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Beneficial effects of tuna hydrolysate in poultry by-product meal diets on growth, immune response, intestinal health and disease resistance to *Vibrio harveyi* in juvenile barramundi, *Lates calcarifer*

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ABSTRACT

This study was conducted to investigate the effects that tuna hydrolysate (TH) supplementation in poultry by-product meal (PBM) diets would have on growth, immunity and resistance to *Vibrio harveyi* infection in juvenile barramundi, *Lates calcarifer*. Five isonitrogenous and isocaloric diets containing fishmeal (FM) without TH supplementation (control) and four diets with 10% TH supplementation viz. a FM protein diet (FMBD + TH), a 75% PBM protein diet (LPBM + TH) and two 90% PBM protein diets, either bioprocessed (BPBM + TH) or unprocessed (HPBM + TH), were formulated for juvenile barramundi, *Lates calcarifer*. The diets were fed to triplicate groups of juvenile barramundi (average pool weight 12.63 ± 0.11 g) for 10 weeks. Significantly ($P < 0.05$) higher final body weights and specific growth rates were noted in fish fed with FMBD + TH and BPBM + TH diets when compared to the control. Transmission electron microscopy observation of fish distal intestines revealed a significant enhancement of microvilli length in fish fed FMBD + TH and BPBM + TH whereas scanning electron microscopy analysis found no significant difference in microvilli density. A bacterial challenge with *Vibrio harveyi* was conducted for 14 days after the growth trial to test the immune response and survival of barramundi. In the pre-challenge condition, a significant reduction in blood glucose was found in BPBM + TH compared to the control, and fish in the post-challenge at 24 h had higher glucose levels compared to fish in the pre- and post-challenge conditions at 72 h. The serum lysozyme activity was significantly higher in FMBD + TH and BPBM + TH compared to the control and fish at 72 h post-challenge exhibited higher lysozyme activity in each treatment compared to all dietary groups in the post-challenge condition at 24 h and to HPBM + TH and BPBM + TH in the pre-challenge condition. Fish fed FMBD + TH, LPBM + TH and BPBM + TH diets had significantly higher survival to the bacterial challenge than fish in the control and HPBM + TH. These results showed that PBM supplemented with TH could successfully replace FM without compromising growth, however, bioprocessed PBM supplemented with TH (BPBM + TH) may significantly improve growth performance, immune response, intestinal health and disease resistance in juvenile barramundi.

1. Introduction

In recent years, there has been a growing disparity between the demand and supply of raw FM materials and the ecological and economic issues associated with its use, which has exerted pressure on aquaculture nutritionists to evaluate viable alternative protein sources to FM [1]. With this aim, aquaculture scientists have tended to not only focus on finding suitable alternatives to FM but also on improving the applicability of the already existing alternative products through innovative approaches such as fermentation and advanced bioprocessing for nutritional enrichment [2]. A number of studies have reported that fermentation, as an environmental friendly and cost effective method, improves the digestibility and amino acid profile of animal by-products [3,4] and thus enhances the suitability of their inclusion in fish feed formulations [4,5]. Another advantage of the fermentation process is that it breaks down carbohydrates into lower molecular-weight compounds that can potentially enhance innate energy and mineral absorption [6,7]. Fagbenro, Kim Jauncey [8] observed that feeds made from fermented products tend to have higher stability in water, thereby allowing more time for fish to ingest the feed and maximise nutrient intake. Fermentation is also used to overcome many of the other inherent problems of animal waste products including high moisture,

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indigestible particles and microbial contaminants [9–11].

Among the animal waste by-products used in aqua-diets, PBM is a good source of protein but is limiting in some of the essential amino acids [12]. Another limiting factor in the use of PBM, as with many animal by-product meals, is that digestibility varies highly from batch to batch in the rendering process and among suppliers [13]. Therefore, success has only been reported when PBM only partially replaced FM in the diet of fish. PBM successfully replaced FM at a level of 25% with juvenile tench, *Tinca tinca* [14], 67% with both totoaba juveniles, *Totoaba macdonaldi* [15], and rainbow trout, *Oncorhynchus mykiss* [16], and 70% with Florida pompano, *Trachinotus carolinus* [17]. However, the complete replacement of FM by PBM has resulted in depressed growth performance of Florida pompano, *Trachinotus carolinus* [18]. Therefore, to enable a qualitative improvement of PBM in fish diet, it may be refined through fermentation to potentially improve the biological value of the raw material as well as improve the utilization of the finished product. Furthermore, PBM may be supplemented with a fish protein hydrolysate (FPH) during the preparation of diets to minimise the limiting amino acids as well as to improve the quality of the final meal. As a supplement, FPH is a highly nutritious product made from whole fish or fish by-products, and it has been used as a supplement in many aqua-diets to improve immunity [19–22] and as an attractant to increase diet palatability [23,24]. Many studies have reported that FPH consists of low molecular weight bioactive peptides that may have immune-stimulating and antibacterial properties [25–27]. The improvement of cellular and/or humoral immune function with heightened disease resistance of various fish species due to the bioactive peptides in FPH has already been established [26,28,29]. For example, red sea bream, *Pagrus major*, fed different hydrolysate diets (krill, shrimp and tilapia hydrolysate) exhibited significant improvement in disease resistance against *Edwardsiella tarda* [21].

Barramundi or Asian Seabass, *Lates calcarifer*, a catadromous species widely distributed in the Indo-Pacific region and Australia [30] is progressively becoming a major commercial species in aquaculture because of the species-wide range of salinity tolerance and adaptability to versatile farming systems, its tendency to readily consume pelleted feeds, and its highly appreciated meat among consumers [31]. To date, no research has been done to prove whether fermentation and TH in combination further improve the inclusion level of PBM in fish diets. Therefore, the present study was designed to investigate the effect of complete replacement of FM with TH supplementation in PBM diets, on growth performance, biochemical response, gut health and disease resistance of juvenile barramundi.

2. Materials and methods

2.1. Ethic statements

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Australia. The protocol was approved by the Ethics Committee in Animal Experimentation of the Curtin University (Approval number AEC_2015_41).

2.2. Diet preparation

Five experimental diets were prepared using Feed-soft Professional® providing 47.0% crude protein (CP) and 20.0 MJ kg^{-1} gross energy (GE) with commercial ingredients to fulfil the nutrient requirements of juvenile barramundi according to NRC [32]. The PBM was fermented following the technique described in our earlier study [3]. In short, PBM was weighed and then Baker's yeast, *Saccharomyces cereviceae* (Instant dried yeast, Lowan®), and *Lactobacillus casei* in the form of skim milk product (Yakult® @ cell density of 3×10^6 CFU mL^{-1}), were added at 10% and 5% of the weight of PBM, respectively. Distilled water was then added to approximately 70% of the total weight of the meal mixture and ingredients were thoroughly mixed in a food mixer. The mixture was then placed in an Erlenmeyer flask covered with aluminium foil and incubated at 30 °C for 4 days. Then, the fermented product was dried in the oven at 60 °C for 24 h and used as a feed ingredient. All diets were prepared isonitrogenous and isocalorific, therefore, quantities of individual ingredients were adjusted so as to provide equal protein and energy contents. Wheat and wheat starch were used to provide NFE and binding strength respectively to the diets. Nevertheless, care is taken to minimise different ingredients concentration and therefore, casein, a pure source of protein, was used to balance the protein in the diet formulation.

