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EFFECTS OF EXOGENOUS PROTEASE SUPPLEMENTATION IN PALM KERNEL MEAL-BASED DIETS ON GROWTH PERFORMANCE AND HEALTH CONDITION OF PACIFIC WHITE SHRIMP (*Penaeus vannamei*) CULTURED IN HAPA NETS

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ABSTRACT

The effect of exogenous protease complex (Jefo Protease, Jefo Nutrition Inc., Saint-Hyacinthe, QC, Canada) was investigated for 90 days on the forty hapa nets (2 m × 2 m × 1 m, 200 shrimps per hapa net) with five replicates for each treatment groups to the growth, health condition, and also histological condition of the distal intestine and hepatopancreas of the shrimp Penaeus vannamei. Treatments were generally grouped into feed groups supplemented with protease and without protease. The first diet in the protease group was formulated using 40% soybean meal (SBM) and supplemented with protease (0JPKM). Then, the following three experimental feeds were formulated with reduced inclusion levels of SBM, replaced with 5, 10, and 15% palm kernel meal (PKM), supplemented with protease, and labeled as 5, 10, and 15 JPKM. For feed without protease, a similar formulation was used as the protease group without adding protease and labeled as 0, 5, 10, and 15% PKM. In our study, adding protease improved shrimp growth performance; even the inclusion of 5% PKM to replace SBM supplemented with protease (5JPKM) had the same growth as the shrimp fed with SBM without protease (0 PKM). Data on the total hemocyte count (THC) of shrimp showed an increase in the group with protease supplementation compared to those without protease supplementation. Meanwhile, lysozyme activity did not significantly differ. Histomorphology evaluation data in the hepatopancreas and intestine of shrimp showed better morphology in shrimp supplemented with protease compared to the group of shrimps that were not supplemented with protease. These results show that protease supplementation in feed protease with an inclusion level of 0.018% could significantly improve growth performance, THC, and digestion in P. vannamei shrimp.

KEYWORDS: Protease; Palm kernel meal; Growth; Health; *Penaeus vannamei*

INTRODUCTION

Shrimp production is experiencing significant challenges related to production inefficiency due to increasing feed prices (Nguyen *et al*., 2019; Villarreal, 2023). The increase in feed prices was undoubtedly triggered by the increase in the primary protein source ingredient in feed formulations, such as fish meal (FM) and soybean meal (SBM) (Banaszkiewicz, 2011; Hulefeld *et al*., 2018; Villarreal, 2023). Therefore, feed formulation is now starting to be directed at reducing formulation costs while still meeting the digestible energy requirements sourced from balanced protein, fat, and carbohydrates to optimize growth (Cho & Bureau, 2001; De Blas & Mateos, 2020). Several alternative ingredients to replace FM or SBM have been analyzed, such as poultry meal (PM), corn meal (CM), and insect meal (IM). However, the use of these alternative ingredients, apart from increasing the production efficiency, is also expected to consider several factors, including digestibility, palatability, and nutritional profile as the strategies to use any of particular ingredient in the diet formulation (Glencross, 2020; Glencross *et al*., 2007; Kaushik, 2000).

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According to Pastore *et al*. (2012) and Suresh (2016) formulators in feed mills work with a least-cost formulation software to target the lowest economical cost possible while maintaining the targeted nutrient requirement and digestibility levels in feed. Using low economical value ingredients with inconsistent levels of digestibility, such as feather meal, (Adelina *et al*., 2021), will certainly impact feed quality and the growth of the aquatic organisms (Kikuchi *et al*., 1994). Another low economic value ingredients that can be considered to be used for aquafeed is palm kernel meal (PKM) (Sangavi & Betsy, 2020). However, even though it is a source of protein in animal feed, the utilization of the protein in PKM is a challenge for aquatic organisms (Al-Maadhedy *et al*., 2020). Some causes of difficulty in utilizing protein in PKM are due to several factors, including: Complex protein structures, making PKM challenging to digest (Al-Maadhedy *et al*., 2020); the presence of anti-nutritional factors, such as protease inhibitors, lectins, and tannins, which can interfere with protein digestion and absorption (Shi-hang *et al*., 2024), and high fiber content that may reduce the digestibility of its protein content (Azizi *et al*., 2021; da Silva *et al*., 2020). However, considering the existence of PKM in large quantities, especially in Indonesia and Malaysia (Ibrahim, 2013), the potential on the use of PKM has begun to be widely discussed as one of the raw materials in aquafeed production (Ng, 2003; Sangavi & Betsy, 2020; Thongprajukaew *et al*., 2015).

To increase the nutritional profile of PKM protein for aquatic organisms, several strategies can be implemented, such as formulating feeds with a combination of protein sources, including those with higher digestibility, can help offset the challenges associated with PKM (Shamsuddin *et al*., 2021; Wattanakul *et al*., 2021), or include protease in the diet formulation to break down proteins into smaller peptides and amino acids (Hasanthi *et al*., 2023; Shah *et al*., 2024). This can improve nutrient utilization efficiency and satisfy shrimp nutrient requirements to support optimum growth and health conditions. Protease has been used in aquafeed formulations to optimize feed efficiency and reduce environmental impact by improving the digestibility of feed ingredients (Gopalraaj *et al*., 2024; Lee *et al*., 2020; Saleh *et al*., 2022). However, it is essential to carefully select the form and balance the protease's inclusion level with other ingredients in feed formulation to ensure optimal performance and cost-effectiveness.

