intake, PER – protein efficiency ratio, FCE – feed conversion efficiency.

Table 3. Correlation analysis between indices influencing the growth of black tiger shrimp *Penaeus monodon* supplemented with squid by-product hydrolysate.

	%WG	SGR	TFI	PER	FCE
SGR	r = **1.00** P = <0.01				
TFI	r = **0.90** P = <0.01	r = **0.89** P = <0.01			
PER	r = **0.80** P = <0.01	r = **0.81** P = <0.01	r = 0.49 P = 0.06		
FCE	r = **0.65** P = <0.01	r = **0.66** P = <0.01	r = 0.24 P = 0.39	r = **0.91** P = <0.01	
Survival	r = 0.05 P = 0.85	r = 0.04 P = 0.89	r = -0.18 P = 0.52	r = 0.05 P = 0.83	r = 0.12 P = 0.63

Statistically significant results are in bold. R – Pearson correlation coefficient, P – P-value, %WG – per cent weight gain, SGR – specific growth rate, TFI – total feed intake, PER – protein efficiency ratio, FCE – feed conversion efficiency.

Table 4. Nutrient composition of the black tiger shrimp *Penaeus monodon* carcass (%, dry weight).

Treatments	Crude protein	Crude lipid	Ash
Initial	69.87 ± 1.50	4.91 ± 0.16	18.50 ± 0.44
CTRL	75.88 ± 0.46^{b}	7.67 ± 0.15^{a}	12.78 ± 0.48
SH25	73.93 ± 0.75^{b}	9.14 ± 0.06^{b}	12.77 ± 0.40
SH50	73.12 ± 1.14^{b}	9.33 ± 0.47^{b}	12.47 ± 0.01
SH75	71.25 ± 1.06^{a}	9.58 ± 0.32^{bc}	13.34 ± 0.12
SH100	69.66 ± 0.03^{a}	10.94 ± 0.13^{c}	13.60 ± 0.09

Values are mean ± SEM (n = 3). Different superscript letters indicate significant differences among treatments ($P < 0.05$).

similar to those obtained in the SH25 and SH50 groups. The lowest carcass lipid content was recorded in the CTRL.

Nutrient retention

The highest numerical value on protein retention was observed in treatment SH25, but it did not differ significantly from that of treatments SH50, SH75 and the CTRL. The SH100 group exhibited a protein retention value statistically similar to those obtained in SH50 and the CTRL group. However, the protein retention value in SH100 was significantly lower than those obtained in treatments SH25 and SH75. The CTRL group exhibited a protein retention value that was statistically similar to those obtained in all the treatment groups (Table 5).

Shrimp lipid retention indicates that all the treatments containing squid by-product hydrolysate exhibited higher lipid retention values than the CTRL. The CTRL group recorded the lowest lipid retention among the treatments (Table 5).

Table 5. Nutrient retention in black tiger shrimp *Penaeus monodon* fed with squid by-product hydrolysate as fishmeal replacement for 8 weeks.

Treatments	Parameters	
	Protein retention (%)	Lipid retention (%)
CTRL	13.98 ± 0.65^{ab}	5.51 ± 0.25^{a}
SH25	14.32 ± 0.37^{b}	7.03 ± 0.18^{b}
SH50	13.94 ± 0.59^{ab}	6.93 ± 0.28^{b}
SH75	14.09 ± 0.29^{b}	7.28 ± 0.14^{b}
SH100	12.02 ± 0.27^{a}	7.06 ± 0.16^{b}

Values are mean ± SEM (n = 3). Different superscript letters indicate significant differences among treatments ($P < 0.05$).

Digestive enzyme activities

Protease activity was found highest in the CTRL compared to the treated groups (Table 6). No significant differences in the digestive protease activities were observed in SH75, SH50 and SH25 treatment groups. Treatment SH100 exhibited a protease activity that is not significantly different to SH25 but lower than treatments SH75, SH50 and the CTRL. Amylase and lipase activities were not significantly different among the treatments (Table 6).

Hepatopancreas histology

The hepatopancreas tubule of shrimp in the CTRL group was dominated by B-cells, while in treatment SH25 the tissue is mostly composed of R-cells (Fig. 1). Both B- and R-cells were observed in the hepatopancreatic tubule of the SH50 group. Moreover, the presence of R-cells in the hepatopancreas of shrimp in SH75 was evident but B-cells were also noted. In SH100 treatment, the hepatopancreatic tubules were also dominated by R-cells.

