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Effects of various levels of squid hydrolysate and squid meal supplementation with enzyme-treated soy on growth performance, body composition, serum biochemistry and histology of Florida pompano *Trachinotus carolinus*

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A R T I C L E I N F O

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A B S T R A C T

The purpose of this study was to evaluate the impact of plant-based diets supplemented with various levels of enzyme-treated soybean meal (ESBM) and squid products on the growth, proximate composition, serum biochemistry, and liver and intestinal histological alterations in pompano (*Trachinotus carolinus*). A 15% poultry by-product meal (PBM) based practical diet was used as a reference and basal diet was produced by replacing PBM with ESBM. Basal diet was then modified to contain varying levels of squid products. Squid hydrolysate (SH) and squid meal (SM) were added to basal diets, to produce diets containing 1, 2 and 4% of the squid products. A total of eight experimental diets were each fed to quadruplicate groups of 20 pompanos (mean initial wt = 7.68 ± 0.1 g) in a recirculating rearing system for 56 d. Results from the growth trial indicate that fish fed with basal diet exhibited significantly lower growth performance and feed utilization as compared to fish fed with PBM. The addition of 4% SH improved the response of basal diet and did not show any significant difference in terms of growth performance as compared to PBM. Whole body proximate and amino acids composition of fish were not significantly different among fish reared on any of the diets. Data on mineral composition of fish showed that the content of phosphorus and calcium were significantly higher in fish fed with basal diet compared to other treatments. Total protein and cholesterol level of fish fed PBM were significantly lower compared to basal diet. Total albumin, alanine transaminase, aspartate transaminase, and bile acids were similar among the dietary treatments. Fish fed with basal diet showed disordered vacuolization in the liver and decreased the lamina propria thickness in the intestine. The inclusion of 4% SH partly prevented the alteration of liver and distal intestine of Florida pompano and findings were similar to fish fed with PBM. Based on these results, combination of ESBM and 4% SH has the potential to serve as an alternative protein source and attractant to improve the efficacy of plant-based diet for pompano.

1. Introduction

Considerable research has been conducted to evaluate the effect of soybean meal (SBM) inclusion to partially or completely replace the use of animal meal in the aquatic animal feed formulations (Hertrampf and Piedad-Pascual, 2000; Sales, 2009). However, it has been suggested that when a substantial amount of fish meal (FM) is replaced by SBM and other plant protein sources, feed intake generally declines (Morales et al., 1994) and affecting the growth performance of fish (Nunes et al., 2006; Watanabe et al., 1998). Supplementation with attractants or palatability enhancers, such as krill meal (Gaber, 2005), blue mussel meal (Nagel et al., 2014), tuna by-product meal (Hernández et al., 2011), algal meal (Kissinger et al., 2016), nucleotides (Barnard, 2006) and chemo-attractants derived from hydrolysis process of seafood waste and by-products (Barry et al., 2017; Refstie et al., 2004) may enhance the palatability and feed intake of plant-based diet. However, according to De la Higuera (2001) low inclusion level of animal meal is still needed to increase the feed intake in some fish species.

Previous findings in Florida pompano *Trachinotus carolinus* highlighted that reducing animal protein from 15% to 0% in the diet resulting a linear decrease in the growth performance of fish (Rhodes et al., 2017; Rossi and Davis, 2012). In addition, with 100% replacement of animal meal, supplementation of glycine, histidine and fish protein concentrate did not significantly affect the growth performance

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or feed utilization of pompano (Rhodes et al., 2017). However, when 5% squid hydrolysates (SH) was added to the animal free diet, fish had significantly higher final weight, percentage weight gain and thermal growth coefficient (TGC) in comparison to fish fed with plant-based diet, 5% chemical attractant mix and 5% poultry by product meal (PBM) (Rhodes et al., 2017). The increase in feed intake and growth performance might be due to the presence of chemoattractant properties in squid products, such as glycine and betaine, that have potential impact to activate fish feeding behavior (Meyers, 1986). In addition, well balanced amino acid profile, high protein value and the presence of highly unsaturated fatty acids (HUFA) proportion of total fat in squid products would also be beneficial factors for its use as an ingredient in aquaculture diets (Hertrampf and Piedad-Pascual, 2000; Lian et al., 2005). Hence, there is an opportunity to improve the performance and utilization of the plant-based diet by proper supplementation of these ingredients.

It has also been suggested that when animal meal is replaced by traditional solvent extracted SBM, the anti-nutritional factors (ANFs) may play a role in decreasing performance (Batal and Parsons, 2003; Iwashita et al., 2008; Krogdahl et al., 1994). Different processing technique have been reported to be an effective method to denature and reduces several ANFs, including fermentation, enzyme treatment and alcohol extraction (Hong et al., 2004; Papagianni et al., 1999; Riche and Williams, 2011). However, little is known about the value of novel enzyme-treated soybean meal (ESBM) produced by using a combination of non-alcohol extraction process and enzymatic treatment. Thus, the aim of this study was to evaluate the use of ESBM and squid products to completely replace PBM on the growth performance, body composition, serum biochemistry and histology of Florida pompano.

