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*Research Article*

# Effects of Dietary Tuna Viscera Hydrolysate Supplementation on Growth, Intestinal Mucosal Response, and Resistance to *Streptococcus iniae* Infection in Pompano (*Trachinotus blochii*)

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The effects of tuna viscera hydrolysate (TVH) on juvenile pompano *Trachinotus blochii*, growth performance, nutritional response, intestinal and liver health, and resistance to *Streptococcus iniae* were investigated in this study. Five isonitrogenous and isocaloric diets (protein 46.0%, lipid 10.0%) were formulated in which TVH was added to replace fishmeal protein at levels of 0 (control), 30, 60, 90, and 120 g $kg^{-1}$, labelled as TVH0, TVH05, TVH10, TVH15, and TVH20, respectively. Triplicate groups of pompano were fed the respective diets for ten weeks. The results showed that fish fed diets containing TVH10 produced significantly higher final body weight and specific growth rate in comparison to the fishmeal control ($P < 0.05$). Dietary TVH did not produce any effect on feed utilisation, somatic indices, and proximate composition of juvenile pompano ($P > 0.05$). While most amino acids were unchanged by the dietary inclusion of TVH, phenylalanine and valine levels were significantly lower in the fish fed TVH20 diet compared to the control. Fish fed the TVH20 diet had significantly lowered total serum protein compared to the TVH10 treatment, whereas other biochemical parameters in the blood did not show any difference among treatments. The intestinal histology indicated a significant increase in goblet cell numbers in fish fed TVH10 diet. Fish fed diet supplemented with TVH showed the highest disease resistance against *Streptococcus iniae* after 14 days of challenge. Based on a quadratic regression between final body weight and dietary TVH levels, the optimum TVH was calculated to be 10% or 60.0 g $kg^{-1}$ for maximum growth performance when fed to pompano.

## 1. Introduction

Snubnose pompano, *Trachinotus blochii*, is widely farmed throughout Asia, including Vietnam because of its fast-growing, high market price, and appropriateness for both pond and commercial sea-cage farming [1]. Current commercial aquafeeds for juvenile pompano in Vietnam contain relatively high protein contents (44–48%), in which fishmeal is used as the major protein source. However, the rapid development of global aquaculture has led to an increase in the demand for fishmeal in synchrony with the well-documented static supply of this raw material [2]. In addition to the issues faced around the supply marine-derived raw materials for aquafeed production, intensive farming of pompano is challenged by disease outbreaks, especially bacterial diseases, which are commonly reported in pompano cultured in sea-cage systems in several farming areas of Vietnam [3]. Bacterial species isolated from diseased pompano include *Streptococcus* spp., *Escherichia coli*, *Salmonella* spp., *Pseudomonas* spp., *Norcadia seriolae*, and *Vibrio* spp. [4, 5]. As such, identifying fishmeal alternatives that contain bioactive compounds that improve growth and health via the enhancement of nonimmune specific systems against pathogens is identified as a high priority for the sustainable growth of pompano farming.

Seafood processing industries generate over 60% of by-products as waste, including head, skin, fins, viscera, and bones, which may cause serious pollution and disposal problems in both developed and developing countries [6]. Several biotechnologies have been established to retrieve the essential nutrients and bioactive compounds from these waste streams to produce high biologically active fish protein hydrolysates (FPH) [7]. The enzymatic hydrolysis converts native proteins in fish waste into smaller peptides. These contain 2-20 amino acids with a low molecular weight, increasing intestinal assimilation given their good functional properties ([8]: [9]). The utilisation of protein hydrolysates as an alternative protein source to traditional fishmeal has been investigated in some marine fish species. Siddik et al. [10] indicated that the replacement of 100 g kg$^{-1}$ fishmeal protein with tuna hydrolysate improved growth, immunity, intestinal mucosal morphology, and resistance against bacterial infection in juvenile barramundi *Lates calcarifer*. A significant improvement in apparent digestibility coefficients and growth has also been observed in Atlantic salmon *Salmo salar* [11], red seabream *Pagrus major* [12], olive flounder *Paralichthys olivaceus* [13, 14], and barramundi [10, 15–17] fed diets containing appropriate levels of FPH supplementation.

However, excessive dietary FPH inclusion can also impair the growth, digestibility, and immune response in fish. Siddik et al. [17] found that barramundi fed tuna hydrolysates at dietary inclusion of 415 or 590 g kg$^{-1}$ exhibited reduced growth and nutrient digestibility in comparison to fish fed fishmeal-based diets. Fish fed high dietary FPH also recorded a reduction in glutathione peroxidase activity, increased lipid accumulation, and necrosis in the liver tissue [17]. Significant depression in growth and feed utilisation were also observed in olive flounder fed diets containing more than 160 g kg$^{-1}$ of FPH [18], whereas this threshold in turbot *Scophthalmus maximus* was found to be 200 g kg$^{-1}$ [19]. Recently, Tejpal *et al.* [20] demonstrated enhanced growth performance and feed efficiency in snubnose pompano fed FPH; however, in comparison to commercial growth rates, the performance of fish was low regardless of the experimental treatment. Moreover, the amino acid profile, feed digestibility, and histological change of pompano fed dietary FPH were not evaluated. Notably, considering diverse nutritional values of FPH derived from different native protein sources, differences in enzymatic specifications, and hydrolysis conditions [6, 7], the investigation of alternative FPH requires further investigation to ascertain efficacy. Therefore, the aim of the current study was to determine the effects of including tuna viscera hydrolysate (TVH) in practical diets on growth, nutritional response, intestinal and liver health, and resistance against bacterial pathogen juvenile pompano.

## 2. Materials and Methods

*2.1. Experimental Site.* The experiment was carried out at Nha Trang University (NTU), Khanh Hoa, Vietnam. Animal care was fully compliant with the Vietnamese Code of practice for the care of animals for scientific purposes.

*2.2. Fish Hydrolysate Preparation.* Yellow fin tuna *Thunnus albacares* viscera was provided from the Hai Vuong Co., Ltd (Khanh Hoa, Vietnam) and minced and stored in a freezer for TVH preparation. Alcalase provided from Novozymes (Novozymes, Bagsvaerd, Denmark) with a declared activity of 2.4 AU/g and a density of 1.18 g/mL was used for the hydrolysis. The hydrolysate was prepared following the protocol detailed by Ovissipour et al. [21] with minor modifications as mentioned in Pham *et al.* [22]. Briefly, the minced viscera were mixed with 0.1 M sodium phosphate buffer and homogenised in a blender for 2 min. The optimum pH (8.5) for activity of Alcalase was adjusted using 0.2 N NaOH. Enzyme was added at 5 g/kg of the total protein of raw material. All reactions were performed in a shaking incubator with constant agitation (150 rpm) at 50°C in 4 hours. The solution was then heated at 90°C for 15 min to deactivate enzyme. The solution was then centrifuged at 7500 rpm at 4°C for 40 min to collect supernatant (TVH) and then stored at -20°C until analysis. The nutritional profiles of fishmeal and TVH are displayed in Table 1. The dried TVH contains 61.07% protein, 7.80% lipid, and 9.17% ash.