All the experimental diets, except the control, were supplemented with 10% TH. Thus, the dietary treatments were: FM-based basal diet devoid of TH (control), FM-based diet supplemented with 10%TH (FMBD + TH), a diet of 75% PBM and 15% FM supplemented with 10%TH (LPBM + TH), 90% PBM supplemented with 10% TH (HPBM + TH) and 90% bioprocessed PBM supplemented with 10% TH (BPBM + TH). The diet codes were allotted as described in Table 1. All dry ingredients were homogeneously mixed before adding fish oil and warm water in a food mixer (Hobart Food equipment, Australia) to form a dough. The dough was then passed through a mincer to produce 3 mm pellets. The moist pellets were then dried in an oven at 60 °C for 36 h, cooled at room temperature, broken up by hand, sealed in plastic bags and stored in refrigerated conditions prior to use in the feeding trial. The proximate composition and amino acids (AAs) profile of the tested FM, PBM and BPBM are presented in Table 2. The formulation and proximate composition of the experimental diets are presented in Table 3.

Table 1
Dietary codes for feeds used in the article.

Codes	Diet
Control	Basal diet without tuna hydrolysate supplementation
FMBD + TH	Basal diet supplemented with 10% tuna hydrolysate
LPBM + TH	75% PBM and 15% FM supplemented with 10% tuna hydrolysate
HPBM + TH	90% PBM supplemented with 10% tuna hydrolysate
BPBM + TH	90% bioprocessed PBM supplemented with 10% tuna hydrolysate

Table 2
Nutrient composition (%) and amino acid contents (g 100 g^{-1} protein) of FM, PBM and BPBM used in the feed formulation.

	FM	PBM	BPBM
DM	94.32	97.7	95.40
Crude protein	64.00	67.13	66.98
Crude Lipid	10.76	13.52	11.70
Ash	19.12	13.34	14.68
Gross energy (MJ/kg)	20.30	21.05	20.42
Essential amino acid			
Arginine	4.35	5.43	5.24
Phenylalanine	2.68	2.65	2.65
Leucine	4.64	5.00	4.97
Lysine	4.77	4.17	4.21
Methionine	1.81	1.46	1.43
Isoleucine	3.04	2.67	2.66
Histidine	1.79	1.47	1.39
Threonine	2.53	2.81	2.86
Valine	3.20	3.08	3.11
Non-essential			
Alanine	4.42	4.62	4.89
Aspartic acid	6.20	5.95	6.03
Glutamic acid	8.25	9.55	9.84
Glycine	4.73	6.53	6.79
Proline	3.81	5.63	5.94
Serine	3.05	3.01	3.08

Table 3
Formulation and proximate composition of the experimental diets for juvenile barramundi.

Ingredients (g kg^{-1})	Experimental diets				
	Control	FMBD + TH	LPBM + TH	HPBM + TH	BPBM + TH
FM	610.0	549.0	91.5	518.5	488.0
PBM	–	–	457.5	549.0	–
BPBM	–	–	–	–	549.0
TH[a]	–	61.0	61.0	61.0	61.0
Wheat	266.0	264.0	268.0	267.0	265.0
Wheat starch	20.0	20.0	20.0	20.0	20.0
Fish Oil	30.0	30.0	30.0	30.0	30.0
Calcium carbonate	2.0	2.0	2.0	2.0	2.0
Sodium chlorite	2.0	2.0	2.0	2.0	2.0
Vitamin premix	1.0	1.0	1.0	1.0	1.0
Casein	63.0	65.0	61.0	63.0	64.0
Cellulose	6.0	6.0	6.0	6.0	6.0
Dry matter	92.72	89.36	90.90	91.22	91.03
Crude protein	47.17	46.86	47.44	47.79	46.85
Crude lipid	9.99	9.40	10.69	10.94	9.53
Ash	13.04	12.06	9.26	8.70	8.71
Gross energy (MJ kg^{-1})	19.98	19.92	19.89	19.90	19.91

FM: fishmeal, PBM: poultry by-product meal, BPBM: bioprocessed poultry by-product meal.
[a] The dried TH contains 58.4% protein, 1.05% lipid and 11.3% ash.

2.3. Fish and feeding trial

Juvenile barramundi were obtained from the Australian Centre for Applied Aquaculture Research, Fremantle, Australia, and reared in three fibreglass tanks (300 L water capacity) to attain a desirable size of approximately 10 g. The fish were then graded and 300 juvenile barramundi were selected for experiment purposes. Before commencing the feeding trial, fish were acclimated to experimental conditions at Curtin Aquatic Research Laboratories for two weeks. During the acclimation period, fish were fed three times a day with a commercially formulated diet (470 g protein kg^{-1} diet and 20.0 MJ kg^{-1} dietary gross energy). After that, juvenile barramundi with an initial pool weight of average pool weight 12.63 ± 0.11 g were randomly distributed into fifteen independent tanks (300-L water capacity) at a stocking density of 20 fish per tank.

Each tank in the rearing facility was equipped with constant aeration, a water heater and an external bio-filter (Fluval 406, Hagen, Italy) to maintain water recirculation at a rate of 10 L min^{-1} throughout the experimental period. Fish were reared in seawater with a salinity of 32.71 ± 2.17 ppt. The water quality parameters such as temperature, pH and dissolved oxygen were monitored daily using a portable multiparameter meter (YSI, USA) and were maintained at 26.0 ± 2.8 °C, pH 6.51 ± 0.43 and 7.10 ± 0.6 mg L^{-1}, respectively. Total ammonia and nitrite were monitored using commercial kits twice a week with the resulting mean values of 0–2.0 mg L^{-1} and 0–1.0 mg L^{-1}, respectively. Fish were held under a 14-h light/10-h dark cycle using an automatic indoor light switch (Clipsal, Australia). Fish tanks were randomly assigned with three tanks for each individual diet. Throughout the 10-weeks of the feeding trial, fish were hand-fed to apparent satiation three times a day at 0800, 1200 and 1700 h. After 30 min of feeding, uneaten feed was removed from the bottom of the tank by siphoning, transferred to aluminium cups, and oven dried at 60 °C for 36 h in order to determine the daily feed intake. At the end of the growth trial, total numbers of fish in each tank were counted and individually weighed after starving them for one day. Growth and feeding performances of juvenile barramundi were assessed at the end of the feeding trial according to the equations described in Ref. [22].