Like other animals, shrimp require proteins and amino acid balances for growth and maintain their metabolism and physiological functions (Kureshy & Davis, 2002; Nunes *et al*., 2019; Zhou *et al*., 2013). However, they lack the necessary enzymes to break down complex proteins into smaller, absorbable units. For this reason, feed formulations using raw materials with high inhibiting factors, such as PKM, will require the addition of external enzymes in the feed. In this research, we evaluate the use of commercial exogenous protease complex (Jefo Nutrition Inc., Saint-Hyacinthe, QC, Canada) as a unique heat stable alkaline protease that is active on a wide range of protein sources, in diet of Pacific white shrimp *Penaeus vannamei* cultured in an outdoor condition using hapa nets installed within the earthen ponds lined with high-density polyethylene (HDPE) plastics. Therefore, the present study was designed to evaluate the effect of exogenous protease on performance, health condition, and also histological status of the distal intestine and hepatopancreas of the shrimp *Penaeus vannamei* fed diets with different inclusion levels of PKM. The results of the study were then compared with the performance of shrimp fed with PKM-containing feed without exogenous enzyme supplementation.

MATERIALS AND METHODS

Experimental Diets

Two groups of test feeds consisted of four experimental feeds in the first group, with all feeds supplemented with 0.018% protease (Jefo Nutrition Inc., Canada), and four diet in the second group without protease supplementation. In the first group, the control feed (0 JPKM) was designed without palm kernel meal (PKM) containing 10% FM, 12% PM, and 40% SBM, supplemented with protease. The experimental diets were prepared by utilizing palm kernel meal (PKM) at the inclusion levels of 5, 10, and 15% supplemented with protease to partially replace the inclusion of SBM and labeled as 5, 10, and 15 JPKM, respectively. The second group of experimental diets utilized 0, 5, 10, and 15% PKM to partially replace SBM without protease supplementation and labeled as 0, 5, 10, and 15 PKM, respectively (Table 1). Before production, all ingredients, including protease, were crushed and passed through < 200 mesh sieve (Jinan Shengrun, China), weighted, and mixed in a paddle mixer (Marion Mixers, Inc., Marion, IA, USA). The cooking-extrusion temperature was kept at 110°C for approximately 14 seconds in five-barrel sections, and the last section was maintained at 62°C. All feeds were extruded into 1- and 2-mm diameter and 2 – 4 mm lengths. Diets were air-dried in a pulse bed dryer (Jinan Shengrun, China) and stored at 4°C in sealed containers until further use. The experimental diets' proximate and amino acid profiles were analyzed at Saraswati Indo Genetech Laboratory, Bogor, West Java, Indonesia, and summarized in Table 2.

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Table 1. Composition (% *as is*) of diets consisting of protease (Jefo Protease, Jefo Nutrition Inc., Canada) in commercial diet formulation by utilizing palm kernel meal (PKM) replace soy-protein and fed to *P. vannamei* for 90 days (1CP = Crude protein)

Diet name	0JPKM	5JPKM	10JPKM	15JPKM	0PKM	5PKM	10 PKM	15PKM
Menhaden fishmeal	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Poultry meal	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Soybean meal (CP[1]: 44%)	40.00	35.00	30.00	25.00	40.00	35.00	30.00	25.00
Palm Kernel Meal (CP[1]: 17%)	0.00	5.00	10.00	15.00	0.00	5.00	10.00	15.00
Menhaden fish oil	3.30	3.30	3.30	3.30	3.30	3.30	3.30	3.30
Lecithin (soy)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Corn Starch	14.37	14.12	13.82	13.47	14.39	14.14	13.84	13.49
Whole wheat	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00
Mineral premix (shrimp)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix (shrimp)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride (0.2% all diets)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Rovimix Stay-C 35%	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
CaP-dibasic	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Lysine (78.8%)	0.10	0.20	0.30	0.50	0.10	0.20	0.30	0.50
Methionine	0.10	0.25	0.45	0.60	0.10	0.25	0.45	0.60
Protease (JEFO)	0.018	0.018	0.018	0.018	0.00	0.00	0.00	0.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[1] PT FKS Multi Agro, Tbk. Jakarta, Indonesia

[2] PT Rajawali Mitra Pakanindo, Banten, Indonesia

[3] PT Pundi Kencana, Cilegon, Banten, Indonesia

[4] Trace mineral premix (g/100 g premix): cobalt chloride, 0.004; cupric sulfate pentahydrate, 0.550; ferrous sulfate, 2.000; magnesium sulfate anhydrous, 13.862; manganese sulfate monohydrate, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193; alpha-cellulose, 69.664.

[5] Vitamin premix (g/kg premix): thiamin·HCL, 4.95; riboflavin, 3.83; pyridoxine·HCL, 4.00; Ca-pantothenate, 10.00; nicotinic acid, 10.00; biotin, 0.50; folic acid, 4.00; cyanocobalamin, 0.05; inositol, 25.00; vitamin A acetate (500,000 IU/g), 0.32; vitamin D3 (1,000,000 IU/g), 80.00; menadione, 0.50; alpha-cellulose, 856.81

Feeding Trial

The feeding trials were conducted at the Business Service Center for Aquaculture Production (BSCAP), Karawang (West Java, Indonesia). Pacific white shrimp post larvae (PL) were obtained from Salira Teknik Benur (Serang, Banten, Indonesia) and acclimatized to the culture system. After seven days, shrimp (0.97 ± 0.01 g initial mean weight) were randomly distributed into 40 hapa nets (2 m × 2 m × 1 m, 200 shrimps per hapa net). The hapa nets were in the outdoor ponds, and the water source was obtained from the surrounding sea-water environment that had been treated and sterilized in a reservoir before use for the research. Five replicate groups of shrimps per dietary treatment were administered different types of experimental diets for 90 days and fed by hand four times daily, at 07:00, 11:00, 15:00, and 20:00h. Daily feed inputs were pre-programmed assuming the average growth of shrimp and feed conversion ratio of 1.5. Daily allowances of feed were adjusted based on weekly sampling for the weight.