*2.3. Diet Preparation.* A basal diet (TVH0) containing 46.5% crude protein derived largely from fishmeal, and 11.0% lipid from fish oil was formulated and utilised as a control diet. Four other diets were formulated by substitution of fishmeal in the control diet with TVH at levels of 0 (control), 30, 60, 90, and 120 g kg$^{-1}$, labelled as TVH05, TVH10, TVH15, and TVH20, respectively. All feed ingredients were purchased from Long Sinh Feed Company (Khanh Hoa, Vietnam), except fishmeal which was supplied from TC Union Viet Nam (Tien Giang, Vietnam). The test diets were produced at NTU following the methods described by Pham et al. [23]. The formulation, proximate, and amino acid profiles of the test diets are presented in Table 2.

*2.4. Fish Rearing.* A commercial marine fish hatchery provided the juvenile pompano (Nha Trang, Khanh Hoa, Vietnam). During acclimation, fish were fed a commercial pelleted feed (NRD G12, INVE Ltd, Thailand) providing 53.0% crude protein, 12.0% lipid, and 21.0 MJ/kg gross energy for two weeks. After acclimation, juveniles (average weight of 3.14 g/fish) were randomly dispersed into 15 350 L tanks with three replicates for each treatment, at a density of 20 fish in each replicate. For ten weeks, the fish were fed till they were satisfied twice a day, at 0800 and 1600 hrs. The following parameters were monitored within a reasonable range for pompano culture: dissolved oxygen was maintained higher than 5.0 mg/L, average temperature was 29.0°C, total ammonia was less than 0.25 mg/L, and salinity ranged from 29.0 to 32.0 ppt.

Table 1: Proximate composition and amino acid profiles of viscera tuna hydrolysates.

| | Fishmeal | Viscera tuna hydrolysates |
|---|---|---|
| Proximate composition (% dry matter) | | |
| Crude protein | 60.30 | 61.07 |
| Crude lipid | 10.20 | 7.80 |
| Ash | 20.90 | 9.17 |
| Histamine (ppm) | <500 | — |
| Essential amino acids (g/100 g dry weight) | | |
| Arginine | 3.95 | 4.19 |
| Phenylalanine | 2.32 | 2.42 |
| Leucine | 4.24 | 4.29 |
| Lysine | 4.37 | 4.48 |
| Methionine | 1.84 | 1.49 |
| Isoleucine | 2.91 | 2.61 |
| Histidine | 1.83 | 2.95 |
| Threonine | 2.63 | 2.98 |
| Valine | 3.11 | 3.35 |

*2.5. Sample Collection.* Fish were individually weighed to record final weight at the end of the trial, after 24 hours of feed deprivation. Three fish were taken from each tank for proximate composition study and another three for amino acid analysis. A heparinised syringe was used to draw blood from the caudal vein, and an aliquot was extracted and preserved for whole blood haematological analysis. Serum was sampled by centrifuging the remaining blood aliquot at 5000 g for 10 min at 4°C for biochemical measurement. The hepatic and intestinal tissues of pompano in each tank were cut and immersed in 10% buffered formalin. Faeces samples were collected from the eighth week using the settlement method expressed by Kim et al. [24] with modifications. The faecal substrate was separated by centrifugation from the whole sample for 20 min at 10,000 rpm, and then stored at −20°C until further use.

*2.6. Chemical Analysis.* Protein, lipid, moisture, and ash analyses were performed following standard methods [25]. Moisture by baking the samples at 105°C to a constant weight; protein by nitrogen analysis using the Kjeldahl method; lipid by ether extraction using Soxhlet determination; ash by burning at 550°C in 24 h. Muscle amino acids were determined after acid hydrolysis by gas chromatography with GC 2010 Plus (Shimadzu, Kyoto, Japan). The fatty acid composition of the whole body was subjected to a methylated ester method following the procedures detailed by O'Fallon et al. [26]. Using a spectrophotometer UV-1201 (Shimadzu, Kyoto, Japan), chromatic oxide was measured using the technique of Bolin et al. [27]. A Cobas Roche 6000 blood analyser was used to assess the haematological and biochemical characteristics of the blood.

*2.7. Growth Parameters and Digestibility Analysis.* The growth parameters and somatic indexes were calculated using the following equations:

$$
\begin{aligned}
&\text{Specific growth rate (SGR, \%/day)} \\
&\quad = \left[\frac{\ln\ (\text{final body weight}) - \ln\ (\text{pooled initial body weight})}{\text{days}}\right] \times 100, \\
&\text{Feed intake (TFI, g)} = \left[\frac{\text{dry feed consumed}}{\text{fish number}}\right], \\
&\text{Feed conversion ratio (FCR)} = \left[\frac{\text{dry feed fed}}{\text{wet weight gain}}\right], \\
&\text{Protein retention (PR)} = \left[\frac{\text{protein gain}}{\text{protein fed}}\right], \\
&\text{Protein efficiency ratio (PER)} = \left[\frac{\text{weight gain}}{\text{protein fed}}\right], \\
&\text{Hepatosomatic index (HSI, g liver/100g body weight)} \\
&\quad = \left[\frac{\text{liver weight}}{\text{body weight}}\right] \times 100, \\
&\text{Viscerasomatic index (VSI, g viscera/100g body weight)} \\
&\quad = \left[\frac{\text{viscera weight}}{\text{body weight}}\right] \times 100, \\
&\text{Survival (\%)} = \left[\frac{\text{number of final fish}}{\text{number of initial fish}}\right] \times 100.
\end{aligned} \tag{1}
$$

The apparent digestibility coefficient (ADC) of dry matter, protein, and lipid was assessed by the following equation:

Apparent digestibility coefficient (ADC) was determined according to Cho et al. [28]:

$$
\text{ADC} = \left[1 - \left(\frac{Cr_2O_2 \text{ in diet}}{Cr_2O_2 \text{ in faeces}}\right) \times \left(\frac{\text{Nutrient in faeces}}{\text{Nutrient in diet}}\right)\right] \times 100. \tag{2}
$$

*2.8. Histological Examination.* After dehydration in ethanol, fixed intestine and liver were equilibrated in xylene before embedding in paraffin wax. Sections (5-6 $\mu$m) were cut and

Table 2: Dietary ingredients and the nutrient compositions of the tested diets.