2. Materials and methods

2.1. Experimental diets

The proximate analysis of dietary protein sources and formulation of the experimental diets are presented in Tables 1 and 2, respectively. Diets were designed to be iso-nitrogenous and isolipidic (40% protein and 8% lipid). The basal diet contained no animal-based protein sources and was formulated using de-hulled solvent extracted soybean meal (SBM, Bunge Limited, Decatur, AL, USA), enzyme-treated soybean meal (ESBM, Nutrivance™, Midwest Ag Enterprises, Marshall, MN, USA), and corn protein concentrate (CPC, Empyreal 75™, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA) as the dietary protein sources. The next six diets were formulated to contain 1, 2, and 4% squid hydrolysates (SH) or squid meal (SM), designated as 1%SH, 2% SH, 4% SH, 1% SM, 2% SM, and 4%SM, respectively, at the expense of ESBM. Additionally, a poultry-based practical diet which has been run in numerous trials was included as a reference diet and was produced utilizing 15% poultry by product meal (PBM, Griffin Industries, Inc., Mobile, AL, USA) to completely remove ESBM in the basal diet. Test diets were produced in the Aquatic Animal Nutrition Laboratory, School of Fisheries, Aquaculture and Aquatic sciences, Auburn University, AL, USA, using standard procedures for Florida pompano. Briefly, diets were made by mixing preground dry ingredients and fish oil in a food mixer (Hobart, Troy, OH, USA) for approximately 15 min. Boiling water was then blended into the mixture to attain a consistency appropriate for pelleting. The moist mash from each diet was passed through a 3 mm die in a meat grinder, and the pellets were then placed into a fan-ventilated oven (< 45 °C) overnight to attain a moisture content of < 10%. Diets were stored at − 20 °C, and prior to use, each diet was ground and sieved to an appropriate size. The experimental diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate analysis (Table 2) and amino acid profile (Table 3).

Table 1
Proximate composition (g/100 g as is) of primary protein sources.

Ingredient	Protein	Moisture	Lipid	Ash
SBM[a]	46.72	10.99	2.46	6.44
SH[b]	72.22	8.16	10.53	6.03
SM[c]	72.19	10.19	9.03	9.66
ESBM[d]	62.55	7.25	1.48	4.31
PBM[e]	67.72	5.02	15.07	10.35

[a] SBM (de-hulled Solvent Extracted Soybean Meal), Bunge Limited, Decatur, AL, USA.
[b] SH (Squid hydrolysates), Produced for this research (Lian et al., 2005).
[c] SM (Squid meal), Foodcorp S.A., Chile.
[d] ESBM (Enzyme-treated soybean meal), Nutrivance™, Midwest Ag Enterprises, Marshall, MN, USA.
[e] PBM (Poultry by-product meal), Griffin Industries, Inc., Mobile, AL, USA.

2.2. Preparation of squid products

Dry squid hydrolysates (SH; 71.6% Crude protein, special select, Rhode Island University, RI, USA) produced from enzymatic hydrolysis of squid processing by-products (SPB) (heads, viscera, cut offs, fins and small tubes) and squid meal (SM; 70% Crude protein, Foodcorp S.A., Chile) served as the supplement in the plant-based diet formulation. For dry squid hydrolysates, preparation of raw material and hydrolysis process were carried out at the Food Science and Nutrition Research Center-Seafood Lab at the Rhode Island University, RI, USA and processed according to Lian et al. (2005).

2.3. Growth trials

The growth and feeding trials were conducted in the Claude Peteet Mariculture Development Center (CPMC), Gulf Shores, AL, USA. Florida pompano fingerlings were purchased from Troutlodge Marine Farms LLC, (Proaquatix) Vero Beach, Florida, USA, nursed in an indoor recirculating system facility of CPMC, and fed with commercial diet until reaching a suitable size. The trial was conducted in a recirculating system consisted of thirty-two 700-L polyethylene open top tanks equipped with reservoir tank, biological filter, supplemental aeration (provided using a central line, regenerative blower and air diffusers) and circulation pump. At the start of experiment, twenty fish (mean weight = 7.68 ± 0.1 g) were stocked into each tank and assigned to quadruplicate tanks in a completely randomized design. Fish were maintained under a natural photoperiod with water temperature, salinity, pH, and the dissolved oxygen concentration of the culture water at 26.6 ± 1.9 °C, 30.1 ± 2.8‰, 7.9 ± 0.2, and 5.9 ± 0.6 mg L^{-1}, respectively. A subsample of fish from the initial stocking was retained for proximate, amino acid and mineral profile analysis. Fish were fed four times per day and the daily ration was adjusted to apparent satiation weekly throughout the trials. Additionally, feed inputs were calculated on a two-weeks basis after each sampling to adjust for growth and mortalities. The growth trial lasted for 56 d. At the end of growth trials, fish were grouped and individually weighed to obtain the final biomass, final weight, feed conversion ratio (FCR) (feed offered/wet weight gain), percentage survival [100 × (final number / initial number)], and thermal unit growth coefficient (TGC), calculated as: $100 \times [FBW^{1/3} - IBW^{1/3} / \Sigma DT]$, where FBW is final body weight, IBW is initial body weight, D is number of days and T is water temperature (°C).

2.4. Body composition analysis

Upon termination of the trial, twenty fish per treatment (five fish per replicate) were randomly selected from each group and stored at − 80 °C for body composition analysis. Prior to proximate, amino acid and mineral analysis, dried whole fish were rigorously blended and chopped in a mixer according to methods described by Association of Official Analytical Chemist (AOAC). All parameters were analyzed at

Table 2
Composition (as is g kg^{-1}) of diets containing various levels of squid hydrolysates (SH) and squid meal (SM) added into the basal diet and fed to juvenile Florida pompano for 8 weeks.