Water quality analysis and sample collection

Water quality parameters were collected four times daily for pH, dissolved oxygen (DO), water temperature, total dissolved solids, and salinity using sensors (Aqua TROLL 500 Multiparameter Sonde instrument), and the data were stored in an application (AquaEasy apps, Bosch, Singapore) for traceability. Meanwhile, Total ammonia-nitrogen (TAN), alkalinity, nitrate, nitrite, ammonia, and phosphate were measured weekly using absorption spectrophotometry (DR890, HACH, USA). At the termination of the feeding period, the shrimp in each hapa net were counted as a group and a random sub-sample of 20 shrimps per hapa nets was measured for individual weight.

Group and individual weighed were used to calculate the final biomass, final weight, percentage weight gain (PWG), feed conversion ratio (FCR), percentage survival (SR), and thermal unit growth coefficient (TGC) as follows:

$$PWG = \frac{(average\ individual\ final\ weight - average\ individual\ initial\ weight)}{(average\ individual\ initial\ weight)} \text{ x } 100$$

$$FCR = \frac{feed\ given\ (g)}{alive\ weight\ gain\ (g)}$$

$$SR = \frac{final\ number\ of\ shrimp}{initial\ number\ of\ shrimp} \text{ x } 100$$

$$TGC = \frac{FBW^{1/3} - IBW^{1/3}}{\sum TD} \text{ x } 100$$

Where FBW is final body weight, IBW is initial body weight, T is water temperature (^{0}C) and D is number of trial days.

Whole-Body composition analysis

Whole-body shrimp samples (n = 20 per dietary treatment) were collected and stored at - 20 ^{o}C for the whole-body composition analysis. Before proximate, energy, and amino acid analyses, dried whole shrimp were rigorously blended and chopped in a mixer according to methods described by (Helrich, 1990). All proximate and amino acid profiles of the whole shrimp body were analyzed at the Saraswati Indo Genetech Laboratory (Bogor, West Java, Indonesia).

Table 2. Proximate and amino acid (AA) composition (% *as is*, *dry matter basis*) of experimental diets utilized in the trial

Composition	Unit	0JPKM	5JPKM	10JPKM	15JPKM	0PKM	5PKM	10 PKM	15PKM
Ash Content	%	9.56	9.64	9.62	9.88	9.77	10.03	9.87	10.02
Total Fat	%	6.54	6.62	6.77	6.71	6.58	6.59	6.68	6.61
Moisture Content	%	11.55	11.43	11.39	11.42	11.12	11.17	11.27	11.29
Protein Content	%	33.49	33.11	33.17	33.14	33.38	32.84	32.75	32.66
Crude Fiber	%	1.56	1.93	1.98	2.11	1.62	1.92	1.99	2.03
L-serine	%	2.17	2.09	2.18	2.19	2.11	1.98	1.93	1.82
L-Glutamic Acid	%	4.29	4.27	4.26	4.23	4.24	4.19	4.15	4.12
L-Phenylalanine	%	2.91	2.92	2.93	2.94	2.77	2.64	2.58	2.55
L-Isoleucine	%	1.73	1.72	1.73	1.77	1.72	1.64	1.65	1.63
L-Valine	%	2.08	2.07	2.14	2.11	2.08	1.99	1.95	1.94
L-Alanine	%	1.71	1.72	1.74	1.68	1.73	1.62	1.64	1.64
L-Arginine	%	2.01	2.02	2.11	2.08	2.94	2.85	2.77	2.74
Glycine	%	2.25	2.28	2.33	2.31	2.34	2.24	2.28	2.11
L-Lysine	%	1.41	1.42	1.44	1.39	1.44	1.31	1.32	1.29
L-Aspartic Acid	%	1.71	1.72	1.71	1.74	1.73	1.66	1.69	1.68
L-Leucine	%	2.88	2.82	2.91	2.89	2.95	2.97	2.83	2.82
L-Tyrosine	%	0.26	0.25	0.27	0.29	0.31	0.23	0.24	0.23
L-Proline	%	2.02	2.03	2.09	2.05	2.08	1.97	1.98	1.95
L-Threonine	%	1.84	1.83	1.89	1.81	1.86	1.72	1.73	1.68
L-Histidine	%	1.25	1.13	1.15	1.23	1.22	1.11	1.14	1.12
L-Tryptophan	%	0.32	0.29	0.31	0.32	0.32	0.28	0.28	0.27
L-Cystine	%	1.19	1.18	1.16	1.15	1.22	1.23	1.19	1.17
L-Methionine	%	0.36	0.34	0.35	0.35	0.32	0.29	0.31	0.29

* Analysis conducted by the Saraswanti Indo Genetech Laboratory, Bogor, West Java, Indonesia. Website www.siglaboratory.com

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Total haemocyte counts and lysozyme activity analysis

At the end of the growth trial, fifteen shrimps per dietary treatment were sampled for total hemocyte count (THC) analysis, and another fifteen shrimp per dietary treatment for lysozyme activity analysis. The procedure for THC and lysozyme activity analysis was measured following protocol described in (Novriadi *et al*., 2024b).

Histology of hepatopancreas and distal intestine

For hepatopancreas and distal intestine histology, after 48 h of fixation with Davison's fixative solution at room temperature (Bell & Lightner, 1988), sample were then transferred to 50% ethanol solution (VWR, Radnor, PA, USA) until processed by standard histological analysis procedures. Samples were dehydrated through a standard ethanol series to 100%, embedded in paraffin wax, and sectioned (4 - 6 µm) were stained with Hematoxylin-Eosin (H&E, Merck, Darmstadt, Germany). Finally, the stained sections were photographed and observed under digital imaging microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan), then the results were evaluated with double-blinded evaluation with a grading scale of 1 to 5, where score1 was considered the normal condition, and subsequent scores accounted for higher levels of alteration.