2.4. Proximate analysis

After termination of the feeding trial, two fish from each tank (six samples per dietary treatment) were randomly selected for analysis of dry matter, protein, ash and gross energy whilst one fish from each tank (three samples per dietary treatment) were considered for lipid analysis. The muscles sample of fish were analysed for proximate composition based on the Association of Official Analytical Chemists procedures [33]. Briefly, the carcass dry matter was determined by oven drying to constant weight at 105 °C; crude ash by combustion at 550 °C; crude protein content (nitrogen × 6.25) by the Kjeldahl digestion method; crude lipid content by the Soxhlet technique; and gross energy content by an IKA oxygen bomb calorimeter (Heitersheim, Germany).

2.5. Challenge with Vibrio harveyi

Vibrio harveyi is a virulent pathogen infecting wide range of marine vertebrates and invertebrates including fish. It is a primary pathogen for barramundi, *Lates calcarifer* too and has been used in many studies as a challenge pathogen to investigate the immune status of the species. The bacterial challenge study was performed by infecting juvenile barramundi with *Vibrio harveyi,* after the 10-week feeding trial. The pathogen, *Vibrio harveyi* was obtained from the Bacteriology Laboratory, Department of Agriculture & Food, Perth, Australia. The bacteria were grown in trypticase soy broth (Oxoid, Basingstoke, UK) at 24 °C for 24 h and the broth containing the culture was centrifuged at 5000 g for 15 min. The supernatant was discarded and the pellets were washed twice in phosphate-buffered saline (pH 7.2) for experimental use. The LD_{50} of the bacteria was adjusted to 1.7×10^8 cells/mL.

At the end of the growth trial, 10 fish from each replicate tank was relocated to each of 20 × 100 L capacity glass aquaria for 14 days of bacterial challenge. Of the 20 aquaria, five were used for blood sampling and 15 were utilized for survival counting. Following three days of acclimation, fish were subjected to intraperitoneal injection (IP injection) with *Vibrio harveyi* by removing the fish from the tank and bathing them in mild anaesthetic (5–10 ppm). A dose of 0.1 mL of *Vibrio harveyi* suspension in PBS media (1.7×10^8 CFU/mL) was administered to individual fish using a 1-mL syringe and 27-gauge needle according to the procedure described by Ref. [34]. After IP injection all the challenged fish were returned to their respective rearing tanks and fed once daily for a further 2 week period with the same experimental diet that was assigned before the challenge. As a part of constant monitoring, fish with signs of infection were recorded immediately and the infected fish were removed from the tank and euthanised with AQUI-S at 175 mg L^{-1} for 20 min, following the protocol of the Curtin University Standard Operating Procedure CARL01 Euthanasia of Fish. Fish

were euthanised as soon as clinical signs, including lethargic swimming, flared opercula, skin lesions, moribund and loss of equilibrium, were obvious. Under the bacterial challenge conditions, fish health and condition were monitored three times a day at around 7:00am, 2:00pm and 9.00pm.

2.6. Blood and serum biochemical indices

Blood and serum samples for assessing biochemical and immunological indices were conducted before the bacterial challenge and then again 24 h and 72 h post challenge. Two sets of blood from mildly anaesthetized fish (AQUI-S, 8 mg l^{-1}) were withdrawn by caudal vein puncture with a 1 mL non-heparinised syringe at the end of the growth trial. The first aliquot of extracted blood sample was collected in heparinised tubes for the determination of blood glucose level. The second aliquot of the blood sample was collected in non-heparinised tubes and allowed to sit for 24 h. The clotted blood samples were then centrifuged at 3000 rpm for 15 min at 4 °C for serum extraction and the extracted serum was stored at −80 °C for the measurement of the serum biochemical indices including serum total cholesterol (TC), triglyceride (TG), total protein (TP) and albumin using an automated blood analyser (SLIM; SEAC Inc, Florence, Italy) and following the methods of Blanc, Neveux [35]. The total globulin content was determined by subtracting the albumin values from the total serum protein values. The albumin and globulin ratio (A/G ratio) was obtained by dividing albumin values by globulin values.

2.7. Biochemical assessment of liver damage

To investigate liver health, serum aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) activities were examined at pre-challenge and post-challenge conditions with an automatic biochemical analyser (Mindray BS-400, Mindray Medical International Ltd., Shenzhen, China) and attached kit (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan).

2.8. Serum immunological indices

Serum immunological indices, including lysozyme and bactericidal activity, were assayed in pre- and post-challenge fish at 24 h and 72 h. Serum lysozyme activity was examined based on the method previously described by Siddik, Howieson [22] and the bactericidal activity according to Le and Fotedar [36].

2.9. Intestinal mucosal morphology

Three randomly selected fish per treatment after 10-weeks post feeding was considered for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis. For TEM analysis, intestinal samples were dissected into ~1 mm long transverse sections and then processed using a microwave-assisted protocol. Briefly, samples were excised from freshly collected specimens and then immediately bathed in 2.5% glutaraldehyde buffered in 1x PBS at pH 7.4 and stored at 4 °C for over 24 h. Samples were then briefly rinsed in PBS prior to secondary fixation using 1% OsO_4 (80 W 2 min on, 2 min off, 2 min on), dehydration in ethanol (50, 70, 95 and 100% at 250 W, 40 s each) then acetone (100% 2x at 250 W, 40 s each) and finally infiltration with epoxy resin in acetone (Procure 812, Proscitech) (1:3, 1:1, 3:1 ratios at 250 W, 3 min each) was undertaken using a laboratory microwave (Pelco, Biowave® with cold spot and vacuum chamber). Samples were left in the final 3:1 ratio overnight before two further overnight changes in 100% resin. Samples were then mounted in BEEM™ capsules and polymerized in an oven at 70 °C overnight. Semi- and ultra-thin transverse sections of the gut were cut from trimmed blocks using an ultramicrotome (UC6, Leica Microsystems) at thicknesses of 1 μm and 120 nm using a Histo and Ultra diamond knife, respectively (Diatome, Switzerland). Semi-thin sections were mounted on glass slides and stained with toluidine blue, whilst ultra-thin sections were mounted onto carbon filmed copper finder TEM grids (EMS, Hatfield, USA). TEM imaging was carried out on a LaB_6 TEM (JEOL 2100, Japan) operating at 120 kV. Conventional bright field images were acquired on an 11 Mpx charge coupled device camera (Gatan ORIUS1000, Pleasanton, USA). The electron micrographs were investigated with Photoshop CS6 (Adobe, USA), and ImageJ (National Institute of Health, USA) to determine microvilli length, as described elsewhere (Ran et al., 2015). TEM images (magnification × 30,000) were examined to measure the microvilli length.

For SEM analysis, the intestinal samples were removed and cleaned by dipping in normal saline water. The samples were then dissected, the interior cleaned and cut into pieces measuring 5 mm. They were then washed in 1% S-carboxymethyl-L-cysteine for 30 s to remove mucus and then preserved in 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M pH 7.2). Samples were processed as described elsewhere [37] and screened with a JSM 6610 LV (Jeol, Tokyo, Japan) SEM or JEN 1400 (Jeol, Tokyo, Japan). The SEM images (magnification × 30,000) were analysed to assess the number of microvilli present on the surface of enterocytes standardised to 1 μm^2 region [38].