Lactic acid bacterial count

The lactic acid bacterial count was observed to be highest in SH50, but the numerical value was not significantly different from SH25. Both SH25 and SH50 exhibited higher gut content of lactic acid bacteria than the other treatment groups. The CTRL, SH75 and SH100 exhibited similar counts of lactic acid bacteria (Fig. 2).

Discussion

The potential of utilising squid by-product hydrolysate to replace fishmeal in the plant protein meal-based diet of *P. monodon* was evaluated in this study. Results showed that dietary fishmeal could be completely replaced with squid by-product hydrolysate without affecting the growth performance and feed conversion of *P. monodon*. Similar to our findings, it has been shown in salmon (*Salmo salar* Linnaeus, 1758) that fishmeal can be replaced with fish hydrolysate at low inclusion levels (Espe et al., 1999; Refstie et al., 2004; Hevroy et al., 2005). In the case of shrimp, particularly *P. vannamei*, supplementation of hydrolysates was reported to improve growth performance. The growth-promoting effects of the hydrolysate was associated with the type of raw

Table 6. Digestive enzyme activities of black tiger shrimp *Penaeus monodon* fed with squid by-product hydrolysate as fishmeal replacement for 8 weeks.

Treatments	Protease units	Amylase units	Lipase units
CTRL	16.55 ± 0.00^{c}	444.27 ± 19.42	611.81 ± 29.84
SH25	11.92 ± 0.00^{ab}	447.16 ± 8.39	544.39 ± 128.93
SH50	12.84 ± 0.86^{b}	438.93 ± 68.55	374.68 ± 16.47
SH75	13.24 ± 0.60^{b}	450.33 ± 34.83	507.98 ± 0.00
SH100	9.31 ± 1.03^{a}	524.59 ± 67.00	572.01 ± 77.70

Values are mean ± SEM (n = 3). Different superscript letters indicate significant differences among treatments ($P < 0.05$).