Statistical analysis

Data of growth parameters, total hemocyte counts, and lysozyme activity were analyzed using regression and one-way analysis of variance (ANOVA) to compare differences among treatments, followed by Tukey's multiple comparison tests to differences between the means. Score data for the histomorphological condition of the hepatopancreas and distal intestine of shrimp were treated as categorical data, tested for normality and homoscedasticity, and subsequently analyzed using a linear regression model. All statistical analyses were conducted using the SAS system (V9.4. SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Proteases, also known as peptidases or proteinases, are enzymes that break down proteins into smaller peptide fragments composed of 2 to 20 amino acids of free amino acids (Islam *et al*., 2022; López-Otín and Bond, 2008). Protease play a crucial role in the digestion of dietary proteins in the gastrointestinal tract and the degradation of certain proteinaceous antinutrients (Chen *et al*., 2023), thus promotes the health and growth of farmed aquatic organisms. Indeed, there is a challenge in the commercial application of exogenous proteases due to their sensitivity to heat, which reduces their efficacy during fee processing, especially when using the extrusion process (Espinosa *et al*., 2020). However, Li *et al*. (2016) mentioned that the protease complex produced by Jefo Nutrition Inc. (Saint- Hyacinthe, QC, Canada) has the unique characteristic of being resistant to high temperatures, allowing it to be used in the production of shrimp feed using pelleting or extrusion process. Current research shows the efficacy of this unique protease complex in the shrimp *Penaeus vannamei* diet utilizing palm kernel meal (PKM).

Recently, regarding to the optimal use of local ingredients in Indonesia, PKM has received considerable attention as an aquafeed ingredients in line with the concern for continued availability and the rising costs of conventional feedstuffs in some parts of the world (Sundu *et al*., 2006). PKM is increasingly attractive because of its low economic value, making aquaculture production more competitive and efficient. However, there are some challenges on the use of PKM, including the amino acid profile, especially the availability of methionine and lysine as two essential amino acids for shrimp (Sundu *et al*., 2006; Xie *et al*., 2012; Ye *et al*., 2012). In shrimp, ensuring the adequate level of lysine and methionine in the diet is essential for promoting growth, maximizing feed efficiency and maintaining overall health. (Ye *et al*., 2012).

From the nutritional profile of the experimental diet (Table 2), the inclusion of palm kernel meal (PKM) to partially replace the use of soybean meal (SBM) has an impact on the protein and crude fiber levels in the diet. In both groups, feed with the addition of PKM has lower crude protein levels than without PKM. Meanwhile, fiber content increases with increasing use of PKM in feed. According to Grieshop *et al*. (2003), crude protein (CP) characteristics of SBM produced in 55 processing plants in the United States range from 51.6 – 54.6%, and the CP of PKM ranges from 14 – 21% (Akpodiete *et al*., 2006; Allen *et al*., 1997; Sekoni *et al*., 2008). From a comparative study using different sources of protein, Nwokolo & Bragg (1977) indicated that the crude fiber content of SBM was at the level of 6,5%, while PKM had 17.5% crude fiber content. Indeed, this will affect the growth performance of the shrimp.

In this research, the final mean weight (FMW) and percentage weight gain (PWG) were significantly higher in shrimp fed with 0 JPKM and 5 JPKM than in other dietary treatments (Table 4). The growth of shrimp fed with PKM without protease supplementa-

tion was significantly poorer with regard to FMW, PWG, and thermal growth coefficient (TGC). Adding PKM, both with and without protease supplementation, into the diet did not affect the survival rate (SR, %). Meanwhile, for the feed efficiency, the lowest feed conversion ratio (FCR) was found in the 0 JPKM group, with no significant difference from 5 JPKM. The poorest FCR was found in the shrimp group fed with PKM at all inclusion levels without protease (Table 4). Shrimp growth in this research proves that without PKM, shrimp had a better final weight than shrimp-fed PKM. Interestingly, in the protease-supplemented group, shrimp growth with 5% PKM (5 JPKM) did not significantly differ from 0 JPKM (control diet). This was possible since the use of protease in combination with a proper blending strategy with other protein sources can potentially reduce the need for expensive ingredients without compromising the quality of the feed (Bhowmik *et al*., 2015; Córdova-Murueta *et al*., 2017; Saetoo *et al*., 2019). The use of protease at higher levels in PKM has not yielded optimal results for shrimp growth, due to PKM's relatively high fiber content (Novriadi *et al*., 2025; Pangesti *et al*., 2023).

During the growth trial, water quality parameters recorded including pH, dissolved oxygen (DO), temperature, and salinity, were 8.20 ± 0.12; 7.40 ± 1.08 mgL^{-1}; 28,63 ± 1.06 °C; and 28.96± 0.93 ‰, respectively (Table 3). Additionally, Ammonia (NH_3-N), Nitrite (NO_2-N), Nitrate (NO_3-N) and Phosphate (PO_4-N) were 0.014±0.006 mgL^{-1}; 0.022 ± 0.007 mgL^{-1}; 4.659± 1.248 mgL^{-1}; and 0.023 ± 0.006 mgL^{-1}, respectively (Table 3). Overall, all water quality is still within the acceptable range for shrimp growth under outdoor pond conditions and not negatively impact the growth of Vannamei. It is important to note that monitoring water quality is crucial in nutritional studies, as the study must ensure that the growth of aquatic organisms is influenced solely by the intervention in the feed and not by other variables.