2.10. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's tests was applied in growth and muscle tissues composition to compare the control diet against each dietary groups containing TH. The biochemical and immune responsive parameters were analysed by multifactorial analysis of variance (ANOVA). The survival graph was constructed using the Kaplan–Meier method and the differences among different dietary groups were performed using the log-rank test. All results were expressed as means and standard errors (SE), and p-values less than 0.05 were considered statistically significant.

3. Results

3.1. Growth performance and feed utilization

Dietary TH supplementation in fish diets had a significant effect on the growth performance and feed utilization of juvenile barramundi (Table 4). Fish fed FMBD + TH and BPBM + TH diets demonstrated the highest growth performance in terms of final body weight (FBW) and weight gain (WG) than the control ($P < 0.05$). However, FBW and WG were not significantly ($P > 0.05$) different with the treatment of HPBM + TH. The highest feed intake was observed in fish fed FMBD + TH when compared to the control and the rest of the dietary groups. The survival rate, feed conversion ratio (FCR) and daily weight gain (DWG) of fish were not significantly ($P > 0.05$) affected by any dietary groups.

3.2. Proximate composition

Fish fed with HPBM + TH and BPBM + TH had a higher lipid content than fish with the control and other diets. The moisture content of barramundi showed a tendency to decrease in response to increased lipids and hence significantly lower moisture levels were registered in HPBM + TH and BPBM + TH when compared to the control. No significant differences were observed in protein, energy and ash content among the dietary groups (Table 4).

3.3. Blood and serum biochemical indices

The blood and serum constituents of juvenile barramundi fed the experimental diets are presented in Table 5. A significant decrease ($P < 0.05$) in glucose with BPBM + TH and triglyceride levels occurred in all dietary groups at pre-challenge condition over the control.

Table 4
Growth performance and muscles nutrient composition of juvenile barramundi fed tuna hydrolysate (TH) included diets at various levels for 10 weeks.

Parameters	Experimental diets					P-value
	Control	FMBD + TH	LPBM + TH	HPBM + TH	BPBM + TH	
Growth performance						
Initial body weight (g)	12.71 ± 0.11	12.65 ± 0.01	12.69 ± 0.07	12.49 ± 0.14	12.61 ± 0.09	0.526
Final body weight (g)	106.32 ± 5.34^{b}	127.19 ± 9.13^{a}	120.63 ± 3.01^{ab}	105.27 ± 6.49^{b}	127.06 ± 0.53^{a}	0.046
Weight gain (g)	93.61 ± 5.45^{b}	114.54 ± 9.13^{a}	107.95 ± 2.95^{ab}	92.78 ± 6.60^{b}	114.46 ± 0.58^{a}	0.048
Specific growth rate (%/d)	3.31 ± 0.08	3.60 ± 0.11	3.52 ± 0.03	3.33 ± 0.10	3.61 ± 0.02	0.065
Feed intake (g/fish/day)	1.63 ± 0.04^{bc}	1.70 ± 0.01^{a}	1.68 ± 0.01^{ab}	1.61 ± 0.01^{d}	1.69 ± 0.01^{ab}	0.028
Feed conversion ratio	1.12 ± 0.07	1.13 ± 0.07	1.13 ± 0.03	1.19 ± 0.07	1.20 ± 0.03	0.152
Survival (%)	96.67 ± 1.67	95.00 ± 2.88	91.67 ± 4.41	93.33 ± 4.41	98.33 ± 1.67	0.365
Muscles nutrient composition (% wet weight basis)						
Moisture (%)	72.42 ± 0.37^{a}	73.22 ± 0.26^{a}	72.63 ± 0.13^{a}	75.07 ± 0.37^{b}	75.40 ± 0.32^{b}	0.000
Protein (% WW)	15.96 ± 0.34	16.68 ± 0.37	15.34 ± 0.51	14.89 ± 0.39	15.01 ± 0.61	0.056
Lipid (% WW)	2.58 ± 0.02^{a}	2.13 ± 0.02^{b}	2.35 ± 0.01^{ab}	1.88 ± 0.01^{c}	1.87 ± 0.01^{c}	0.000
Ash (% WW)	3.59 ± 0.07	3.74 ± 0.05	4.03 ± 0.22	4.18 ± 0.28	4.32 ± 0.30	0.122
GE (MJ Kg^{-1})	0.61 ± 0.04	0.56 ± 0.00	0.59 ± 0.04	0.55 ± 0.00	0.56 ± 0.00	0.501

Values are mean ± SE of three replicate tanks per treatment. Values in the same row with different superscript letters (a,b,c) are significantly different based on Tukey's multiple range test (One-way ANOVA, $P < 0.05$).
Weight gain (g) = [(mean final body weight – mean initial body weight)/mean initial body weight].
Specific growth rate (%/d) = [(ln final body weight - ln (pooled initial body weight))/days] × 100.
Feed intake (g/fish/day) = dry feed consumed/fish number.
Feed conversion ratio = dry feed fed/wet weight gain.
Survival (%) = (number of final fish-number of initial fish)/number of initial fish × 100.

Table 5
Blood/serum biochemical parameters of juvenile barramundi fed FM and PBM diets supplemented with tuna hydrolysate for 10 weeks pre-challenge, post-challenge at 24 h and post-challenge at 72 h.