Ingredients (g kg^{-1} as is)	PBM	Basal	1%SH	2%SH	4%SH	1%SM	2%SM	4%SM
Poultry by product meal[1]	150.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Soybean meal[2]	472.1	472.1	472.1	472.1	472.1	472.1	472.1	472.1
Enzyme-treated soybean meal[3]	0.0	148.0	136.5	125.1	102.2	136.8	125.6	103.2
Squid hydrolysates[4]	0.0	0.0	1.0	2.0	4.0	0.0	0.0	0.0
Squid meal[5]	0.0	0.0	0.0	0.0	0.0	1.0	2.0	4.0
Corn protein concentrate[6]	63.0	80.0	80.0	80.0	80.0	80.0	80.0	80.0
Menhaden fish oil[7]	47.4	63.7	63.5	63.3	62.9	63.3	63	62.3
Corn starch[8]	7.0	4.7	6.6	8.4	12.1	6.9	8.3	11.9
Whole wheat[8]	220.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0
ASA trace mineral premix[10]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
ASA vitamin premix w/o choline[11]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Choline chloride[8]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Stay C 35%[12]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
CaP-dibasic[8]	20	31	30.8	30.6	30.2	30.4	30.5	30
Lecithin (soy commercial)[9]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Taurine[8]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Lysine[8]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Proximate analyses (g kg^{-1} as is)								
Crude protein	426.0	414.9	411.5	399.4	422.3	417.6	426.3	408.1
Moisture	72.9	83.6	93.1	110.2	76.6	80.5	59.1	108.7
Crude fat	95.6	82.8	79.5	85.3	89.4	90.8	82.9	80.4
Crude fiber	27.5	32.4	29.1	28.8	30.8	29.8	31.7	31.3
Ash	64.9	64.4	63.7	61.6	63.9	62.9	64.6	62.2

[1] Griffin Industries, Inc., Mobile, AL, USA.
[2] De-hulled Solvent Extracted Soybean Meal, Bunge Limited, Decatur, AL, USA.
[3] Nutrivance™, Midwest Ag Enterprises, Marshall, MN, USA.
[4] Produced for this research (Lian et al., 2005).
[5] Foodcorp S.A., Chile.
[6] Empyreal 75™ Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[7] Omega Protein Inc., Houston, TX, USA.
[8] MP Biomedicals Inc., Santa Ana, CA, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] ASA Premix (g 100 g^{-1} premix): cobalt chloride, 0.004; cupric sulfate pentahydrate, 0.250, ferrous sulfate heptahydrate, 4.0, manganous sulfate anhydrous, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193, and α-cellulose 81.826.
[11] ASA Premix (g kg^{-1} Premix): thiamin HCL, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Ca-pantothenate, 20.0; niacin, 40.0; biotin, 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}), 2.40; vitamin D_3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; and α cellulose, 834.258.
[12] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA.

Table 3
Amino acid (AA) profile (as is g kg^{-1}) of experimental diets.

Composition[1]	PBM	Basal	1%SH	2%SH	4%SH	1%SM	2%SM	4%SM
Taurine	6.5	5.9	6.1	6.3	7.5	5.9	6.2	6.1
Hydroxyproline	3.4	1.0	0.5	0.5	0.1	0.5	0.4	0.8
Aspartic acid	39.1	40.4	38.7	39.6	40.7	40.2	41.9	39.6
Threonine	15.2	14.6	14.2	14.5	15.2	14.8	15.5	14.7
Serine	17.1	16.9	16.3	16.7	17.1	17.4	17.9	16.7
Glutamic acid	77.2	79.0	76.4	77.2	79.3	80.2	82.0	76.3
Proline	26.1	24.9	24.3	24.8	25.7	26.1	26.1	24.0
Glycine	21.9	17.1	16.9	16.6	17.8	17.2	18.2	17.4
Alanine	21.8	20.0	19.7	19.5	20.9	20.9	21.5	20.2
Cysteine	5.9	5.9	5.7	5.8	6.1	5.8	6.1	5.6
Valine	20.8	20.4	19.7	20.1	20.7	20.5	21.4	20.0
Methionine	7.1	6.5	6.4	6.6	7.1	6.6	7.1	6.7
Isoleucine	18.2	18.5	18.0	18.4	18.9	18.6	19.3	18.1
Leucine	35.7	36.5	35.6	35.9	37.5	38.1	38.9	35.9
Tyrosine	13.6	13.4	13.5	13.8	14.0	14.4	14.6	11.7
Phenylalanine	20.5	21.2	20.4	20.7	21.2	21.5	22.0	20.7
Hydroxylysine	0.8	0.4	0.5	0.5	0.6	0.4	0.5	0.5
Ornithine	0.3	0.3	0.3	0.3	0.6	0.3	0.3	0.5
Lysine	22.2	21.2	20.4	20.8	21.6	20.8	22.1	21.1
Histidine	10.0	10.2	9.8	9.9	10.1	10.1	10.5	9.9
Arginine	26.1	25.3	24.7	25.2	25.5	25.6	26.6	24.8
Tryptophan	5.8	5.8	5.5	5.5	5.8	5.6	5.6	5.1

[1] Ingredients were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Midwest Laboratories (Omaha, NE, USA) and the mean of each value were taken.