Table 3. Water quality data during the feeding trials for 90 days in the out-door ponds. Data were presented as mean± standard deviation (range)

Parameters	Unit	Analysis results
Dissolved oxygen	mg L^{-1}	7.40 ± 1.08
Temperature	^{0} C	28.63 ± 1.06
Salinity	‰	28.96 ± 0.93
pH		8.20 ± 0.12
Ammonia (NH_3-N)	mg L^{-1}	0.014 ± 0.006
Nitrite (NO_2-N)	mg L^{-1}	0.022 ± 0.007
Nitrate (NO_3-N)	mg L^{-1}	4.659 ± 1.248
Phosphate (PO_4-N)	mg L^{-1}	0.023 ± 0.006

Table 4. Growth performance of pacific white shrimp *Penaeus vannamei* (0.97 ± 0.01 g initial mean weight) fed experimental diets for 90 d in out-door ponds. Values represent the mean of five replicates. Results in the same columns with different superscript letter are significantly different (*P<0.05*) based on analysis of variance followed by Tukey's multiple comparison test

Diet code	Final Biomass (g)	Final Mean Weight (g)	Survival (%)	PWG[1] (%)	FCR[2]	TGC[3]	ADG[4]
0 JPKM	2872.00	13.86^{a}	74.06	1493.36^{a}	1.40^{d}	0.0568^{a}	0.1540^{a}
5 JPKM	2866.00	13.71^{a}	73.90	1476.14^{a}	1.41^{de}	0.0565^{ab}	0.1524^{a}
10 JPKM	2848.00	13.48^{b}	73.44	1448.97^{b}	1.44^{cd}	0.0559^{bc}	0.1497^{a}
15 JPKM	2834.00	13.39^{b}	73.08	1439.31^{b}	1.45b^{c}	0.0558^{c}	0.1488^{a}
0 PKM	2774.00	13.44^{b}	71.53	1445.06^{b}	1.44^{c}	0.0559^{c}	0.1494^{a}
5 PKM	2724.00	13.14^{c}	70.24	1410.34^{c}	1.48^{ab}	0.0552^{d}	0.1460^{a}
10 PKM	2726.00	12.96^{c}	70.29	1389.43^{c}	1.50^{a}	0.0547^{d}	0.1440^{b}
15 PKM	2720.00	12.94^{c}	70.14	1387.33^{c}	1.50^{a}	0.0547^{d}	0.1438^{b}
P-value	0.8450	<0.0001	0.8950	<0.0001	<0.0001	<0.0001	<0.0001
PSE	95.9961	0.0435	2.2986	4.9984	0.0053	0.0001	0.0011

Note: [1] PWG = Percentage weight gain; [2] FCR = Feed conversion ratio; [3] TGC = Thermal growth coefficient; [4] ADG = Average daily growth in gram per day, [5] PSE = Pooled standard error

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Table 5 presents the shrimp's whole body's proximate and amino acid profile at the end of the growth trial. In this research, the entire body protein in shrimp fed with control and PKM supplemented with protease was higher than that of shrimp fed with PKM without protease. Consequently, most amino acid levels were higher in the shrimp fed with a diet supplemented with protease than non-protease feed, especially for Arginine, Lysine, and cysteine. However, whole body fat was not affected by the dietary treatment, and the ash content increased with increasing levels of PKM.

Table 5. Nutritional profile of shrimp at the end of the growth trial[1]

Composition	Unit	0JPKM	5JPKM	10JPKM	15JPKM	0PKM	5PKM	10 PKM	15PKM
Ash Content	%	1.68	1.61	1.69	1.61	1.76	1.71	1.87	1.73
Total Fat	%	1.02	1.04	1.04	1.06	1.02	0.98	1.01	1.01
Moisture Content	%	72.51	73.57	72.55	71.84	72.68	72.48	70.72	75.21
Total Calories	Kcal/100 g	103.28	99.30	104.51	105.42	102.26	104.31	109.66	92.26
Carbohydrate (By Difference)	%	2.47	2.59	0.97	1.75	1.64	1.31	1.98	2.28
Protein Content	%	24.35	24.24	24.52	24.61	23.93	23.31	20.44	20.79
Crude Fiber	%	0.61	0.67	0.55	0.53	0.57	0.20	0.20	0.23
L-Glutamic Acid	%	3.87	3.85	3.78	3.54	3.60	3.72	3.42	3.14
L-Phenylalanine	%	0.99	1.00	1.16	1.37	1.51	1.36	1.26	0.78
L-Isoleucine	%	0.95	0.99	0.98	0.98	1.01	0.98	0.84	0.79
L-Valine	%	0.97	0.91	1.01	1.00	1.04	0.99	0.88	0.82
L-Alanine	%	1.55	1.51	1.59	1.48	1.51	1.61	1.51	1.30
L-Arginine	%	1.85	1.84	2.00	2.14	1.88	1.60	1.54	1.17
Glycine	%	1.78	1.64	1.85	1.93	1.89	2.00	1.78	1.45
L-Lysine	%	1.71	1.74	1.65	1.34	1.26	1.35	1.33	1.33
L-Aspartic Acid	%	2.21	2.21	2.16	1.97	1.86	2.03	1.95	1.74
L-Leucine	%	1.57	1.57	1.62	1.61	1.67	1.63	1.45	1.35
L-Tyrosine	%	0.68	0.63	0.78	0.96	1.05	0.97	0.88	0.58
L-Proline	%	1.41	1.39	1.24	1.33	1.41	1.30	1.26	1.11
L-Threonine	%	0.91	0.98	0.98	1.06	1.14	1.05	1.10	0.77
L-Histidine	%	0.59	0.59	0.71	0.78	0.85	0.77	0.59	0.49
L-Tryptophan	%	0.16	0.16	0.16	0.16	0.17	0.16	0.16	0.18
L-Cysteine	%	0.78	0.79	1.42	1.22	0.78	0.64	0.51	0.43
L-Methionine	%	0.39	0.37	0.36	0.39	0.36	0.35	0.35	0.36

[1]Analysed at Saraswanti Indo Genetech laboratory (Bogor, West Java, Indonesia)