Parameter	Diets	Pre-challenge	Post-challenge-24 h	Post-challenge-72 h
Glucose	Control	5.40 ± 0.04^{a}	6.97 ± 0.13*	5.43 ± 0.22
	FMBD + TH	5.29 ± 0.07^{a}	6.76 ± 0.03***	5.21 ± 0.10
	LPBM + TH	5.35 ± 0.06^{a}	6.84 ± 0.05***	5.24 ± 0.05
	HPBM + TH	5.39 ± 0.04^{a}	6.86 ± 0.18*	5.33 ± 0.17
	BPBM + TH	5.11 ± 0.03^{b}	6.67 ± 0.10***	5.23 ± 0.13
Cholesterol	Control	6.03 ± 0.46**	3.20 ± 0.21^{ab}	3.57 ± .49 ^{ab}
	FMBD + TH	5.67 ± 0.70*	2.63 ± 0.30^{b}	2.20 ± .00^{b}
	LPBM + TH	5.37 ± 0.69	3.45 ± 0.15^{a}	4.27 ± 0.39^{a}
	HPBM + TH	5.20 ± 0.60	3.77 ± 0.07^{a}	3.80 ± 0.35^{a}
	BPBM + TH	6.27 ± 0.81*	3.70 ± 0.26^{a}	4.37 ± 0.48^{a}
Triglyceride	Control	3.53 ± 0.41^{a}**	0.40 ± 0.06	0.30 ± 0.00
	FMBD + TH	1.70 ± 0.06^{b}**	0.40 ± 0.12	0.90 ± 0.29
	LPBM + TH	1.50 ± 0.26^{b}*	0.20 ± 0.00	1.10 ± 0.51
	HPBM + TH	1.20 ± 0.15^{b}*	0.23 ± 0.09	0.70 ± 0.20
	BPBM + TH	1.00 ± 0.12^{b}*	0.30 ± 0.06	0.83 ± 0.22*
Total protein	Control	43.33 ± 6.36	31.00 ± 2.08	37.00 ± 1.73
	FMBD + TH	38.33 ± 2.33	33.33 ± 3.84	35.00 ± 1.00
	LPBM + TH	40.00 ± 3.00	35.00 ± 1.00	37.00 ± 2.00
	HPBM + TH	41.00 ± 1.53	34.00 ± 1.15	37.00 ± 5.13
	BPBM + TH	40.67 ± 3.71	32.67 ± 3.18	36.00 ± 1.15
Albumin	Control	12.00 ± 2.00	9.00 ± 0.58	11.33 ± 0.67
	FMBD + TH	10.67 ± 0.88	9.33 ± 0.88	9.67 ± 0.33
	LPBM + TH	10.67 ± 0.67	11.50 ± 0.50	9.67 ± 0.33
	HPBM + TH	12.33 ± 0.33	8.67 ± 0.88	10.00 ± 1.53
	BPBM + TH	11.00 ± 1.00	9.33 ± 0.88	9.67 ± 0.67
Globulin	Control	31.33 ± 4.37	22.00 ± 1.53	25.67 ± 1.20
	FMBD + TH	27.67 ± 1.45	24.00 ± 3.00	25.33 ± 0.88
	LPBM + TH	29.33 ± 2.33	23.50 ± 0.50	27.33 ± 1.67
	HPBM + TH	28.67 ± 1.33	25.33 ± 0.88	27.00 ± 3.61
	BPBM + TH	29.67 ± 2.73	23.33 ± 2.40	26.33 ± 0.67
A/G	Control	0.38 ± 0.01	0.41 ± 0.01	0.44 ± 0.03
	FMBD + TH	0.38 ± 0.01	0.39 ± 0.02	0.38 ± 0.02
	LPBM + TH	0.36 ± 0.01	0.49 ± 0.01**	0.35 ± 0.01
	HPBM + TH	0.43 ± 0.02	0.34 ± 0.04	0.37 ± 0.01
	BPBM + TH	0.37 ± 0.01	0.40 ± 0.03	0.37 ± 0.02

The values are expressed as mean ± SE of three replicate fish per treatment. Mean values bearing different lowercase (a,b,c) letters among different dietary treatments were statistically significant ($P < 0.05$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ denote significant level among pre-challenge, post-challenge-24 h and post-challenge- 72 h in each treatment.

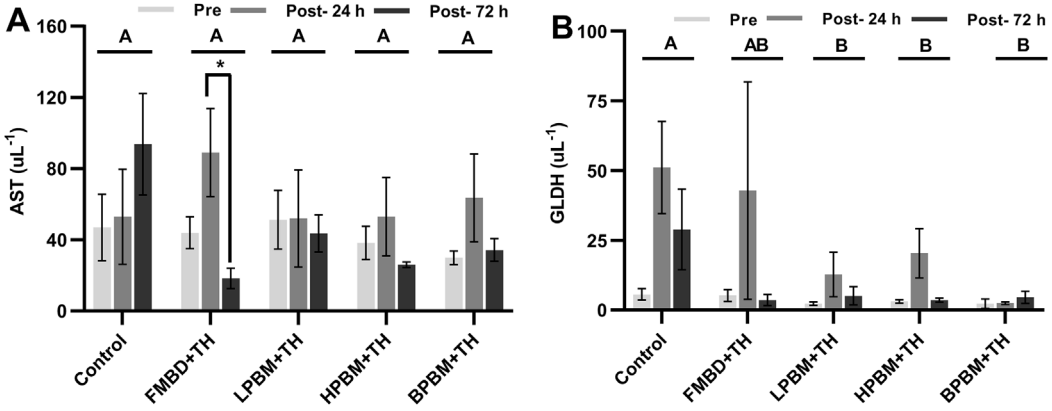

Fig. 1. Serum aspartate aminotransferase, AST (A) and glutamate dehydrogenase, GLDH (B) in barramundi fed with different experimental diets supplemented with TH. Data were expressed as mean ± SE, n = 3. Different uppercase (A,B,C) letters among different dietary treatments were statistically significant (two-way ANOVA; Tukey post-hoc test; $P < 0.05$). *$P < 0.05$ denotes significant level among pre-challenge, post-challenge-24 h and post-challenge- 72 h in each treatment (one-way ANOVA; Tukey post-hoc test).

But the glucose and triglyceride levels among the treatments were not significantly different in the post-challenge condition at 24 h and 72 h. The highest glucose level was observed in the post-challenge condition at 24 h when compared to fish at the pre-challenge and post-challenge condition at 72 h. However, the triglyceride level was found to be significantly higher at the pre-challenge condition when compared to post-challenge fish at 24 h and 72 h. Although the cholesterol level remained consistent among the dietary groups in the pre-challenge condition, a significantly lower level was observed with the control and FMBD + TH when compared to the rest of the diets in the post-challenge condition at 24 h and 72 h. Neither dietary supplementation of TH nor fermentation had the significant effect on the total protein, albumin, globulin and A/G ratio in juvenile barramundi ($P > 0.05$) in either the pre-challenge or post-challenge conditions.

3.4. Assessment of liver damage

The serum AST activity of fish was not significantly affected, either by the tested diets or challenge periods ($P > 0.05$), except in FMBD + TH where the AST level in post-challenge fish at 72 h was significantly lower when compared to post-challenge fish at 24 h (Fig. 1A). However, except for FMBD + TH, the cumulative results (pre, post-24 h and post-72 h) of serum GLDH activity was significantly lower in the experimental diets when compared to the control. No significant changes were observed between challenge periods in each treatment for GLDH activity (Fig. 1B).

3.5. Lysozyme activity

Serum lysozyme activity was significantly influenced, both by dietary groups and the duration of time after the challenge (Fig. 2A). The highest overall lysozyme activity was found in fish fed FMBD + TH and BPBM + TH when compared to the control. Fish at 72 h post-challenge exhibited higher lysozyme activity in each treatment compared to the post-challenge condition at 24 h in all dietary groups, and to the pre-challenge condition in HPBM + TH and BPBM + TH.

3.6. Bactericidal activity

In terms of bactericidal activity, the higher the efficiency of immune cells to kill pathogens, the lower the bactericidal colonies observed in cells. In the present study, the accumulative serum bactericidal activity in all dietary groups was not significantly different ($P > 0.05$) when compared to the control (Fig. 2B). However, fish at 72 h post-challenge demonstrated the highest bactericidal activity compared to the pre-challenge condition in FMBD + TH and BPBM + TH. Fish that received the control, LPBM + TH and HPBM + TH diets showed no significant differences in bactericidal activities among the challenge periods.