| Ingredients | Experimental diets (g kg$^{-1}$, dry weight) | | | | |
|---|---|---|---|---|---|
| | TVH0 | TVH05 | TVH10 | TVH15 | TVH20 |
| Fishmeal[1] | 630.0 | 598.5 | 567.0 | 535.5 | 504.0 |
| TVH | 0 | 30.0 | 60.0 | 90.0 | 120.0 |
| Soybean meal[2] | 90.0 | 90.0 | 90.0 | 90.0 | 90.0 |
| Wheat gluten[2] | 50.0 | 50.0 | 50.0 | 50.0 | 50.0 |
| Fish oil[2] | 50.0 | 51.0 | 52.0 | 53.0 | 54.0 |
| Cellulose[2] | 2.0 | 2.5 | 3.0 | 3.5 | 4.0 |
| Vitamin premix[2] | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Wheat flour[2] | 161.0 | 161.0 | 161.0 | 161.0 | 161.0 |
| $CaCO_3$[3] | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Dicalcium phosphate[3] | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| NaCl[3] | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| $Cr_2O_3$[3] | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| *Proximate compositions (%)* | | | | | |
| Gross energy (MJ/kg) | 21.05 | 21.17 | 21.65 | 22.04 | 22.30 |
| Dry matter | 90.70 | 90.34 | 89.71 | 88.29 | 90.44 |
| Crude protein | 46.73 | 46.82 | 46.35 | 46.73 | 46.21 |
| Crude lipid | 11.27 | 11.76 | 11.41 | 11.23 | 10.87 |
| Ash | 9.60 | 8.18 | 7.51 | 6.58 | 5.20 |
| *Essential amino acids (g/100 g)* | | | | | |
| Arginine | 3.41 | 3.43 | 3.44 | 3.47 | 3.51 |
| Histidine | 1.25 | 1.26 | 1.25 | 1.27 | 1.27 |
| Isoleucine | 2.05 | 2.12 | 2.09 | 2.11 | 2.10 |
| Leucine | 3.54 | 3.56 | 3.61 | 3.59 | 3.63 |
| Lysine | 3.86 | 3.88 | 3.85 | 3.88 | 3.88 |
| Methionine | 1.38 | 1.39 | 1.39 | 1.41 | 1.43 |
| Phenylalanine | 1.75 | 1.78 | 1.81 | 1.79 | 1.83 |
| Threonine | 2.03 | 2.11 | 2.20 | 2.28 | 2.31 |
| Valine | 2.34 | 2.37 | 2.32 | 2.36 | 2.34 |

[1]Fishmeal: tuna fishmeal, TCnUnion, Tien Giang, Vietnam; [2]LongSinh Feed, Khanh Hoa, Vietnam; [3]Thermo Fisher Scientific, Vietnam; TVH: tuna viscera hydrolysate.

stained with H&E solutions and observed under light microscopy (Leica) at different magnifications. Image J was used to evaluate and measure the fold height, microvilli, and enterocyte number following the methods of Escaffre et al. [29].

*2.9. Bacterial Challenge. Streptococcus iniae* acquired from the Center for Aquatic Animal Health and Breeding (Nha Trang University, Vietnam) was used as the pathogenic agent for assessment in the current study. The bacterium was isolated from infected pompano and then cultured in a 250-mL Erlenmeyer flask, containing 100 mL tryptic soy broth (TSB) and 25 g kg$^{-1}$ NaCl at 28°C for 8 h. After 10 weeks, 10 juvenile pompano per tank were transferred to another 100 L fiber-glass tank for a bacterial challenge. Fish were injected intraperitoneally (IP) with 100 $\mu$L of *S. iniae* containing $1 \times 10^5$ CFU/mL ($1 \times 10^5$ CFU per fish). After the IP challenge, fish were restocked into their respective tanks and fed their respective test diets once daily. The challenge test lasted for 2 weeks.

*2.10. Statistical Analyses.* Data were analysed with one-way ANOVA using SPSS 21.0 (IBM, New York, USA). The significance of differences between treatments was determined using Tukey's HSD post hoc test. The SGR was regressed against dietary TVH inclusion levels using a quadratic regression model. The Kaplan-Meier method was used to create the survival graph. The significant difference was evaluated at $P < 0.05$.

## 3. Results

*3.1. Growth Performance, Feed Utilisation, and Biometric Indices.* Fish readily accepted all experimental diets and no significant differences were observed in the survival rate of pompano following the ten weeks of experimentation. Final biomass, feed utilisation, and somatic indices of pompano fed the various dietary TVH inclusion levels are shown in Table 3. Fish fed TVH10 (60 g kg$^{-1}$) had higher ($P < 0.05$) FBW and SGR compared to those fed the control diet. Statistically, dietary TVH did not influence FI, FCR, PR, PER, VSI,

Table 3: Growth performance, somatic indices, and feed intake of juvenile pompano fed diets supplemented with TVH for 10 weeks.

| | Experimental diets | | | | | SEM | P-value |
|---|---|---|---|---|---|---|---|
| | TVH0 | TVH05 | TVH10 | TVH15 | TVH20 | | |
| FBW (g/fish) | $32.74^{a}$ | $35.00^{ab}$ | $37.53^{b}$ | $35.37^{ab}$ | $33.00^{a}$ | 0.533 | 0.003 |
| SGR (%/day) | $3.35^{a}$ | $3.44^{ab}$ | $3.54^{b}$ | $3.46^{ab}$ | $3.36^{a}$ | 0.022 | 0.003 |
| FI (g/fish) | 37.86 | 40.36 | 40.51 | 40.07 | 39.53 | 0.393 | 0.192 |
| FCR | 1.16 | 1.15 | 1.08 | 1.13 | 1.20 | 0.015 | 0.154 |
| PR (%) | 29.43 | 30.15 | 31.33 | 29.92 | 27.89 | 0.483 | 0.262 |
| PER (%) | 1.70 | 1.72 | 1.84 | 1.74 | 1.63 | 0.026 | 0.089 |
| VSI (%) | 8.26 | 7.95 | 7.96 | 7.78 | 7.90 | 0.069 | 0.287 |
| HSI (%) | 1.18 | 1.14 | 1.11 | 1.09 | 1.04 | 0.020 | 0.200 |
| Survival (%) | 93.33 | 93.33 | 96.67 | 96.67 | 93.33 | 1.031 | 0.707 |

Values presented in the table were mean of triplicate groups. Means with the various superscript letters within the same row indicated significant differences at $P < 0.05$. FBW: final body weight; SGR: specific growth rate; FI: feed intake; FCR: feed conversion ratio; PR: protein retention; PER: protein efficiency ratio; VSI: viscerasomatic index; HSI: hepatosomatic index.

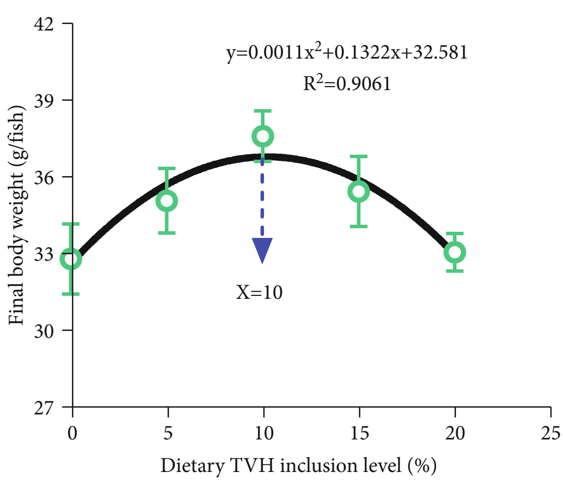



Figure 1: Final body weight (FBW) of juvenile pompano fed varied levels of TVH for 10 weeks was analysed using quadratic regression. The TVH threshold for the highest FBW for juvenile pompano is indicated by an asterisk "X." Each nutritional treatment's data point in the graph was the average of three replicate tanks. The optimal TVH level obtained with the quadratic regression analysis for the highest FBW was 10.0% in the diet.

and HSI of juvenile pompano. The optimum dietary TVH was estimated to be 60.09 g $kg^{-1}$ diet based on quadratic regression analysis (Figure 1).

Dietary TVH inclusion significantly enhanced the ADC of protein and lipid. Fish fed the TVH10 diet had higher protein and lipid ADC than fish fed the control diet ($P < 0.05$). However, the protein and lipid ADC showed significantly reducing trend with increasing TVH inclusion level resulting in significantly lower protein and lipid ADC of fish fed the TVH20 treatment in comparison to those fed TVH10 ($P < 0.05$). Dietary TVH had no effect on dry matter ADC ($P > 0.05$) (Figure 2).