Exogenous supplementation of protease not only maximizes the availability of digestible protein and growth metrics of aquatic species but also can support health and decrease the cumulative mortality after being challenged with the pathogen (Oladipupo *et al.*, 2023). In this research, the inclusion of protease complex significantly increases the total haemocytes count (THC) in shrimp but not lysozyme activity. In shrimp, haemocytes play a crucial role in the immune response of shrimp (Ji *et al.*, 2009), as they are involved in non-self-recognition, phagocytosis (ingestion of foreign particles), encapsulation of pathogens, and production of antimicrobial peptides (Burge *et al.*, 2007; Liu *et al.*, 2020; Miyata *et al.*, 1989). In this research, the number of THC was significantly higher in the group of shrimps fed with 0, 5, and 10 JPKM. The lowest THC was found in the group of shrimps fed with 15 PKM (Figure 1) Typically, an increase in THC may suggest an immune response to infection, while a decrease may indicate immunosuppression or stress (Novriadi *et al.*, 2021a; Novriadi *et al.*, 2021b). Proteases can indirectly contribute to improving the immune system in shrimp through their role in enhancing protein digestion and nutrient absorption, alleviate stress on the digestive system and maintaining gut health in shrimp by preventing the accumulation of undigested proteins and reducing the risk of gut dysbiosis (Hou *et al.*, 2024; Oladipupo *et al.*, 2023; Wu *et al.*, 2020).

Lysozyme is an enzyme that also plays a role in the non-specific immune system in shrimp (Burge *et al.*, 2007). Lysozyme acts as a natural antibacterial

agent by breaking down bacterial cell walls, thus helping to protect the organism against infections (Burge *et al*., 2007). Proteases may indirectly promote the activity of lysozymes by aiding in the breakdown of bacterial cell walls, thereby enhancing the effectiveness of lysozyme action. In this research, despite there is no statistically significant difference between groups in this research, numerically, an increase can be seen when compared within the same treatment, where feed with the addition of protease complex has better lysozyme levels compared to the non-protease feed. Research in juvenile channel catfish (*Ictalurus punctatus*) also found that the insignificant differences in lysozyme at 30 d and 60 d of growth trial, but numerically, the administration of similar protease complex could increase the activity at 8-days post infection in the challenge catfish with *Flavobacterium covae* (Oladipupo *et al*., 2023).

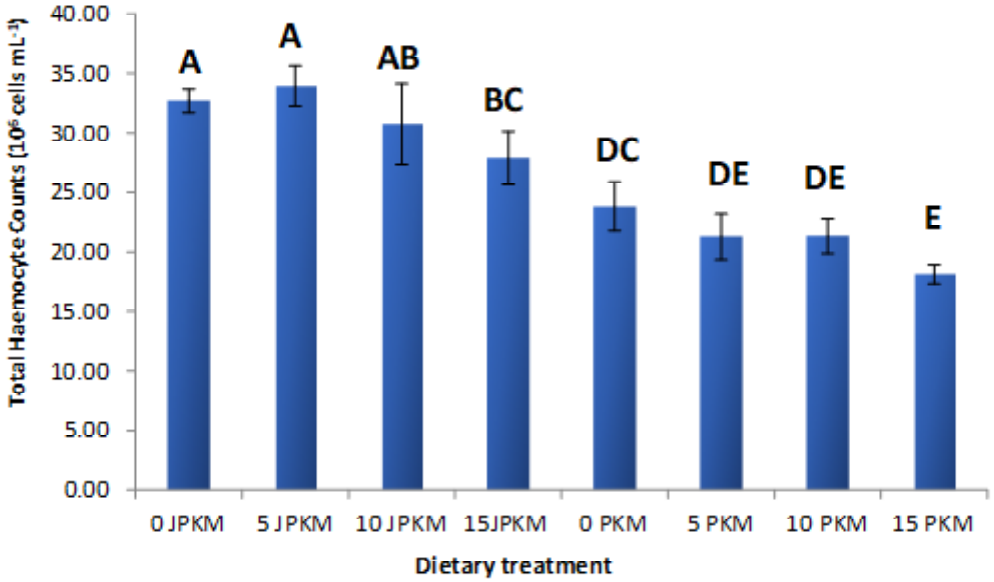


Figure 1. Relationship between inclusion of protease (Jefo Protease, Jefo Nutrition Inc., Canada) in the diet and total hemocyte count (THC) of Pacific white shrimp *Penaeus Vannamei* (10^6 cell mL^{-1}) at the end of the growth trial. Values represent the mean of fifteen replicates per dietary treatment.

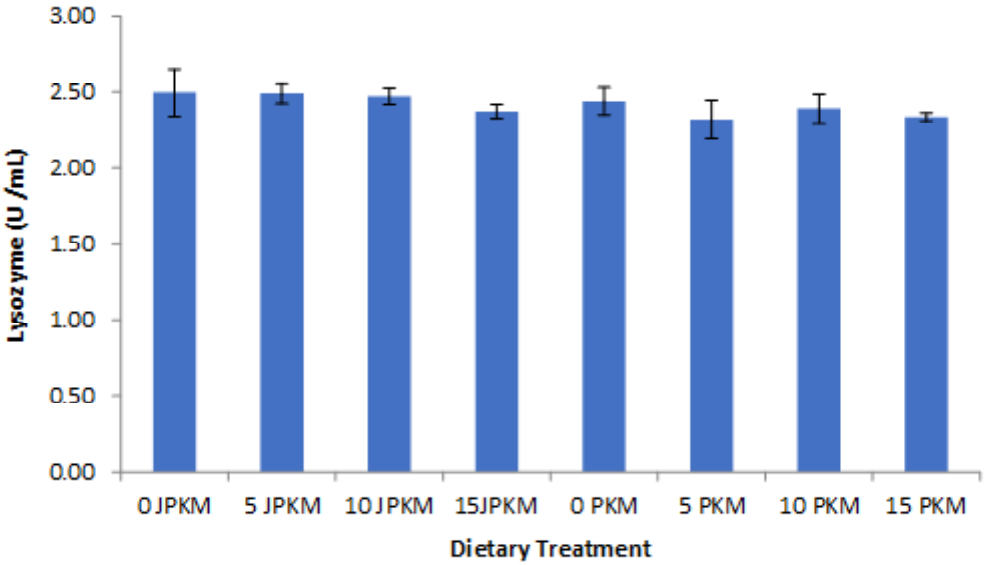


Figure 2. Relationship between inclusion of protease (Jefo Protease, Jefo Nutrition Inc., Canada) in the diet and lysozyme activity of Pacific white shrimp *Penaeus Vannamei* (U mL^{-1}) at the end of the growth trial. Values represent the mean of five replicates.