3.7. Intestinal mucosal morphology

The intestinal mucosal morphology of juvenile barramundi fed the experimental diets was analysed by observation of TEM and SEM (Fig. 3

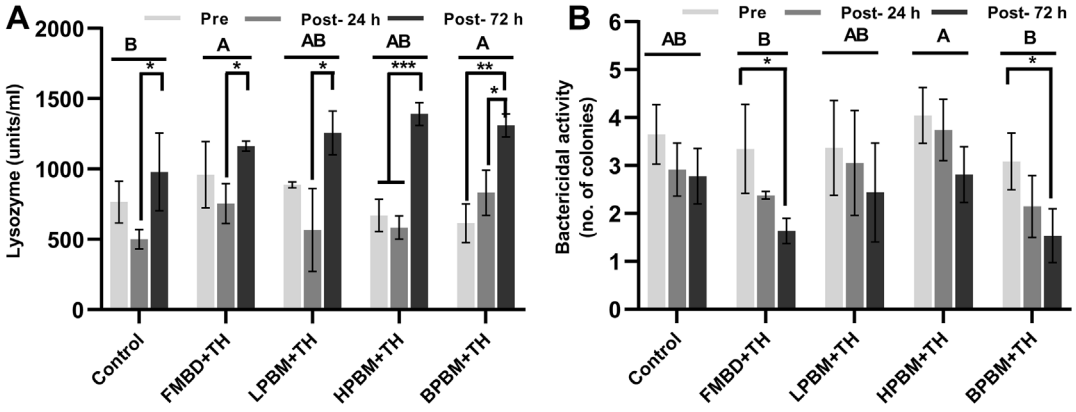

Fig. 2. The serum lysozyme (A) and bactericidal (B) activity in barramundi fed with different experimental diets supplemented with TH. Data were expressed as mean ± SE, n = 3. Different uppercase (A,B,C) letters among different dietary treatments were statistically significant (two-way ANOVA; Tukey post-hoc test; $P < 0.05$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ denote significant level among pre-challenge, post-challenge-24 h and post-challenge- 72 h in each treatment (one-way ANOVA; Tukey post-hoc test).

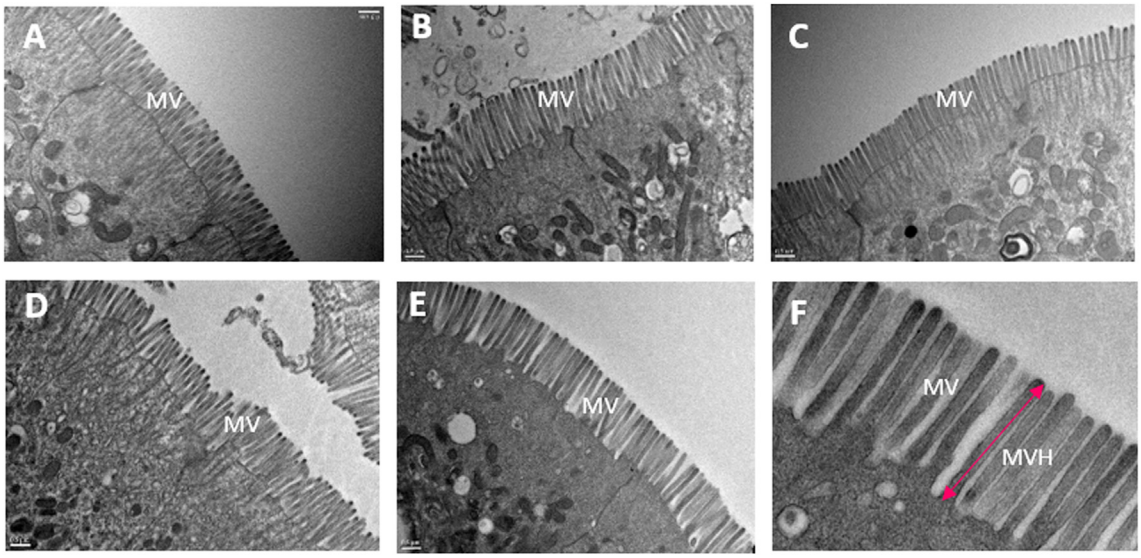

Fig. 3. Comparative transmission electron micrographs (TEM) from the distal intestine of barramundi at the end of the feeding trial. TEM images for microvilli length; A: Control, B: FMBD + TH, C: LPBM + TH, D: HPBM + TH, E: BPBM + TH and F: measurement of microvilli length and diameter. MV: microvilli, MVD: microvilli diameter, MVH: microvilli height. Scale bar = 0.5 μm.

and Fig. 4, respectively) after 10 weeks of the feeding trial. TEM analysis revealed a significant difference ($P < 0.05$) in the microvilli height of the distal intestinal tracts of barramundi reared in different dietary groups (Fig. 5A). The highest microvilli height was found in the FMBD + TH and BPBM + TH, and the lowest value was observed in the HPBM + TH when compared to the control ($P < 0.05$). However, the microvilli height of fish in the LPBM + TH group was similar to that of the control. SEM analysis of the distal intestine demonstrated that microvilli density was not significant among the dietary groups when compared to the control (Fig. 5B).

3.8. Resistance to infection

The survival of *V. harveyi* challenged barramundi was significantly higher in fish fed all TH treated groups (FMBD + TH, LPBM + TH, BPBM + TH) when compared to the control ($\chi^2_{FMBD+TH} = 14.34$, $df = 1$, $P < 0.001$, $\chi^2_{LPBM+TH} = 9.25$, $df = 1$, $P < 0.01$ and $\chi^2_{BPBM+TH} = 22.72$, $df = 1$, $P < 0.001$). However, the HPBM + TH dietary group exhibited no significant difference in survival compared to the control ($\chi^2_{HPBM+TH} = 3.63$, $df = 1$, $P > 0.05$). After 14 days, the BPBM + TH had the highest cumulative survival percent with 90.3%, followed by 80.6%, 67.6%, 50.0% and 33.3% for the FMBD + TH, LPBM + TH, HPBM + TH and control, respectively (Fig. 6).