*3.2. Biochemical Composition.* The whole body and muscle tissue compositions of juvenile pompano were unaffected by the dietary inclusion of TVH ($P > 0.05$) (Table 4). Likewise, with the exception of phenylalanine, no differences were observed in muscle amino acid profiles of fish fed the experimental diets ($P > 0.05$). Fish fed the TVH15 and TVH20 diets had significantly lowered phenylalanine concentrations in the muscle tissue compared to the control ($P < 0.05$) (Figure 3).

The fatty acid composition of pompano fed the tested diets is presented in Table 5. The lowest total saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) were observed in fish fed the control diet and significantly different compared to those fed diets with TVH supplementation ($P < 0.05$), while total monounsaturated fatty acids (MUFA) was not affected by TVH supplementation. The content of highly unsaturated fatty acids (HUFA), particularly eicosapentaenoic acids (20:5n-3, EPA), was significantly higher in fish fed dietary TVH supplementation ($P < 0.05$), whereas docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ARA) have no significant difference among treatments ($P > 0.05$). With respect to fatty acids, the lowest SFA and PUFA concentrations were observed in fish fed the control diet and significantly different compared to those fed diets with TVH ($P < 0.05$). However, no differences were observed in MUFA concentration among the dietary groups. The content of long chain polyunsaturated fatty acids (LC-PUFA), driven particularly EPA, was significantly higher in fish fed the diets supplemented with TVH ($P < 0.05$), whereas DHA and ARA concentrations were largely consistent among the treatments ($P > 0.05$) (Table 5).

*3.3. Haematological Parameters.* Dietary TVH inclusion did not influence haematological parameters (i.e., Ht, Hb, and RBC concentrations) in pompano after ten weeks of feeding trial ($P > 0.05$) (Table 6). Similar trends were also observed in glucose, cholesterol, albumin, triglyceride, aspartate transaminase, and alanine aminotransferase levels of pompano fed experimental diets ($P > 0.05$). However, total protein decreased significantly in fish fed the diets containing 120 g $kg^{-1}$ TVH compared to those fed with 60 g $kg^{-1}$ TVH included diet ($P < 0.5$).

*3.4. Histology.* Juvenile pompano fed diets supplemented with TVH showed normal hepatocytes in all dietary

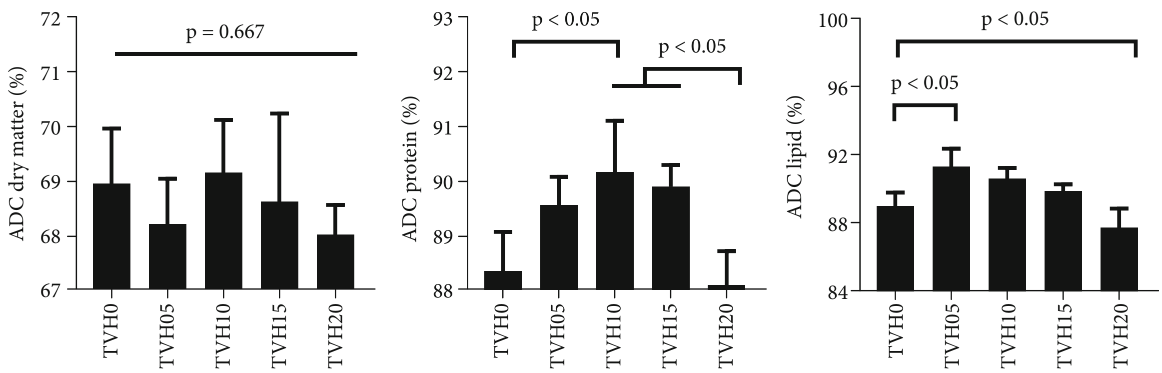
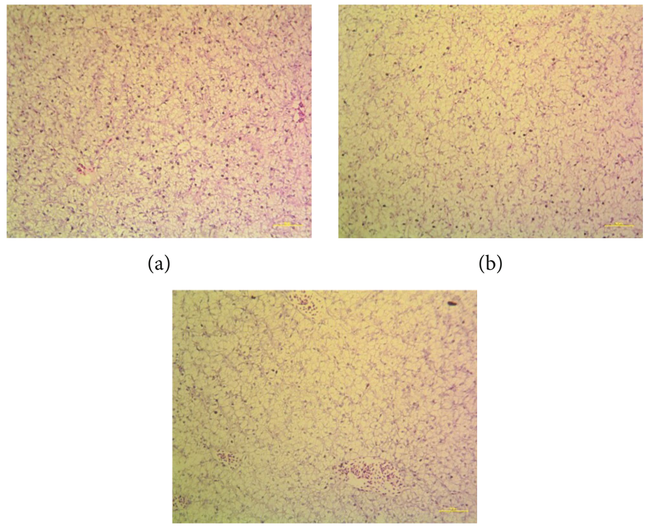



FIGURE 2: Apparent digestibility coefficients (ADC) of dry matter, protein, and lipid of fish fed varying levels of TVH for 10 weeks. Bar holding $p$-value indicates statistically different from the control at $P < 0.05$.

TABLE 4: Nutritional compositions of juvenile pompano fed dietary tuna hydrolysate supplementation for 10 weeks.