2.5. Serum biochemistry analysis

At the end of growth trial, sixteen fish per treatment (four fish per replicate) were immediately euthanized with MS222 (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma), and blood samples were taken from the caudal vein after 12 h starvation. Blood samples were collected from basal diet, PBM, 4% SH and 4% SM treatment using anticoagulant-free centrifuge tubes. Serum was obtained by centrifugation of blood at 3.000 rpm for 10 min and stored at − 80 °C until the analysis. Biochemical parameters in the serum samples were analyzed using an automatic chemistry analyzer (Cobas C311, Roche Diagnostics, IN, USA) for total protein, albumin, activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, cholesterol, and bile acid concentration.

2.6. Histological section

Fish selected for histological sampling and external disease evaluation were individually euthanized in a solution MS222 (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich Co. LLC, USA). Liver and distal intestine of fish was excised and preserved in Bouin's solution for 20 h at room temperature and then transferred to 70% ethanol solution until processed by standard histological procedures. The blocks of designed sample were dehydrated through a standard ethanol series to 100%, embedded in paraffin wax and sectioned at 4 μm intervals for staining with Hematoxylin-Eosin (H & E) stain

(Merck, cat: 1.05174.1000; Scharlau, cat: EO0025). Double blinded evaluation with scoring system from 1 to 5 was used to evaluate the histological condition. Score 1 was considered as the normal condition and subsequent scores accounted for histopathological alteration compared to the normal condition. Liver section was evaluated for hepatocyte vacuolization, nuclear change and glycogen accumulation. Intestinal samples were evaluated for cellular infiltration, presence of goblet cells and widening of the lamina propria within the intestinal folds. Histopathological images were acquired by using an imaging microscope (ECLIPSE 80i, Nikon, Japan).

2.7. Statistical analysis

All data except for histological data were analyzed using one-way analysis of variance to determine the significant difference ($P < 0.05$) among the treatment means followed by the Tukey's multiple comparison test to determine difference between treatments means in each trial. The pooled standard errors were used across growth trials, proximate composition, serum levels and enzyme activities, as the variance of each treatment is the same. Histological scores were treated as categorical data, tested for normality and homoscedasticity and subsequently analyzed using Welch's one-way analysis of variance followed by Games-Howell *post hoc* tests to determine significant differences between treatments. Statistical analyses were conducted using SAS system (V9.3, SAS Institute, Cary, NC, USA).

3. Results

3.1. Nutrient composition of experimental diets

The proximate compositions of each primary protein sources used in this research are presented in Table 1. Both squid products, SH and SM contained high protein levels (72.19–72.22%) and a moderate level of lipid (9.03–10. 53%) and moisture (8.16–10.19%), but differed in ash content, where SH (6.03%) had lower ash content compared to SM (9.66%). For the soy protein sources, ESBM has higher protein level but lower in moisture, lipid and ash content compared to SBM. Regarding to the amino acid and proximate profile of experimental diets, the compositions reflected the dietary protein source (Tables 2 and 3).

3.2. Growth performance

The growth performance data are presented in Table 4. Mean survival of fish in all treatments was not significantly affected ($P = 0.3956$) by dietary treatments and was typical for research with this species. Differences in performance of pompano were observed when PBM was completely replaced by ESBM. Reductions in final biomass (702.3 to 405.8 g), individual final weight (38.6 to 28.17 g) and feed intake (43.99 to 32.54 g fish^{-1}) were observed in fish fed basal diet as compared to fish fed with PBM, respectively ($P < 0.05$). However, performance with PBM was not significantly better when basal diet was supplemented with 4% SH. Feed intake was significantly different with these diets, as the feed intake of PBM was significantly higher compared to 4% SH ($P = 0.0001$). FCR ranged from 1.42 to 2.29 and fish fed with 1% SM had the highest FCR compared to other treatments ($P = 0.0066$). TGC ranged from 0.0522–0.0903 ($P = 0.0001$). PBM had the highest numerical value but did not have any significant difference with 4% SH, 2% SH and 4% SM.

3.3. Proximate, amino acids and mineral composition of Florida pompano fillet

Proximate and mineral composition analysis of whole fish body are presented in Table 5. No significant differences were observed in percentage of protein ($P = 0.2229$), fat ($P = 0.2124$) and ash content ($P = 0.1202$) across all dietary treatments. Phosphorus (P) and calcium (Ca) were significantly higher in fish fed with basal diet compared to other treatments while iron (Fe) and manganese (Mn) in fish fed with basal diet were significantly higher than PBM. Meanwhile, sodium (Na) was significantly higher in fish fed with basal diet and 2% SM compared to PBM. The amino acid (AA) profile of whole fish body is presented in Table 6. Significantly higher cysteine level was found in fish fed with basal diet ($P = 0.0459$) compared to 4% SH, while other amino acids values were not affected by the dietary treatments.