4. Discussion

FPH has been reported as a promising aqua-feed ingredient for aquaculture, not only to improve fish growth but also to enhance immune status, potentially leading to increased disease resistance [22]. The present study investigated the application of 10% TH in experimental diets, and was prompted by the outcomes of our previous study on the use of FPH in juvenile barramundi diets [22]. The results suggest

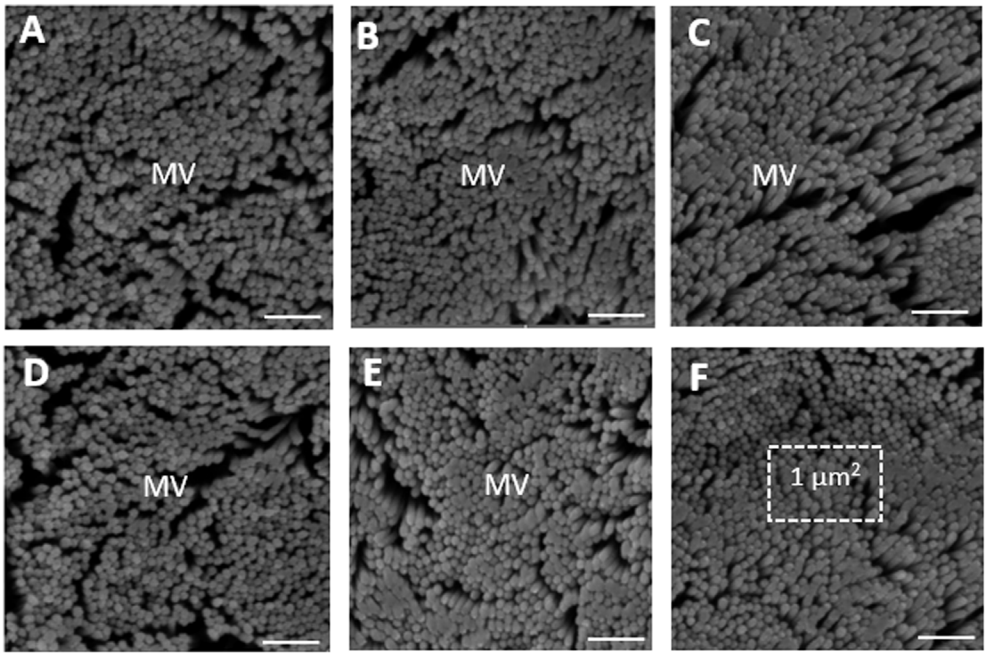

Fig. 4. Scanning electron micrographs (SEM) from the distal intestine of barramundi at the end of the feeding trial. SEM images for microvilli density; A: Control, B: FMBD + TH, C: LPBM + TH, D: HPBM + TH, E: BPBM + TH and F: measurement of microvilli density in each square micrometer from the fish under different dietary groups. MV: microvilli. Scale bar = 0.5 μm.

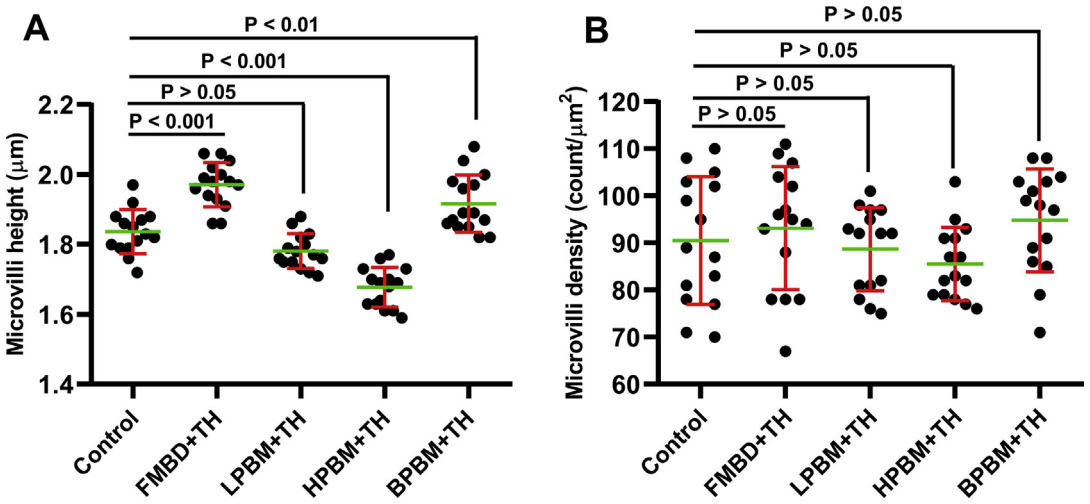

Fig. 5. Intestinal microvilli height (A) and density (B) of barramundi fed with different experimental diets supplemented with TH. Data were expressed as mean ± SE, n = 15. Bar holding P-values denote significant level among the experimental treatments (one-way ANOVA; Tukey post-hoc test; not significant $P > 0.05$; significant $P < 0.05$; $P < 0.001$).

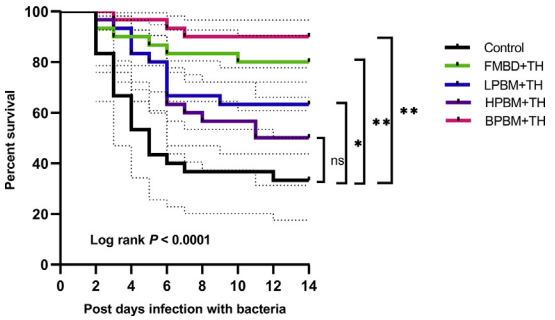

Fig. 6. Survival curve (based on Kaplan Meier's estimates) of barramundi after being challenged with *Vibrio harveyi*. The mortality (moribund condition) was recorded daily for 14 days after the bacterial challenge. Thirty individuals per group i.e.10 per replicate were considered for the survival analysis and comparison among groups were made at the same time point after completion of the challenge trial. Significantly higher survival was observed in all test diets when compared to the control. Asterisks × and ** indicate statistically significant difference between treated group and infected control at $P < 0.01$ and $P < 0.001$, respectively. ns indicates non-significant. The dotted lines indicate 95% confidence intervals.

that supplementation of 10% TH in diets of FMBD + TH and BPBM + TH significantly improve the FBW and SGR of juvenile barramundi. These results are in agreement with past studies in which dietary inclusion of FPH consisting of low molecular weight peptides (< 3000 Da) had positive effects on the growth performance of marine carnivorous species, including Atlantic salmon, *Salmo salar* [39], yellow croaker, *Pseudosciaena crocea* [40], and olive flounder, *Paralichthys olivaceus* [41]. The enhanced growth performance with FMBD + TH and BPBM + TH may be due to the supplementation of TH, which improved the palatability of the diets leading to feed intake of fish [19]. Furthermore, the fermented bacteria, *Lactobacillus casei* and yeast could also have stimulated endogenous enzyme activity for digestion [42,43] and positively affected the gut microbiota through improved feed intake and enhanced nutrient absorption and assimilation [44].

The lipid content of the muscle tissues of juvenile barramundi in the present study was influenced by TH inclusion which was in accordance with many previous studies where the proximate composition of the whole-body and/or individual organs or component parts of the fish were affected by the addition of various alternative animal protein sources in FM containing diets [45–47]. In particular, Wu, Zhou [48] found that crude fat in the whole body and liver of yellow catfish, *Pelteobagrus fulvidraco*, were changed by the low, medium and high inclusions of hydrolysate stickwater at various levels in non-FM diets. Like the present study, protein, energy and ash content of fish were not found significantly different by the dietary inclusion of marine soluble proteins in FM diets [21,49]. However, altered whole-body protein contents were reported in fish with the addition of FPH [19,50]. It is likely a number of factors, including sources of hydrolysates, varying inclusion levels and the size of fish species, may have impacted the effects of protein hydrolysates on the whole-body composition of fish [21,48].