| Parameters | Experimental diets TVH0 | TVH05 | TVH10 | TVH15 | TVH20 | *SEM* | *P -value* |
|---|---|---|---|---|---|---|---|
| Proximate compositions (%, wet weight basis) | | | | | | | |
| *Whole body* | | | | | | | |
| Moisture | 66.87 | 68.05 | 66.95 | 68.42 | 66.90 | 0.246 | 0.093 |
| Crude protein | 17.01 | 17.20 | 16.79 | 16.95 | 16.88 | 0.089 | 0.715 |
| Crude fat | 8.35 | 8.66 | 8.21 | 8.39 | 8.59 | 0.069 | 0.224 |
| Ash | 4.31 | 4.20 | 4.29 | 4.29 | 4.27 | 0.051 | 0.979 |
| *Muscle* | | | | | | | |
| Moisture | 74.17 | 74.28 | 74.48 | 74.14 | 74.40 | 0.090 | 0.762 |
| Crude protein | 20.47 | 20.41 | 20.28 | 20.49 | 20.73 | 0.121 | 0.871 |
| Crude fat | 2.36 | 2.35 | 2.25 | 2.42 | 2.43 | 0.031 | 0.519 |
| Ash | 1.12 | 1.11 | 1.16 | 1.08 | 1.19 | 0.039 | 0.951 |
| Muscle amino acid composition ($g\,kg^{-1}$ dry weight basis) | | | | | | | |
| *Nonessential amino acids* | | | | | | | |
| Alanine | 45.00 | 44.27 | 45.80 | 45.87 | 45.60 | 0.347 | 0.618 |
| Aspartic acid | 71.90 | 72.83 | 73.03 | 74.43 | 74.50 | 0.424 | 0.241 |
| Glutamine | 118.50 | 118.87 | 120.60 | 120.77 | 119.47 | 0.570 | 0.702 |
| Glycine | 34.43 | 34.83 | 35.97 | 35.73 | 35.40 | 0.267 | 0.376 |
| Proline | 30.97 | 31.37 | 35.97 | 35.73 | 35.40 | 0.268 | 0.847 |
| Serine | 30.47 | 29.90 | 29.53 | 29.63 | 30.13 | 0.219 | 0.724 |
| Tyrosine | 23.07 | 22.77 | 22.90 | 22.83 | 23.06 | 0.226 | 0.994 |
| Total NEAAs | 354.33 | 354.83 | 359.30 | 360.82 | 359.11 | 1.028 | 0.154 |
| *Essential amino acids* | | | | | | | |
| Arginine | 42.40 | 44.00 | 44.76 | 43.60 | 43.06 | 0.338 | 0.224 |
| Histidine | 17.97 | 18.10 | 18.33 | 18.16 | 18.06 | 0.069 | 0.594 |
| Isoleucine | 35.20 | 34.89 | 35.56 | 35.20 | 35.30 | 0.119 | 0.583 |
| Leucine | 60.61 | 59.73 | 61.16 | 60.26 | 60.20 | 0.181 | 0.117 |
| Lysine | 63.60 | 62.80 | 62.91 | 62.63 | 62.30 | 0.323 | 0.833 |
| Methionine | 22.00 | 22.10 | 22.61 | 21.60 | 22.00 | 0.159 | 0.447 |
| Phenylalanine | 31.33 | 30.73 | 30.96 | 30.41 | 30.40 | 0.120 | 0.129 |
| Threonine | 30.53 | 30.40 | 31.13 | 30.61 | 30.20 | 0.159 | 0.482 |
| Valine | $38.90^{b}$ | $36.50^{ab}$ | $35.10^{a}$ | $33.80^{a}$ | $33.53^{a}$ | 0.594 | 0.002 |
| Total EAAs | 342.53 | 339.26 | 342.52 | 336.26 | 335.06 | 1.106 | 0.063 |

Values are presented as means of triplicate groups of fish from various tanks. Means values with different superscript letters in a row indicate significant differences at $P < 0.05$.



(a) (b) (c)

Figure 3: Liver microscopic structure of juvenile pompano fed dietary TVH for 10 weeks. (a) control group, (b) TVH10, and (c) TVH20. Stained with haematoxylin and eosin.

treatments (Figure 3). The intestinal fold height (Fh), microvilli height (Mvh), and goblet cells (Gc) number in fish fed the diet containing 60 g kg$^{-1}$ TVH were significantly increased in comparison to the control diet. However, intestinal fold width (Fw) and muscular wall width (Mw) were not influenced by TVH supplementation (Figure 4).

*3.5. Challenge Test.* The first mortality was recorded on the third day following bacterial infection in the TVH0 treatment. Dietary TVH significantly enhanced the survival of juvenile pompano following the challenge with *S. iniae*. Kaplan-Meier analysis indicated a significantly higher survival rate in pompano fed TVH05 and TVH10 diets in comparison to the TVH0 control group ($P < 0.01$) (Figure 5). However, fish fed TVH15 and TVH20 diets did not show any significant difference compared to TVH0 fish.

## 4. Discussion

In fish, the positive benefits of dietary FPH on growth performance and feed efficiency have been thoroughly demonstrated [10, 12, 14, 30–32] and attributed to superior nutritional properties [6, 7] and favourable functional attributes [33]. However, there is limited information about the effect of FPH in pompano species. In the current study, dietary tuna hydrolysate derived from viscera of yellowfin tuna was tested at 5, 10, 15, and 20% in diets for juvenile pompano, where an inclusion level of 10% was found to elicit a significantly higher FBW and SGR than the control. This is consistent with results reported in barramundi [10, 16], red seabream [12, 14], olive flounder [13], Atlantic salmon [34], yellow croaker *Pseudosciaena crocea* [32], Japanese seabass *Lateolabrax japonicus* [35], and other pompano species [20, 36]. The results showed that fish fed appropriate levels of FPH (3–10% in diet) had improved growth performance attributable to increased palatability [34], improved availability, and subsequent uptake of free amino acids and bioavailable peptide fractions produced via the enzymatic hydrolysis process [6, 19].

However, FPH levels beyond a certain threshold have been shown to have deleterious impacts on the growth performance of various fish species. In a study on barramundi fed diets containing 415 and 590 g kg$^{-1}$ FPH exhibited lower FBW than fish fed fishmeal-based diets in a research on barramundi [17]. Similarly, depressed growth performance and reduced feed utilisation have been observed in Japanese flounder fed diets containing FPH above 160 g kg$^{-1}$ [18], whereas this threshold was found at 200 g kg$^{-1}$ in turbot [19]. In the present study, the FBW and SGR of fish fed diets containing more than 10% or 60 g kg$^{-1}$ TVH started to decline in comparison to the lower inclusion levels after 70 days of feeding. An exclusive level of free AAs and low molecular weight peptides, resulting in an imbalance in amino acid intake and saturation of peptide transportation systems, could explain the negative consequences of high FPH inclusion levels in fish growth [37, 38]. In contrast, Tejpal *et al.* [20] found a positive linear relationship between dietary tilapia hydrolysate levels and SGR of pompano. However, tilapia hydrolysate was tested for only 40 days, and juvenile pompano fed the experimental diets demonstrated lowered SGR (1.08–1.47%/day). In the current study, the TVH was evaluated for 70 days, and all fish demonstrated SGRs consistent with the commercial reality (>3.30%/day). However, perhaps more important is the high likelihood that the different native protein sources and enzymatic specification used to produce tilapia hydrolysate and tuna hydrolysate would lead to the variation of nutritional values in FPH, resulting in different growth performance in the fish. Ultimately, different FPH should be tested in unison to further explore the comparative efficacy of these products.

During enzymatic hydrolysis, the native protein is converted into small peptides, which may act as an attractant in fish, resulting in enhanced feed utilisation [39, 40]. In

Table 5: Fatty acid composition (g 100 g$^{-1}$ DW) in the whole body of pompano fed the experimental diets for 10 weeks.