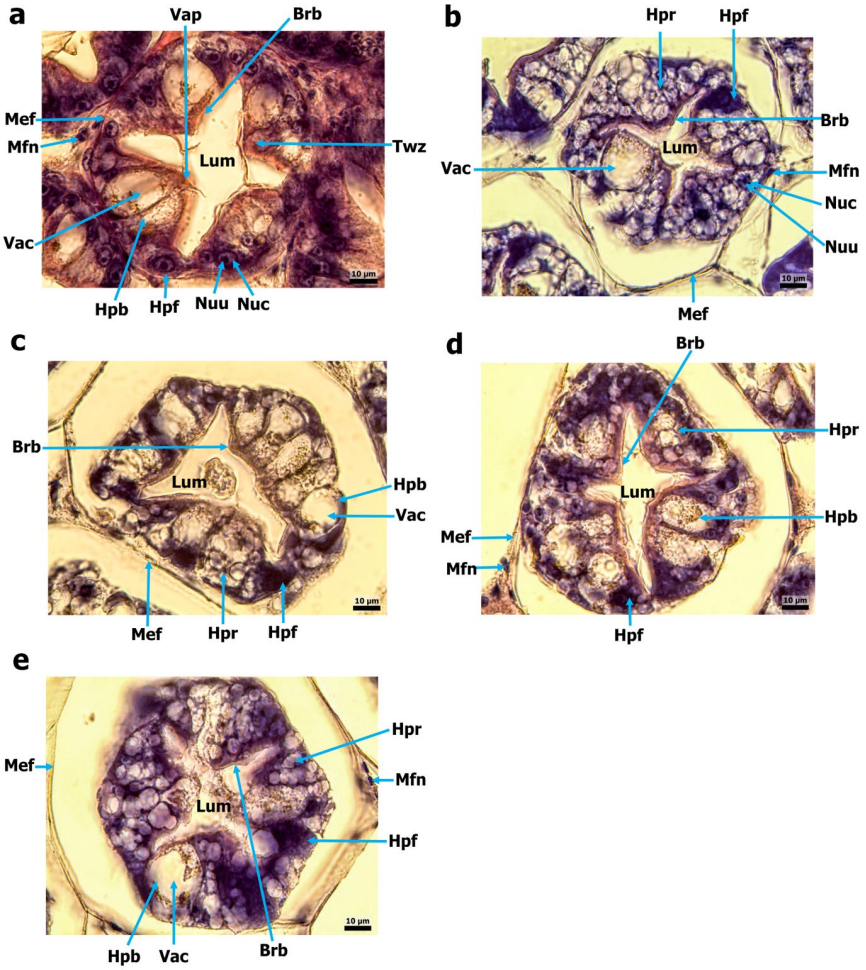

Fig. 1. Hepatopancreatic tubule of black tiger shrimp *Penaeus monodon* fed with the (a) CTRL, (b) SH25, (c) SH50, (d) SH75 and (e) SH100 for 8 weeks (n = 3). The proliferation of R-cells in the hepatopancreas can be noted with the increase in fishmeal replacement. Transverse, 4–5 µm paraffin section, H & E stain, Davidson's fixative, 1000× magnification, bar length = 10 µm. Brb – brush border, Hpb – hepatopancreatic B-cell, Hpf – hepatopancreatic F-cell, Hpr – hepatopancreatic R-cell, Lum – lumen, Mef – myoepithelial fibres, Mfn – myoepithelial fibre nucleus, Nuc – nucleus, Nuu – nucleolus, Twz- terminal web region, Vac – vacuole, Vap – vacuolated apical complex.

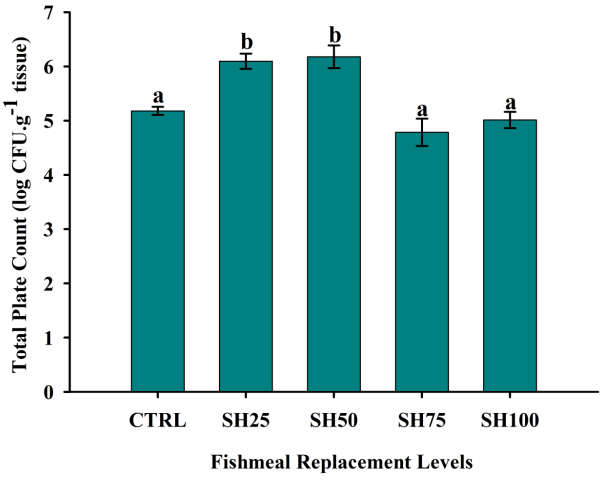

Fig. 2. Lactic acid bacterial count of black tiger shrimp Penaeus *monodon* fed with squid by-product hydrolysate as fishmeal replacement for 8 weeks. Mean ± SEM (n = 3) with different superscript letters is significantly different ($P < 0.05$). CTRL - 5.18 ± 0.07, SH25 - 6.10 ± 0.14, SH50 - 6.18 ± 0.21, SH75 - 4.79 ± 0.25, SH100 - 5.01 ± 0.15.

material used, hydrolysis process and level of dietary incorporation (Hernandez et al., 2011; Quinto et al., 2018).

To the best of our knowledge, the present study is the first to document the complete or total replacement of fishmeal with squid by-product hydrolysate in the plant-based diet of shrimp. Hevroy et al. (2005) stated that the growth of animals fed with protein hydrolysate-containing diets is not compromised if the content of dietary intact protein is in balanced proportion with the protein hydrolysate added in the diet. Protein hydrolysate, composed of free amino acids and di- and tripeptides, if provided at high dietary levels are known to saturate and block the intestinal peptide transporters that impair the general amino acid absorption in animals (Berge et al., 1999; Aragao et al., 2004). In the present study, the low content of fishmeal replaced by squid by-product hydrolysate may explain the total replacement without negatively affecting the shrimp growth performance.

Moreover, in the present study, fishmeal replacement of 25 % squid by-product hydrolysate was found to elicit a growth-promoting effect on this shrimp. The present finding is the first to document the growth enhancement of *P. monodon* as influenced by dietary inclusion of squid by-product hydrolysate. The present study confirms earlier reports elucidating the positive influence of fish-derived protein hydrolysates on the growth and feed utilisation of cultured shrimp (Nguyen et al., 2012; Niu et al., 2014). The growth improvement effects on aquatic animals as influenced by dietary protein hydrolysates have been associated with the presence of highly digestible and bioavailable peptides and amino acids comprising the hydrolysate (Cordova-Murueta and Garcia-Carreño, 2002). The presence of small molecular weight peptides in fish hydrolysate have been found to influence feed palatability, modulate protein and energy metabolic pathways, and overall improve feed utilisation (Aksnes et al., 2006; Nguyen et al., 2012; Zheng et al., 2012).