Blood indices act as indicators of metabolic function, nutritional status and physiological condition of fish [3,30,51]. Previous studies showed that fish hydrolysate and fermented diets with animal protein blend resulted in a lowering of the blood glucose level in fish [52]. Immunostimulants used in aquaculture production are capable of influencing the glucose levels of fish by increasing the insulin level [34,53]. The results presented here suggest that TH produced through enzymatic hydrolysis via the addition of several enzymes, such as alcalase, protease, neutrase, trypsin, α-chymotrypsin, pepsin, protamex and flavourzyme [54] may trigger insulin secretion, accordingly reducing the glucose level, which in turn favourably affects the wellbeing of the fish [34]. In post bacterial challenges at 24 h, elevated glucose levels in all experimental groups were detected. This might have been due to the induced stress by pathogenic bacteria as well as reduced feeding from three times a day to once in the challenge condition. Ardiansyah and Fotedar [55] also found increased blood glucose levels in juvenile barramundi after an ammonia stress challenge.

Cholesterol and triglycerides, important metabolites of blood, are widely used indicators for assessing the nutritional status of fish [56,57]. In the present study, the serum triglyceride levels were significantly reduced in all dietary groups at the pre-challenge condition compared to the control, as well as the cholesterol in the control and the FMBD + TH groups at post-challenge conditions compared to rest of the dietary groups. This result was in accordance with the study of Kader, Koshio [52], who found lower triglyceride and cholesterol in fish fed with fish hydrolysate and an animal protein blend. Furthermore, serum triglycerides and cholesterol in fish varied with the feed depravation time and were usually compensated with body reserves during fasting [58,59]. Accordingly, in the current study, it can be hypothesised that serum triglycerides and cholesterol were decreased after the challenge due to the lower feed supply in the challenge condition.

AST and GLDH are normally measured in fish as indicators of hepatocellular injury, to thereby determine liver health status. Higher

levels of these enzymes in serum might indicate cell damage in the liver or the detrimental effect of feeding regimes. In the present study, AST and GLDH values were found non-significant among the dietary groups, indicating that the tested feeding patterns do not damage the liver in barramundi. These results agreed with the findings of Khosravi, Rahimnejad [21] who reported the serum AST level was not influenced by different FPH inclusions in fish diets.

The structure of the intestinal mucosal morphology is a good indication of the ability of fish to digest and absorb nutrients in the digestive tract [60,61]. The improvements in intestinal micromorphology of fish, including microvillus height and density, is a positive indication of good intestinal health, which is important to boosting the health status of the mucosal epithelium as well as increasing the ability to prevent opportunistic microbial infection [62]. TEM analysis in the present study revealed that microvillus height increased significantly in the distal intestines of the barramundi fed FMBD + TH and BPBM + TH diets. This result was in accord with our previous study in which dietary supplementation of TH in FM-based diets at 10% significantly increased the microvilli height in juvenile barramundi [22]. Similarly, other than in fish, improvements in villus height have been reported in broiler chickens using a low-level (2%) of enzymatically hydrolysed scallop visceral protein [63]. SEM analysis demonstrated that none of the dietary treatments appeared to affect microvillus density in the distal intestines of fish. Despite this, the improvement of microvilli morphology may lead to improved apical brush border integrity which may prevent harmful bacteria and their toxins from impacting intestinal epithelial cells. This, in turn, may improve the growth performance and disease resistance of fish. The longer microvillus of fish fed diets FMBD + TH and BPBM + TH supplemented with TH was also consistent with the observed feed intake and growth performance, indicating that more nutrients may have been digested and absorbed in the intestine. Therefore, the overall analysis of the results obtained from the TEM and SEM images indicate that TH added to diets can induce a significant increase in the absorptive capacity of the intestinal mucosa, with no evidence of morphological alterations.

It has been reported that antimicrobial peptides such as lysozyme may assist in preventing colonisation of micro-organisms in the host body, thereby resulting in the prevention of pathogens [64,65]. Serum lysozyme is one of the antimicrobial enzymes produced by immune cells to fight infection [66]. The bactericidal activity is a mechanism of killing pathogenic microorganisms in fish [67]. Previous studies suggested that dietary inclusion of FPH in fish diets may stimulate the innate immune responses, and this stimulant is strongly influenced by the amount of hydrolysate in the diet [26,68]. The overall improvement of serum lysozyme and bactericidal activities in FMBD + TH and BPBM + TH are an indication of enhanced immune response with these diets in fish when compared to the control. These results are comparable with the increased serum lysozyme activity noted in large yellow croaker fed with FPH with the inclusion of 10 and 15% in a floating case system [40]. In addition, Japanese sea bass fed with a 15% FPH diet had significantly improved lysozyme and complement activities after feeding for 60 days [28].

In the present study, the cumulative mortality of barramundi after the 14-day challenge with *V. harveyi* was significantly lower in all experimental diets when compared with the control and the HPBM + TH (Fig. 6). This may indicate that 10% TH supplementation in FM (FMBD + TH), 75% PBM (LPBM + TH) and 90% bioprocessed PBM (BPBM + TH) diets could protect fish from the impact of bacterial infection. Similarly, dietary administration of FPH increased the disease resistance of various fish, such as red sea bream, *Pagrus major*, and juvenile olive flounder, *Paralichthys olivaceus*, against *Edwardsiella tarda* [19] and European sea bass larvae, *Dicentrarchus labrax*, to *Vibrio anguillarum* [26]. In one of our previous studies, it was demonstrated that TH is able to improve survival rates in barramundi against *Streptococcus iniae* infection [22]. A number of studies in the past have reported that enzymatic FPH with low molecular weight peptides (< 3000Da) have a number of bioactive properties with the potential to influence antioxidative, antimicrobial and anti-inflammatory activity, which may enhance the disease resistance of fish [69]. The reduction of infection in the TH supplemented groups of FMBD + TH, LPBM + TH and BPBM + TH in our present study could be explained either by an improvement in the overall fish health or by the stimulation of the immune system.

In conclusion, the present study revealed that the dietary supplementation of TH in FM (FMBD + TH) and bioprocessed PBM (BPBM + TH) diets in barramundi improved the growth performance and feed intake whilst significantly modulating the gut morphology, lysozyme activity, and disease resistance against *V. harvei*. Although growth performance, biochemical response and disease resistance of barramundi were not significantly improved by the HPBM + TH diet, this diet provided equal performance when compared to the FM based control diet. Therefore, the application of TH in non-FM based diets may present a novel strategy for enhancing growth performance as well as assisting in fish health management in barramundi aquaculture.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.042.

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