| Parameters | Experimental diets TVH0 | TVH05 | TVH10 | TVH15 | TVH20 | *SEM* | *P -value* |
|---|---|---|---|---|---|---|---|
| 14:0 | 1.14 | 1.17 | 1.16 | 1.18 | 1.16 | 0.010 | 0.727 |
| 15:0 | $0.24^{a}$ | $0.28^{c}$ | $0.26^{bc}$ | $0.27^{bc}$ | $0.25^{ab}$ | 0.004 | 0.002 |
| 16:0 | $8.72^{a}$ | $9.66^{d}$ | $9.31^{c}$ | $9.24^{c}$ | $9.09^{b}$ | 0.081 | ≤0.001 |
| 17:0 | $0.41^{a}$ | $0.51^{b}$ | $0.41^{a}$ | $0.49^{b}$ | $0.44^{a}$ | 0.012 | ≤0.001 |
| 18:0 | 1.96 | 2.13 | 1.97 | 2.13 | 2.01 | 0.029 | 0.159 |
| 20:0 | 0.12 | 0.14 | 0.12 | 0.14 | 0.13 | 0.003 | 0.269 |
| 21:0 | 0.04 | 0.06 | 0.04 | 0.06 | 0.05 | 0.003 | 0.153 |
| 22:0 | 0.11 | 0.12 | 0.11 | 0.12 | 0.11 | 0.002 | 0.229 |
| 24:0 | 0.08 | 0.09 | 0.07 | 0.08 | 0.08 | 0.002 | 0.229 |
| ΣSFA | $12.81^{a}$ | $14.15^{d}$ | $13.46^{bc}$ | $13.70^{c}$ | $13.30^{b}$ | 0.122 | ≤0.001 |
| 14:1 | 0.04 | 0.03 | 0.03 | 0.03 | 0.03 | 0.002 | 0.507 |
| 15:1 | 0.04 | 0.04 | 0.03 | 0.05 | 0.03 | 0.002 | 0.055 |
| 16:1n-7 | $1.65^{a}$ | $1.80^{ab}$ | $1.71^{ab}$ | $1.74^{ab}$ | $1.82^{b}$ | 0.020 | 0.032 |
| 17:1 | $0.19^{a}$ | $0.21^{b}$ | $0.19^{ab}$ | $0.21^{ab}$ | $0.21^{b}$ | 0.003 | 0.010 |
| 18:1n-9 trans | $0.12^{ab}$ | $0.14^{c}$ | $0.12^{ab}$ | $0.13^{bc}$ | $0.11^{a}$ | 0.003 | 0.007 |
| 18:1n-9 | $6.43^{ab}$ | $6.98^{v}$ | $6.18^{a}$ | $6.66^{bc}$ | $6.61^{bc}$ | 0.078 | 0.001 |
| 20:1 | $0.68^{b}$ | $0.67^{ab}$ | $0.63^{a}$ | $0.72^{b}$ | $0.70^{b}$ | 0.009 | 0.002 |
| 22:1n-9 | 0.17 | 0.17 | 0.16 | 0.18 | 0.18 | 0.002 | 0.319 |
| 24:1 | $0.28^{a}$ | $0.31^{b}$ | $0.28^{a}$ | $0.33^{b}$ | $0.31^{b}$ | 0.005 | ≤0.001 |
| ΣMUFA | 9.60 | 10.34 | 9.33 | 10.04 | 10.00 | 0.102 | 0.214 |
| 18:2n-6 | $0.63^{a}$ | $0.74^{b}$ | $0.74^{b}$ | $0.71^{b}$ | $0.83^{c}$ | 0.018 | ≤0.001 |
| 18:3n-3 | $0.04^{a}$ | $0.04^{a}$ | $0.06^{b}$ | $0.05^{ab}$ | $0.06^{b}$ | 0.003 | 0.007 |
| 18:3n-6 | 0.04 | 0.03 | 0.03 | 0.04 | 0.03 | 0.002 | 0.274 |
| 20:3n-3 | 0.02 | 0.02 | 0.02 | 0.17 | 0.02 | 0.001 | 0.321 |
| 20:3n-6 | $0.29^{ab}$ | $0.31^{bc}$ | $0.27^{a}$ | $0.32^{c}$ | $0.29^{ab}$ | 0.005 | 0.002 |
| 20:4n-6 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.001 | 0.552 |
| 20:5n-3 | $0.03^{a}$ | $0.06^{b}$ | $0.05^{b}$ | $0.05^{b}$ | $0.08^{c}$ | 0.004 | 0.001 |
| 22:6n-3 | 0.13 | 0.11 | 0.12 | 0.11 | 0.13 | 0.003 | 0.236 |
| ΣPUFA | $1.19^{a}$ | $1.34^{b}$ | $1.33^{b}$ | $1.34^{b}$ | $1.47^{c}$ | 0.025 | ≤0.001 |
| Σn-3 PUFA | $0.20^{a}$ | $0.23^{ab}$ | $0.25^{bc}$ | $0.23^{ab}$ | $0.28^{c}$ | 0.008 | 0.001 |
| Σn-6 PUFA | $0.99^{a}$ | $1.12^{b}$ | $1.08^{b}$ | $1.10^{b}$ | $1.19^{c}$ | 0.017 | ≤0.001 |
| n-3/n-6 PUFA | $0.20^{a}$ | $0.20^{a}$ | $0.23^{ab}$ | $0.21^{ab}$ | $0.24^{b}$ | 0.005 | 0.014 |

All values are presented as means of triplicate groups of fish from three different tanks. Means with different superscript letters within a row indicate significant differences at $P < 0.05$.

the current study, dietary TVH had no effects on FI, FCR, PR, and PER in juvenile pompano, similar to observations recorded in barramundi [10], red seabream [12], and Florida pompano [36]. Tejpal *et al.* [20] also found no significant difference on FI of pompano fed dietary tilapia hydrolysate, although a reduction in FCR was observed. In contrast, cobia *Rachycentron cannadum* fed diets containing 182.2 g kg$^{-1}$ shrimp hydrolysate had significantly higher FCR compared to those fed lower inclusion levels [41]. The reduced feed utilisation was also recorded in turbot fed a high plant-based diet with FPH supplementation [19]. This could be attributed to the lower bioavailability of free amino acids caused by the gastrointestinal absorption rate asynchronism between free amino acids and protein-bound amino acids [42–44].

In this study, the dry matter ADC was not found to differ in pompano fed up to 20% of dietary TVH. However, protein ADC was significantly increased in fish fed diet containing 10% TVH compared to the control. Improvement of nutrient digestibility by protein hydrolysate supplementation has been demonstrated in some marine fish species. Zheng et al. [45] reported significant improvements in protein ADCs in Japanese flounder fed dietary fish protein hydrolysate. Juvenile red seabream fed different dietary FPH as krill concentrate, shrimp and tilapia hydrolysates showed improved protein ADC compared to the control diet [12]. Similar trends were also demonstrated in Atlantic salmon [11], olive flounder [13, 14], and barramundi [10] fed diets containing FPH. These observations are potentially explained by and improved availability and subsequent

Table 6: Blood/serum biochemical parameters of juvenile pompano fed diets containing different levels of tuna hydrolysate for 10 weeks.

| Parameters | Experimental diets | | | | | *SEM* | *P -value* |
|---|---|---|---|---|---|---|---|
| | TVH0 | TVH05 | TVH10 | TVH15 | TVH20 | | |
| Ht (%) | 45.22 | 46.11 | 43.92 | 41.21 | 41.52 | 0.838 | 0.252 |
| RBC (x10$^{12}$/L) | 3.45 | 3.47 | 3.51 | 3.37 | 3.41 | 0.035 | 0.807 |
| Hb (g/dl) | 5.63 | 5.73 | 5.65 | 5.67 | 6.54 | 0.147 | 0.220 |
| Glu (nmol/L) | 5.58 | 5.71 | 5.69 | 5.70 | 5.51 | 0.044 | 0.552 |
| TP (g/L) | 35.02$^{ab}$ | 34.21$^{ab}$ | 36.38$^{b}$ | 34.13$^{ab}$ | 32.46$^{a}$ | 0.405 | 0.010 |
| Cho (nmol/L) | 4.18 | 4.28 | 3.98 | 3.99 | 4.04 | 0.046 | 0.175 |
| Alb (g/L) | 14.05 | 13.37 | 13.55 | 12.98 | 12.92 | 0.159 | 0.133 |
| TGA (nmol/L) | 3.11 | 3.05 | 2.96 | 2.98 | 2.97 | 0.035 | 0.646 |
| ALT ($\mu$/L) | 20.07 | 16.47 | 16.87 | 15.43 | 14.97 | 0.749 | 0.221 |
| AST ($\mu$/L) | 44.30 | 40.50 | 37.50 | 14.53 | 37.73 | 1.754 | 0.531 |