Moreover, in the present study, the growth-promoting effects of dietary inclusion of squid by-product hydrolysate in *P. monodon* is associated with enhanced feed intake that was found to be the highest in SH25. Similar to the present findings, the inclusion of 10–15 $g.kg^{-1}$ low-molecular-weight fish hydrolysate (LWFH) in a soybean-based diet improved the growth, antioxidant activity and innate immunity of *P. vannamei* (Li et al., 2018). Furthermore, feed intake was significantly increased at 20 $g.kg^{-1}$ diet LWFH supplementation level. In addition, enhancement of shrimp growth in terms of weight gain and SGR and efficient feed conversion was observed in *P. vannamei* fed diets supplemented with 5 % tuna by-product hydrolysate (Hernandez et al., 2011). Also, the efficacy of fish and krill hydrolysates to stimulate feed intake in *P. monodon* was reported to be similar to the response evoked during feeding with fishmeal-based diet (Smith et al. 2005). In the red sea bream *Pagrus major* (Temminck & Schlegel, 1843), Kondo et al. (2017) showed enhancement of feed intake and feed conversion efficiency as an effect of dietary supplementation with squid viscera hydrolysate. This improvement in growth and fed utilisation was attributed to increased digestion and activation of appetite-regulating factors in the experimental fish.

The enhancing effects of protein hydrolysates on feeding have been associated with its amino acid and low molecular weight peptide composition that are effective in increasing feed intake, improvement of digestion and overall promotion of growth (Hidaka et al., 2000; Erteken and Nezaki, 2002; Ostaszewska et al., 2013). In the present study, growth response indices including SGR, per cent WG, FCE and PER of the experimental shrimp were found strongly correlated with the total feed intake. These indicate that the growth promotion effects of squid by-product hydrolysate in shrimp is associated with the stimulation of feeding activity, similar to those described in earlier reports in other cultured shrimp species.

Moreover, the use of squid by-product hydrolysate as a replacement of fishmeal in the diet of *P. monodon,* was found to influence only the protease digestive enzyme but not the α-amylase and lipase enzymes. Results suggest declining protease activity in shrimp as the squid by-product hydrolysate dietary inclusion level increases. The observed growth enhancement on SH25 was associated with low protease activity. Similar to our findings, it was also shown in sea bass, *Dicentrarchus labrax* (Linnaeus, 1758), larvae that high dietary level of protein hydrolysate depresses digestive trypsin secretion (Cahu et al., 2004). Moreover, in *P. vannamei,* it was shown that feeding with diets containing 15 % fish protein hydrolysate decreased the total digestive proteolytic activities (Cordova-Murueta and Garcia-Carreño, 2002). This low proteolytic activity had been attributed to the free amino acids and small polypeptides composition of protein hydrolysates. These peptides and free amino acids have been known as poor substrates for proteolysis by endopeptidases since most enzyme-binding sites along the protein chain have been hydrolysed (Cordova-Murueta and Garcia-Carreño, 2002). Also, proteolytic digestion in crustacean is an energy consuming process and since squid by-product hydrolysate is already partially digested and is highly bioavailable, activation of proteolytic activity could be minimal. In the present study, the activities of α-amylase and lipase were not influenced by dietary squid by-product hydrolysate. The present findings agree with the results obtained by Shao et al. (2018), showing that amylase and lipase activities were not enhanced in *P. vannamei* fed diets supplemented with fish by-product hydrolysate. However, Niu et al. (2014) and Shao et al. (2018), documented that moderate supplementation of fish protein hydrolysate could enhance the digestive trypsin activity of cultured shrimp. Several authors have also reported stimulation

of digestive enzyme activities in fish and shrimp larvae fed with low quantities of protein hydrolysates (Zambonino-Infante et al., 1997; Cordova-Murueta and Garcia-Carreño, 2002). These differences in results may have been due to the differences in species response, the type of protein hydrolysate used, and the amount used in the feeds.