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Histological techniques can be used in nutritional studies to assess the effects of diet on the histological structure of shrimp tissues (Novriadi *et al.*, 2024a; Novriadi *et al.*, 2024c). In this research, after being fed with experimental feed, shrimp exhibited minor histological alterations in the hepatopancreas, with vacuolation found in all dietary treatments (Figure 3). Furthermore, large numbers of small vacuoles were found in the hepatopancreas of shrimp fed with PKM without protease. Histological observations also

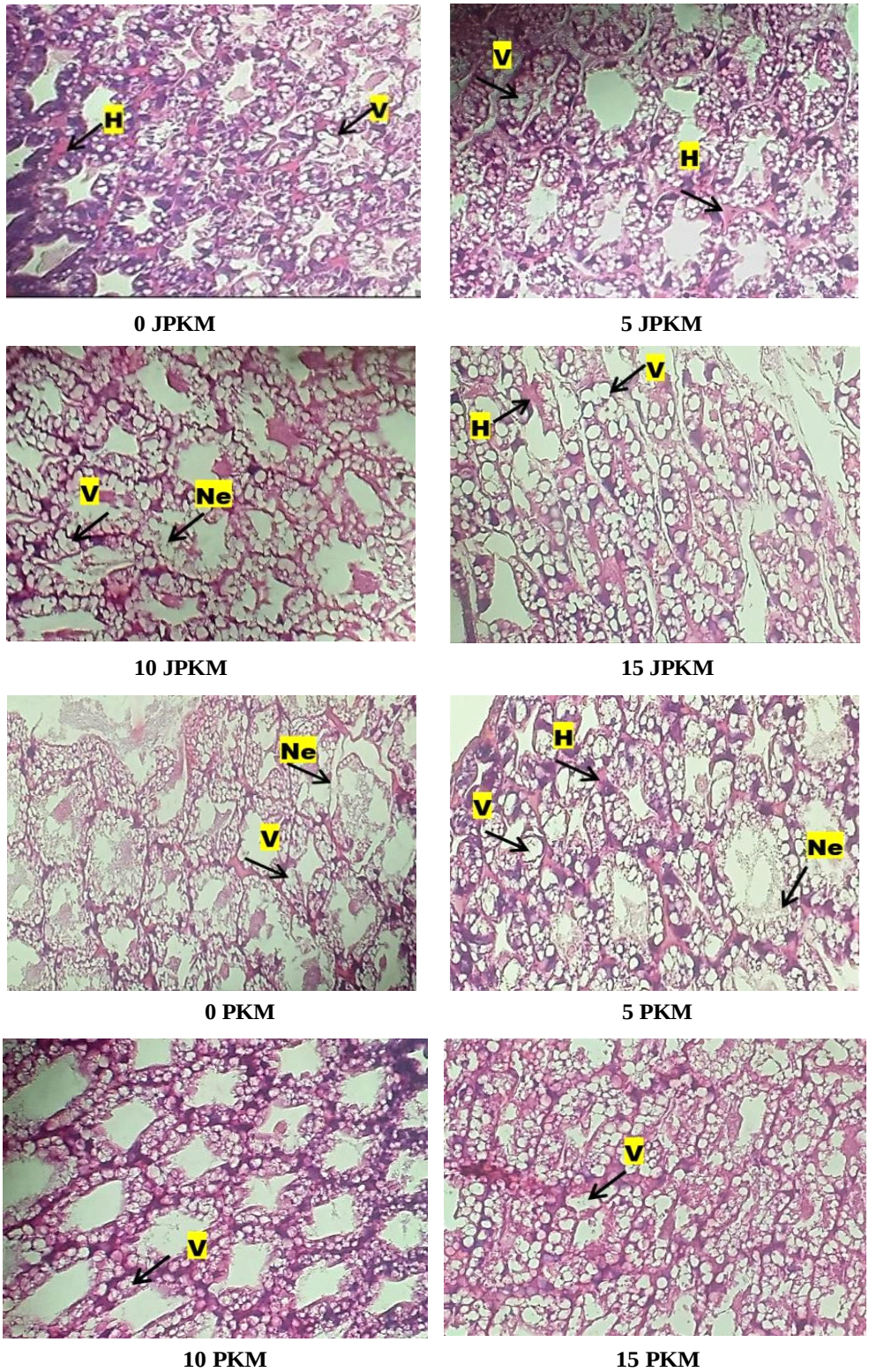


Figure 3. Representative histopathological images of hematoxylin and eosin-stained sections of the hepatopancreas of shrimp *P. vannamei* belonging to the different study groups. Please note that V = Vacuoles and Ne = Necrosis.