3.4. Serum levels and enzyme activities

Effect of different diets on serum levels and enzyme activities in pompano are presented in Table 7. Total protein (g dL^{-1}) and cholesterol (mg dL^{-1}) levels of fish fed with PBM were significantly lower than in fish fed basal diet ($P < 0.05$). Glucose (mg dL^{-1}) level of fish fed with 4% SM was significantly higher in comparison to fish fed with PBM ($P = 0.0198$). Alkaline phosphatase (ALP) activity was significantly lower in fish fed with PBM ($P = 0.0541$). Higher ALP activity was pronounced in fish fed with basal diet supplemented with 4% SM. Serum levels of Alanine transaminase (ALT), Aspartate transaminase (AST) together with the concentration of albumin and bile acid were not significantly different among the dietary treatments ($P > 0.05$).

Table 4
Growth performance of juvenile Florida pompano (Mean initial weight 7.68 ± 0.1 g) fed experimental diets for 56 d. Values represent the mean of four replicates. Results in the same columns with different superscript letter are significantly different ($P < 0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

Diet	Final biomass (g)	Final mean weight (g)	TGC[4]	Feed intake (g × fish^{-1})	FCR[5]	Survival (%)
PBM[1]	702.30^{a}	38.60^{a}	0.0903^{a}	43.99^{a}	1.42^{b}	91.25
Basal	405.75^{bc}	28.17^{bcd}	0.0677^{bcd}	32.54^{bc}	1.69^{ab}	73.75
1% SH[2]	449.47^{bc}	23.65^{cd}	0.0562^{cd}	32.05^{bc}	2.09^{ab}	85.00
2% SH	515.25^{bc}	32.84^{abc}	0.0784^{abc}	36.54^{bc}	1.48^{b}	78.75
4% SH	541.95^{ab}	35.17^{ab}	0.0822^{ab}	38.93^{b}	1.44^{b}	77.50
1% SM[3]	365.35^{c}	21.58^{d}	0.0522^{d}	31.32^{c}	2.29^{a}	85.00
2% SM	431.25^{bc}	27.25^{bcd}	0.0660^{bcd}	32.11^{bc}	1.69^{ab}	80.00
4% SM	512.28^{bc}	30.16^{abcd}	0.0717^{abc}	36.09^{bc}	1.67^{ab}	86.25
P-value	< 0.0001	0.0003	0.0001	0.0001	0.0066	0.3956
PSE[6]	28.1200	1.8458	0.0037	1.2579	0.1220	4.0686

[1] PBM: 15% poultry by-product meal or reference diet.
[2] SH: squid hydrolysates.
[3] SM: Squid meal.
[4] TGC = Thermal growth coefficient.
[5] FCR = Feed conversion ratio.
[6] PSE = Pooled standard error.

Table 5
Proximate and mineral composition (dry weight basis) of whole body of Florida pompano fed experimental diets for 56 d. Values represent the mean of four replicates. Results in the same columns with different superscript letter are significantly different ($P < 0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

Diets[1]	Protein (%)	Fat (%)	Ash (%)	S (%)	P (%)	K (%)	Mg (%)	Ca (%)	Na (%)	Fe (ppm)	Mn (ppm)	Zn (ppm)
PBM	65.43	19.68	15.15	0.93	2.82^{b}	1.21	0.15	4.08^{b}	0.44^{b}	37.70^{c}	15.13^{b}	64.33^{ab}
Basal	60.15	19.65	18.50	0.88	3.76^{a}	1.20	0.19	6.08^{a}	0.65^{a}	58.45^{a}	27.95^{a}	76.00^{a}
1% SH	61.50	25.50	12.31	0.99	2.63^{b}	1.26	0.17	3.70^{b}	0.60^{ab}	56.38^{ab}	23.95^{ab}	61.60^{ab}
2% SH	63.65	22.15	12.98	0.96	2.55^{b}	1.22	0.17	3.59^{b}	0.55^{ab}	53.08^{abc}	22.75^{ab}	64.88^{ab}
4% SH	64.65	20.05	13.95	0.94	2.71^{b}	1.24	0.15	3.95^{b}	0.49^{ab}	46.53^{abc}	19.83^{ab}	60.43^{b}
1% SM	64.93	21.23	13.38	0.95	2.76^{b}	1.25	0.18	3.92^{b}	0.54^{ab}	52.15^{abc}	22.40^{ab}	60.18^{b}
2% SM	63.13	23.30	14.17	0.90	2.87^{b}	1.23	0.18	4.17^{b}	0.65^{a}	49.93^{abc}	25.08^{a}	61.80^{ab}
4% SM	69.63	20.60	12.15	1.04	2.61^{b}	1.43	0.16	3.47^{b}	0.52^{ab}	41.50^{bc}	20.33^{ab}	61.55^{ab}
P-value	0.2229	0.2124	0.1202	0.1283	< 0.0001	0.1041	0.0579	< 0.0001	0.0203	0.0021	0.0090	0.0443
PSE[2]	1.7719	1.2621	1.1316	0.0271	0.0863	0.0401	0.0073	0.1616	0.0329	2.5128	1.5424	2.4817

[1] Analyzed by Midwest Laboratories, Inc. (Omaha, NE, USA).
[2] PSE = Pooled standard error.