Values in the table are presented as means of triplicate groups. Means with different superscript letters within a row indicate significant differences at $P < 0.05$. RBC: red blood cell; Ht: haematocrit; Hb: haemoglobin; Glu: glucose; TP: total protein; Cho: cholesterol; Alb: albumin; TGA: triglyceride; AST: aspartate transaminase; ALT: alanine aminotransferase.

uptake of free amino acids and bioavailable peptide fractions produced in the enzymatic hydrolysis process [10]. In this process, native proteins in fish wastes are converted into smaller peptides which are easily digested and absorbed through intestinal brush borders than the whole proteins, resulting in high nutrient digestibility in fish [8]. However, in the present study, protein ADC significantly reduced in fish fed the diet containing 20% TVH compared to those fed lower dietary TVH levels. Siddik et al. [17] also found that barramundi fed dietary inclusion of 415 or 590 g kg$^{-1}$ tuna hydrolysates had lowered nutrient digestibility compared to those in fish fed fishmeal-based diets. This could be reasoned by an excessive amount of free amino acids and peptides of low molecular weight in high FPH-based diets, resulting in the disturbance of the normal digestion and absorption of nutrients in the ingested diets [17].

The proximate composition in the current study was not influenced by dietary TVH supplementation, similar to the results shown in red seabream [12, 31] and turbot [46]. However, both Japanese flounder and turbot showed increased whole body protein contents when fed upwards of 3.7% of ultrafiltered dietary FPH compared to those fed a fishmeal-based diet [45, 47]. Similarly, the concentrations of lipid and protein contents significantly increased in the whole body of Japanese flounder fed plant-based diets containing 4.5% FPH, whereas fish fed the diets with higher FPH resulted in a reduction of lipid and protein [18]. However, turbot fed high plant-based diet showed reduced lipid and increased protein concentrations in fish fed 12.4% FPH compared to those fed lower FPH inclusion levels [19]. The differences of dietary FPH on proximate composition could be explained by a number of factors, including different FPH sources, their inclusion levels, and the experimental conditions of individual studies [12]. In the present study, dietary TVH had no effect on amino acid profile in muscle tissues of juvenile pompano, with the exception of a marginal reduction in phenylalanine concentration in fish fed to TVH10 diet. Costa-Bomfim et al. [41] reported that cobia fed up to 18.22% shrimp hydrolysate had no effect on the amino acid profile. To date, there are few studies evaluating the effect of FPH on amino acid profiles in fish. However, these studies have shown that high inclusion levels of FPH would increase solubilised protein generated during enzyme hydrolysis, resulting in nonsynchronic absorption of essential compared with nonessential amino acids [11]. Xu *et al.* [19] found that replacing fishmeal protein with FPH changed the fatty acid content of fish muscle in a dose-dependent way. The quantities of saturated fatty acids increased dramatically when FPH was increased from 5% to 20%, but the concentrations of specific PUFA, such as 18:3n-6, 18:3n-3, 20:4n-6, 22:5n-3, and 22:6n-3, fell significantly. In the present study, although the MUFA content was not varied significantly, total PUFA content was increased with the increasing levels of TVH in the muscle of fish. This finding is consistent with Chaklader et al. [30], who found that adding FPH to diets high in poultry by-product meal raised PUFA content considerably when compared to the control. The concentration of long chain polyunsaturated fatty acids such as EPA and DHA in barramundi was negatively influenced by feeding excessive amounts of alternative FM protein diets [30]. However, the current study revealed that total PUFA, Σn-3, and Σn-6 increased significantly in pompano muscle when fed TVH supplemented diets. The modulatory effects of FPH on lipid accumulation and metabolism, as described for turbot [19], and other animals including mice and rats, could explain this retention of n-3 and n-6 PUFA [48].

Despite this, there is very little information known to date to explain how FM diets supplemented with FPH modulate muscle fatty acids. Blood biochemical parameters are considered physiological indicators for assessing the health status of fish [49]. With the exception of total protein, dietary TVH supplementation had no significant effect on the haematological parameters of juvenile pompano in the current study. Bui et al. [12] also found that juvenile rea seabream fed different dietary protein hydrolysate sources showed no significant differences in haematological indices compared to the fish fed the control diet. Likewise, barramundi fed various inclusion levels of FPH did not show any changes in biochemical indices of blood and serum.