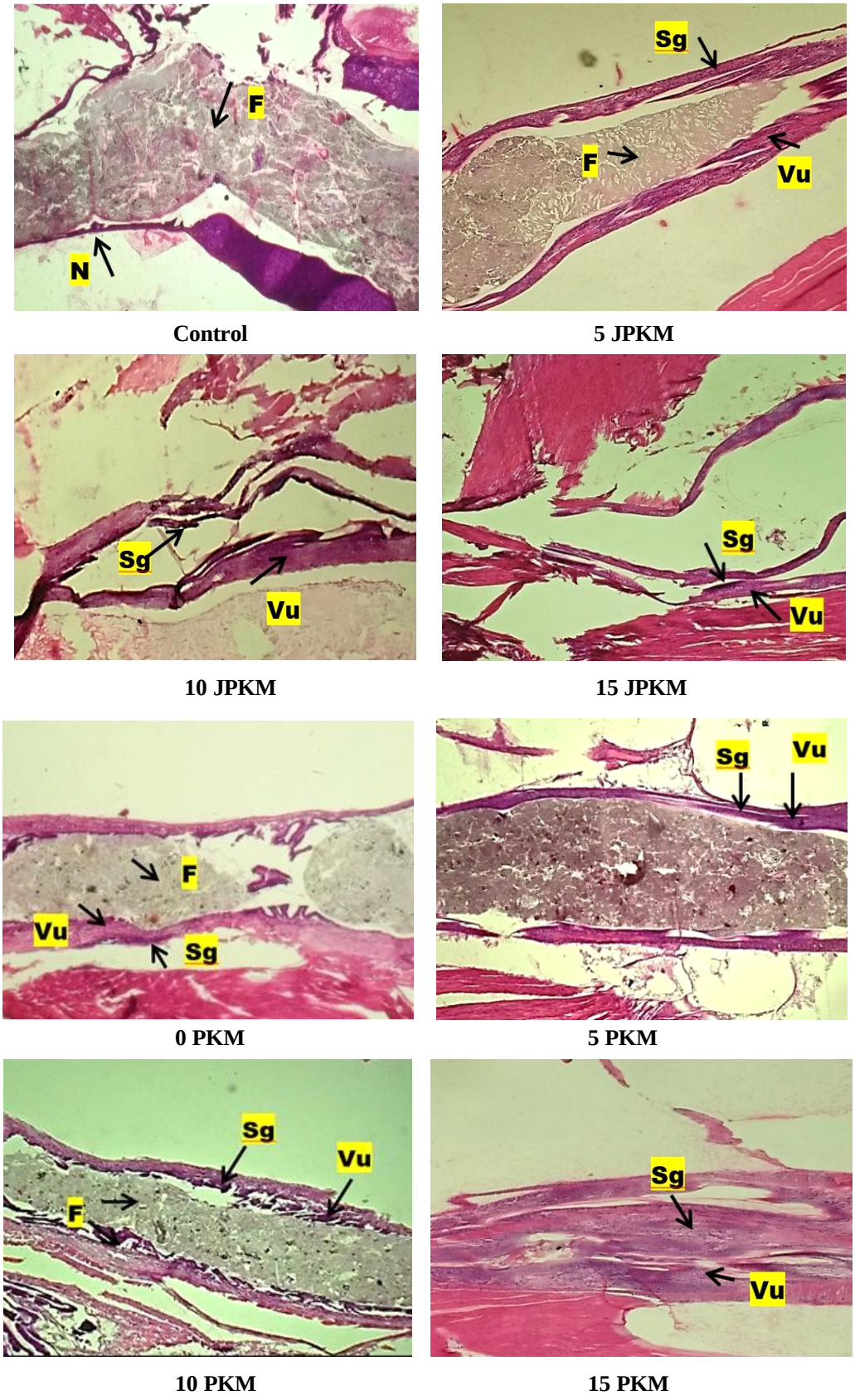


Figure 4. Representative histopathological images of hematoxylin and eosin-stained sections of distal intestines of shrimp belonging to the different study groups. Please note that F = Feces, Sg = Goblet cell, VU = Villi intestinal folds.

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showed that the inclusion of PKM positively influenced the gut structure. A significant increase in intestinal villi and goblet cells was observed in shrimp fed with PKM with protease (Figure 4). In shrimp, vacuolated cells in the hepatopancreas can be regarded as reliable biomarkers of toxic injury and disruption in the detoxification functions. (Novriadi *et al*., 2022; Zhang *et al*., 2023). The better condition of the hepatopancreas in a group of shrimps fed with PKM-diet supplemented with protease might be related to the function of protease in hydrolysis of protein, which breaks down protein macromolecules into peptides and free amino acids, which are more easily absorbed and used as a source of nutrition, thereby improving the performance of the hepatopancreas.

In this research, shrimp fed with PKM with protease have shown significant increase in intestinal villi and goblet cells compared to the digestive system condition in the group of shrimps fed with diet supplemented without protease. In line with our results, Hasanthi *et al*. (2023) reported that the inclusion of increasing levels of protease, resulting with the increase of villi height and relative abundance of heterotrophic marine bacteria, gram positive bacteria, and *Lactobacilli* spp. In addition, Li *et al*. (2016) showed an improvement of apparent digestibility of dry matter and crude protein by dietary protease in diets containing 30 g Kg^{-1} FM supplemented with protease. These findings suggest that protease supplementation in diet is beneficial for the gut health and nutrient absorption in shrimp *P. vannamei.*

CONCLUSION

The addition of 0.018% exogenous protease to feed formulated with 5% PKM to replace SBM provided significant growth of shrimp *P. vannamei* compared to the performance of shrimp fed other experimental feeds and provided performance that was significantly the same as the control diet. However, supplementation of exogenous protease to feed formulated with 10 and 15% PKM to replace SBM, although not providing the same growth as the control, was still better when compared to the performance of shrimp fed with the same amount of PKM without exogenous protease supplementation. In general, for health and histology parameters of the hepatopancreas and digestion, protease supplementation in feed containing PKM maintained conditions similar to the control and better than those of shrimp fed PKM-containing feed without exogenous protease supplementation.

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Conflicts of Interest: Didi Widjaya, Nguyen Ngoc Binh, and Hervé Lucien-Brun are employed by Jefo Nutrition Inc. The remaining authors state no conflict of interest.

Ethical Statement: All procedures and handling process in the present study were approved by the recommendations in the Guide for the Use of experimental Animals of the Jakarta Technical University of Fisheries

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, [RN], upon reasonable request.

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