Table 6
Amino acids analysis (as is $g \times kg^{-1}$) of whole fish. Values represent the mean of four replicates. Results in the same row with different superscript letter are significantly different ($P < 0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

Composition[1]	Initial	PBM	Basal	4%SH	4%SM	*P*-value	PSE
Taurine	1.2	4.1	3.7	3.7	3.7	0.2939	0.0201
Hydroxyproline	3.2	3.5	2.7	3.9	4.0	0.2475	0.0410
Aspartic acid	13.0	15.2	15.5	14.4	14.9	0.2564	0.0382
Threonine	6.0	7.0	7.1	6.7	7.0	0.2910	0.0244
Serine	5.4	6.4	6.2	6.0	6.4	0.2303	0.0149
Glutamic acid	19.1	22.9	23.2	21.6	22.3	0.1456	0.0485
Proline	7.7	8.7	7.8	8.8	9.2	0.1122	0.0341
Glycine	13.4	14.4	12.3	14.2	14.8	0.2064	0.0737
Alanine	9.6	11.3	10.7	11.0	11.4	0.3078	0.0253
Cysteine	1.3	1.5^{ab}	1.6^{a}	1.3^{b}	1.4^{ab}	0.0459	0.0051
Valine	6.9	8.3	8.5	7.8	8.1	0.3014	0.0415
Methionine	4.0	4.7	4.7	4.4	4.6	0.3839	0.0119
Isoleucine	6.1	7.1	7.4	6.7	6.9	0.1711	0.0217
Leucine	10.0	12.0	12.4	11.3	11.6	0.2027	0.0351
Tyrosine	3.3	5.1	5.4	4.8	4.8	0.1596	0.0205
Phenylalanine	5.7	6.6	6.7	6.3	6.4	0.2493	0.0155
Hydroxylysine	0.5	0.6	0.5	0.6	0.7	0.1121	0.0049
Lysine	10.9	13.5	14.0	12.5	12.8	0.0915	0.0398
Histidine	2.9	3.6	3.8	3.4	3.6	0.2027	0.0112
Arginine	9.1	11.0	10.6	10.7	11.0	0.4003	0.0219
Tryptophan	1.1	1.5	1.6	1.4	1.5	0.3377	0.0070

[1] Analyzed by Midwest Laboratories, Inc. (Omaha, NE, USA).

3.5. Histology

Fig. 1 shows histological sections of fish liver, where the vacuolization and glycogen deposition was higher in fish fed with basal diet compared to other dietary treatments. Meanwhile, even there is no statistically significant difference among all dietary treatments, fish fed 4% SM shows a slight increase of enlarged cell with nuclear change. The supplementation of 4% SH and the inclusion of 15% PBM partly improve the liver condition of fish with lower score for vacuolization, nuclear change and glycogen accumulation. Based on the histological score analysis, liver alterations could not be attributed only to the dietary treatment but also to possible individual variations. Regarding to the distal intestine analysis, typical changes were apparent after 56 d of feeding trial (Fig. 2). The Lamina propria (LP) thickness of mucosal folds was decreased with an increase of cellular infiltration in the distal intestine of fish fed with basal diet and 4% SM compared to fish fed with 4% SH and PBM diet. Regarding the goblet cells (GC), differences were noted between basal and PBM. Albeit GC are still present in the distal intestine of fish, the number is lower in fish fed with PBM and 4% SH compared to fish fed with basal diet and 4% SM.

4. Discussion

The growth performance and feed utilization of the pompano fed with basal diet in this study was considerably lower than fish fed with 15% PBM, which is consistent with earlier studies (Rhodes et al., 2017; Rossi Jr and Davis, 2012). A number of findings suggest that palatability, digestibility and/or amino acid (AA) availability become problematic when animal meals are removed from diet formulation (Hajen et al., 1993; Rhodes et al., 2017). However, when 4% SH was added to the basal diet, it improved growth performance, TGC and FCR of fish similar to those maintained on the 15% PBM. The positive impact on the use of SH may be explained by the presence of bioactive peptides produced from enzymatic hydrolysis process, high content of essential amino acids and rich in taurine that is needed to improve the growth performance of fish (Kvåle et al., 2009; Lian et al., 2005). In addition, the hydrolysis process also degrades the protein molecule into smaller

Table 7
Effect of different diets on serum levels and enzyme activities in Florida pompano. Values represent the mean of four replicates. Results in the same columns with different superscript letter are significantly different ($P < 0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

Diets	Total protein ($g\ dL^{-1}$)	Albumin ($g\ dL^{-1}$)	ALP[1] ($U\ L^{-1}$)	ALT[2] ($U\ L^{-1}$)	AST[3] ($U\ L^{-1}$)	Glucose ($mg\ dL^{-1}$)	Cholesterol ($mg\ dL^{-1}$)	Bile acid ($mg\ dL^{-1}$)
PBM	2.72^{b}	0.93	28.47^{a}	32.00	221.50	120.25^{b}	143.75^{c}	2.90
Basal	3.75^{a}	1.21	37.13^{ab}	76.75	289.75	155.50^{ab}	175.25^{b}	4.35
4% SH	3.66^{a}	1.24	35.83^{ab}	34.00	321.75	157.25^{ab}	210.50^{a}	4.00
4% SM	3.99^{a}	1.03	43.20^{b}	56.75	335.00	177.75^{a}	193.25^{ab}	4.35
P-value	< 0.0001	0.4022	0.0541	0.6430	0.9067	0.0198	< 0.001	0.0531
PSE[4]	0.1152	0.1626	3.7933	32.229	137.29	12.540	7.0063	0.4294

[1] ALP = Alkaline phosphatase.
[2] ALT = Alanine transaminase.
[3] AST = Aspartate transaminase.
[4] PSE = Pooled standard error.