In the present study, body protein retention was not affected by dietary squid by-product hydrolysate replacement levels but the significant influence of this dietary ingredient manifests in body lipid retention. Lipid retention in treatments with squid by-product hydrolysate is significantly higher than the CTRL. This high lipid retention must have also contributed to the better growth response in shrimp fed with SH25. The present findings conform to the general observation that dietary inclusion of protein hydrolysate at certain levels can promote fat deposition in cultured aquatic animals. Niu et al. (2014) reported that dietary inclusion levels of fish protein hydrolysate in *P. vannamei* resulted in a dose-dependent increase in body fat content. In juvenile and larval tilapia, *Oreochromis niloticus* (Linnaeus, 1758), similar patterns of increased body fat content were also observed because of feeding with diets containing shrimp head hydrolysates as a replacement for fishmeal (Plascencia-Jatomea et al., 2002; Leal et al., 2010). The biochemical mechanism on how dietary protein hydrolysate can promote tissue fat deposition is currently not understood. However, earlier reports on mice suggest that feeding with fish protein hydrolysate alters body fat synthesis through the activation of the immune cytokines, the interferon gamma (IFN$_{\gamma}$) and tumour necrosis factor-alpha (TNF-α) (Bjorndal et al., 2013). These cytokines are also believed to be found in shrimp (Mekata et al., 2010; Li et al., 2015) and have been known to promote lipid deposition in hepatocytes and macrophage-derived foam cells in mice (Tacer et al., 2007; Persson et al., 2008). It is tempting to speculate that a similar mechanism may be at work in the present study and may explain the higher body fat retention of shrimp fed with diets containing squid by-product hydrolysate. However, this mechanism in shrimp requires a thorough investigation in future studies.

Further, the present results indicate modification of the shrimp hepatopancreatic cells as influenced by dietary squid by-product hydrolysate replacement of fishmeal. Hepatopancreas tubules of shrimp in the CTRL group are dominated by B-cells with lesser numbers of the R-cells. The B-cells are responsible for the secretion of enzyme-rich vacuolar contents (Loizzi, 1971) and these cells are involved in the digestion of complex nutrients. In contrast, the hepatopancreas tubules of shrimp in the treated groups are dominated by R-cells known to be active in the absorption of luminal pre-digested nutrients through contact digestion and molecular transport. Moreover, they are also involved in the storage and metabolism of glycogen and lipids (Loizzi, 1971). The presence of peptides and amino acids in squid by-product hydrolysate may have favoured the dominance of these cells in the shrimp hepatopancreas. The high prevalence of R-cells in the treatment group fed with squid by-product hydrolysate may also account for the increase in body lipid retention with increasing dietary squid by-product hydrolysate levels.

In cultured fish, it has been commonly observed that dietary application of protein hydrolysates could promote the growth of beneficial lactic acid bacteria in the gut. Ha et al. (2019) reported that dietary inclusion of 5 % sardine protein hydrolysate resulted in increased digestive enzymatic activity and significant proliferation of intestinal lactic acid bacteria in South American catfish, *Rhamdia quelen* (Quoy & Gaimard, 1824). Safari et al. (2012) also reported that protein hydrolysate from yellowfin tuna, *Thunnus albacares* (Bonnaterre, 1788) could promote and support the growth of lactic acid bacteria. These findings concur with the present results indicating that fishmeal replacement with squid by-product hydrolysate at 25 % in *P. monodon* diet promoted the growth of gut lactic acid bacteria. Proliferation of these gut associated lactic acid bacteria could be linked to the growth enhancement effect of dietary squid by-product hydrolysate on *P. monodon* in the present study. Promotion of lactic acid bacterial growth by protein hydrolysate has been linked to the easily available nitrogen molecule from the peptides and amino acids comprising the hydrolysates (Safari et al., 2012).

Conclusion

The present study suggests that squid by-product hydrolysate can replace the entire fishmeal in plant-based diets of juvenile black tiger shrimp *Penaeus monodon* without negatively affecting growth, feed conversion and survival. Further, 25 % fishmeal replacement with squid by-product hydrolysate enhances shrimp growth associated with increased feed intake and higher counts of gut lactic acid bacteria. Increasing the inclusion of squid by-product hydrolysate, could increase lipid retention, decrease protease activity, and promotes the hepatopancreatic R-cells' proliferation. The use of squid industry by-product protein hydrolysate could be a practical and efficient way to limit the use of fishmeal in the diet of cultured carnivorous shrimp, *P. monodon*.

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Author contributions: Maila V. Pan: Conceptualisation, conducted the research, data analysis, acquisition of resources and writing of the original draft. Rex Ferdinand M. Traifalgar: Conceptualisation, supervision of the research, data analysis, acquisition of resources, writing, reviewing, and editing of the manuscript.

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