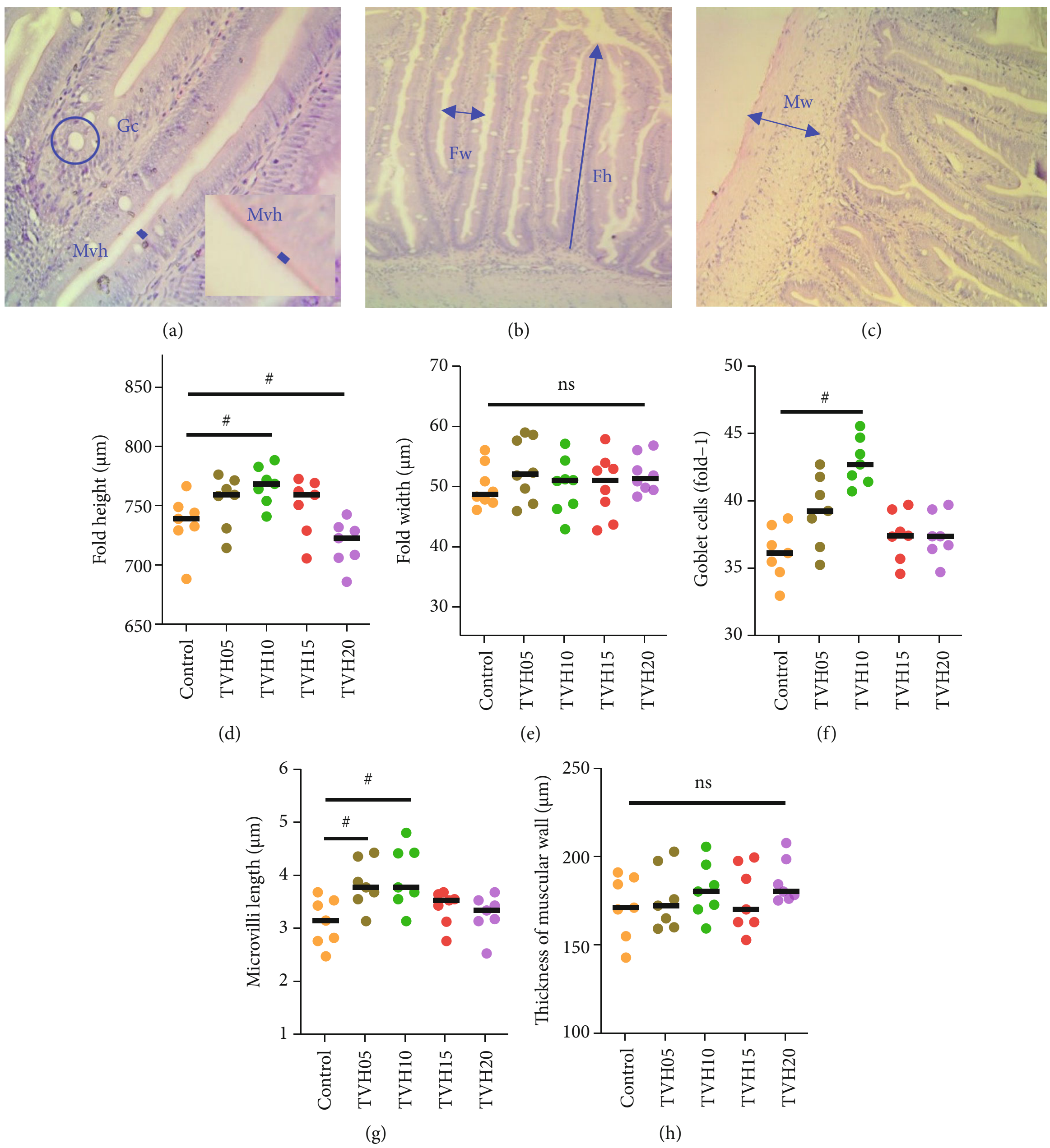



FIGURE 4: Intestinal histo-morphometric photomicrograph and measurement of juvenile pompano fed TVH supplemented diet for 10 weeks (panels a–c). The different measurements include Gc: goblet cells; Mvh: microvillus height; Fh: fold height; Fw: fold width; Mw: muscular wall. In panels (d–h), bar holding asterisk # indicates statistically different from the control at $P<0.05$, while ns indicates not statistically different from the control. Data were represented as mean ± S.E., $n=8$. All sections are stained with H&E. Scale bar, 50 $\mu$m.

However, the fish fed diets containing 61 to 122 g kg$^{-1}$ FPH had significantly lower blood glucose compared to the control [10], similar to those reported in red seabream fed low fishmeal-based diets supplemented with shrimp hydrolysate [31]. No significant difference was recorded in blood glucose of red seabream fed high fishmeal-based diets containing FPH [12]. In the present study, the concentration of blood protein was significantly reduced in pompano fed with 120 g kg$^{-1}$ TVH compared to the fish fed with 60 g kg$^{-1}$. This is inconsistent with observations in barramundi in which blood protein did not show any significant difference among the fish fed with up to 122 g kg$^{-1}$ FPH [10]. The inconsistency in results therefore may be attributed to the experimental conditions, fish size, and handling methods, which strongly influence the fish physiological conditions [50].

Fish intestinal epithelium, consisting of villi, microvilli, and goblet cells (GC), is sensitive to dietary modulation. Insufficient nutrients and subsequent absorption may alter intestinal structure in fish such via a shortening of intestinal fold height and reduced GC numbers, resulting in poor immune function [17]. In the present study, a significant increase of GCs was found in fish fed diets containing 10% TVH compared to the control. These observations are consistent with those reported in barramundi [10] and red

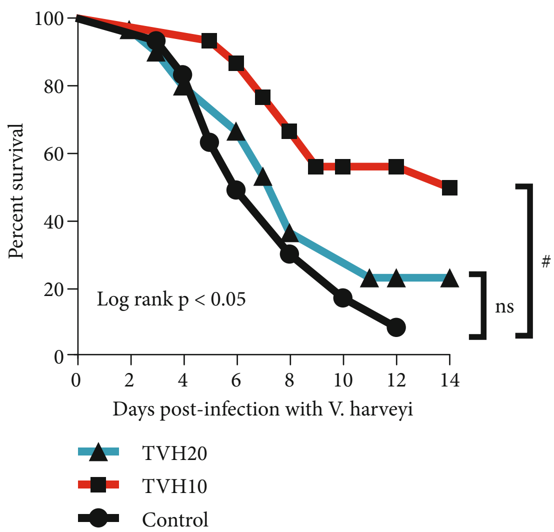



Figure 5: Kaplan-Meier's survival analysis of pompano fed TVH supplemented diets after IP challenge with *Streptococcus iniae* (TVH5 and TVH15 were not considered for the construction of survival graph since they produced no variations with the control and also for the clarity of the graph). Asterisks # indicates statistically significant difference between treated group and infected control at $P < 0.05$ while ns indicates not statistically different between the group.

seabream [31] fed appropriate levels of FPH. Domeneghini *et al.* [51] also revealed a positive relationship between higher GC numbers and elevated mucosal membrane protection. Siddik *et al.* [10] also assumed that the increment of GCs in barramundi fed FPH could be attributed to the enhanced immunity against invading microorganisms. In the present study, histological examination showed increased fold height (Fh) in the intestine of pompano fed the TVH10 diet, potentially attributable to the greater nutrient absorption and utilisation, as reported in barramundi [10]. Moreover, Novriadi *et al.* [36] also demonstrated that the intestinal inflammation observed in Florida pompano fed high plant-based diet could be restored with squid hydrolysate supplementation. Hepatic alterations such as cytoplasmic vacuolisation and increased lipid accumulation have been reported in juvenile barramundi fed >20% FPH [10]. However, the histological evaluation in the current study showed no histopathological hepatic alterations in fish fed TVH diets. Besides, ALT and AST were not significantly different among treatments, indicating that dietary TVH inclusion levels in the current study did not cause any hepatocellular damage in pompano.

The improvement of disease resistance in fish fed appropriate levels of FPH has been demonstrated in many finfish species ([10, 12, 31, 52]: [53]). In the current study, pompano fed with 10% TVH showed significantly lower mortality after the challenge against *Streptococcus iniae* in comparison to the control. Siddik et al. [10] also demonstrated that juvenile barramundi fed tuna hydrolysate modulated the resistant capacity against *Streptococcus iniae*. Increased disease resistance was also recorded in red seabream and olive flounder against *Edwardsiella tarda* [12] and European seabass *Dicentrarchus labrax* to *Vibrio anguillarum* [52]. These studies showed a positive relationship between dietary FPH and lysozyme activity resulting in improved disease resistance against invading pathogens. In contrast, Liang *et al.* [35] reported no significant difference in survival rate of Japanese seabass *Lateolabrax japonicus* fed dietary FPH when exposed to *Vibrio anguillarum* in seawater, while juvenile coho salmon *Oncorhynchus kisutch* and Atlantic salmon *Salmo salar* showed poor survival rates following a challenge test against *Vibrio anguillarum* and *Aeromonas salmonicida*, respectively [54, 55]. The discrepancy in results of FPH in fish disease resistance might be due to the variations of raw protein sources, enzymatic specifications, nutritional profile of hydrolysates, and dietary inclusion levels as well as species-specific differences [6, 56].

## 5. Conclusion

In conclusion, the optimum TVH for juvenile pompano was estimated to be 12.0% or 120 g kg$^{-1}$ in practical diet based on the quadratic regression analysis of FBW level. Although TVH inclusion in the diets had no effect on feed utilisation, proximate composition, and on most blood biochemical parameters, increased serum protein and intestinal goblet cell numbers in fish fed TVH at 10.0% included diet could be linked to improve disease resistance against *S. iniae* infection of juvenile pompano resulting in higher post-challenge survival. Further studies should evaluate the influence of optimised dietary TVH level in alternative plant protein-based diets for the species.

## Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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