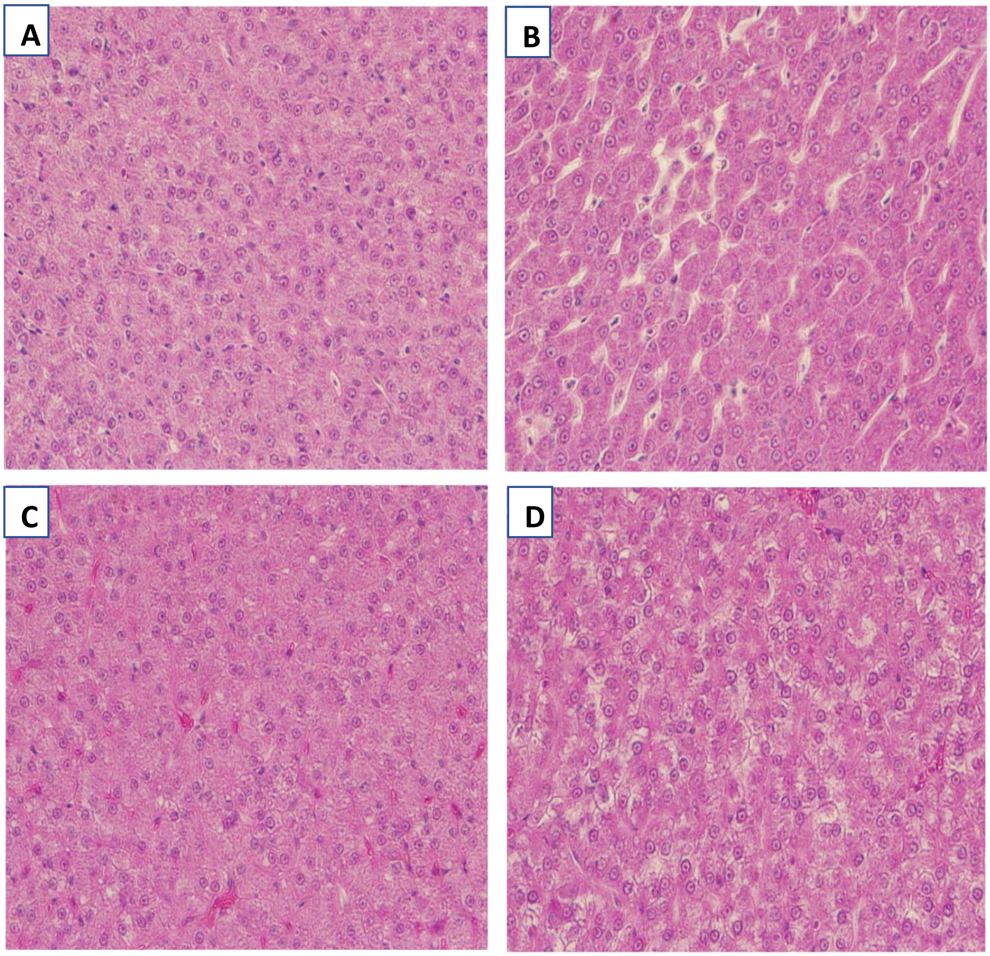

Fig. 1. Representative histopathological images of hematoxylin and eosin-stained sections of liver from Florida pompano after 56 d of being fed with (**A**) PBM, (**B**) basal, (**C**) 4% SH, and (**D**) 4% SM.

material and makes the products become more digestible and could be conveniently used as a potential chemoattractant (Carr, 1982) and growth promoter (Lian et al., 2005). Thus, these results may indicate that with proper inclusion level of SH into the soy based diet, SH could be used as a palatability enhancer and nutrient source, leading to better performance of Florida pompano.

On the contrary, it is unclear why the addition of SM to the basal diet resulted in limited improvements in the growth performance of fish. We expected that with comparable nutritional value to SH, SM would also improve the efficacy of basal diet for pompano. Similarly, Berge et al. (1999) found that there was no significant effect on feed intake and specific growth rate (SGR) of Atlantic halibut *Hippoglossus hippoglossus* when FM and soy protein concentrate (SPC) based diet was coated with SM. Moreover, Kissinger et al. (2016) also indicated that no significant differences were detected in growth performance, hepatosomatic index and survival rate of yellowtail *Seriola rivoliana* fed with SPC and *H. pluvialis* meal supplemented with various level of SM during partial FM replacement study. According to Cordova Murueta and Garcia Carreno (2001), the growth factors contained in SM are often severed by improper drying process to convert it into meal, lead to the reduction of nutritional value and inability to improve the growth performance of fish. In addition, several factors, such as the quality of SM, different diet formulation, rearing condition and species used for the trial may also influence the results (Zhou et al., 2016). However, it should be noted that final mean weight, feed intake and FCR of pompano in the present study was better as SM inclusion level increased. This suggests that with proper processing technique and inclusion level, SM may have potential to serve as an alternative source of protein and improve the efficacy of plant-based diet.

To further investigate the effect of dietary treatment to the whole-body composition of Florida pompano, twenty fish per dietary treatment (five fish per replicate) were randomly selected to be analyzed for proximate and mineral composition profile. No significant differences in terms of percentage protein, fat and ash content in the dry weight basis across all treatments were detected. The percentage composition of P and Ca were higher in the body of fish fed with basal diet compared to other dietary treatments. Similarly, no significant differences were detected in terms of crude protein, crude fat, moisture, and ash content in pompano when PBM was totally replaced by soy protein concentrate and plant-based diet supplemented with valine, glycine, and histidine (Rhodes et al., 2017). In addition, no significant differences were also observed in the final whole body when various levels of SBM were supplemented at moderate and high levels to replace FM in the diets for red sea bream, *Pagrus major* (Kader et al., 2012). This finding indicated that body composition of fish was not negatively affected by total replacement of animal meal with ESBM.

Screening of serum constituents together with biochemical analysis are important tools for indication of general health condition, physiological stress response and metabolic disturbance diagnosis (Blaxhall and Daisley, 1973; Maita et al., 2002). Results of the present study showed that the dietary treatments influence blood serum composition of pompano. Total protein (TP) values tended to be higher in fish fed with soy source diet with or without squid products compared to PBM. On the contrary, several studies concluded that serum TP levels was decrease with an increase of dietary SBM (Takagi et al., 2001; Ye et al., 2011). These contradictory results may have been caused by the use of ESBM, which contains higher levels of protein, low oligosaccharides and minimal levels of soy ANFs compared to SBM (Amezquita and

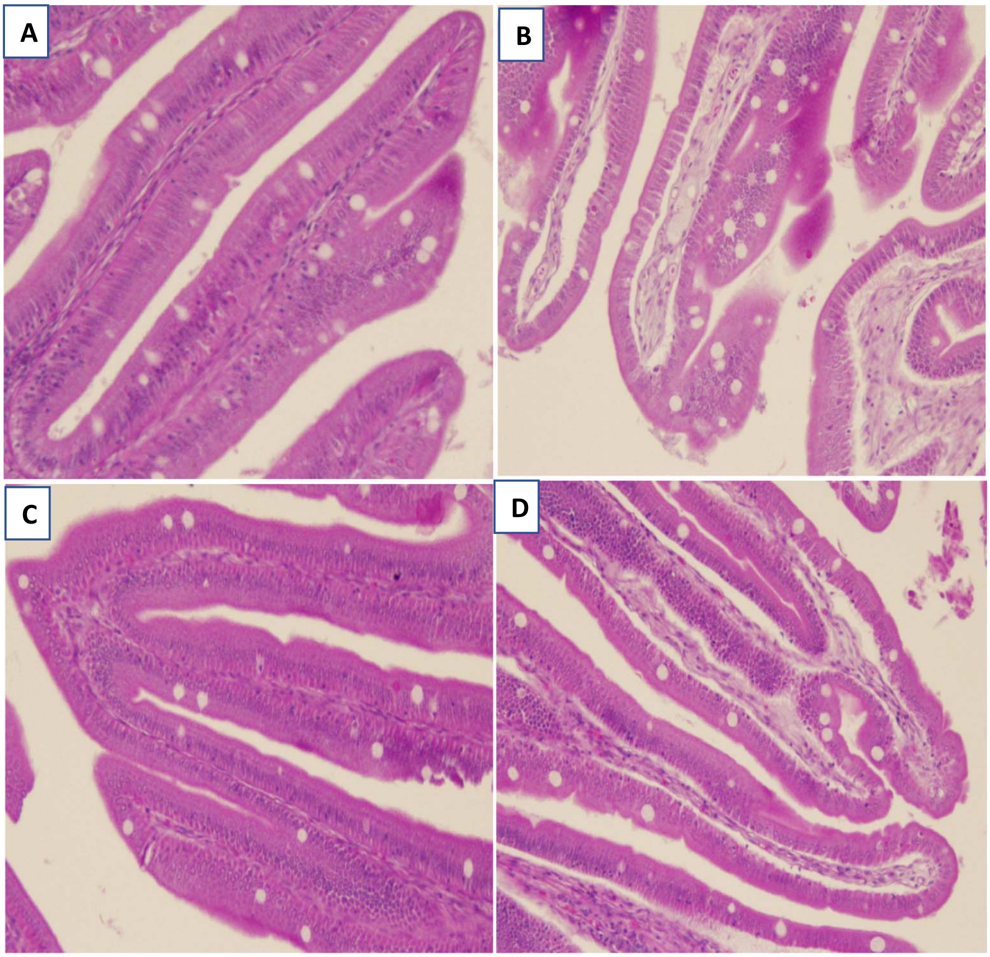

Fig. 2. Representative histopathological images of hematoxylin and eosin-stained sections of distal intestines from Florida pompano after 56 d of being fed with (**A**) PBM, (**B**) basal, (**C**) 4% SH, and (**D**) 4% SM.

Arana, 2015), and the use of squid products in the diet. As albumin constitutes most of the serum protein and it is possible to modulate serum protein both qualitatively and quantitatively, it was suggested that the albumin measurement should be performed together with TP levels analysis (Sandnes et al., 1988). Our present results showed that there is no significant difference in terms of albumin level, indicating that none of diets produced an adverse effect on the health status, nutrient intake and liver function of fish.

Dietary SBM is known as the source of sucrose, oligosaccharides, and complex non-starch polysaccharides (Krogdahl et al., 2005). According to Eldridge et al. (1979) glucose concentration in dehulled SBM is about 8.1%. Further processing of SBM still contains similar sugar composition, but the amount of each sugars was less. In our study, results of blood biochemistry analysis showed that fish fed with PBM had the lowest glucose level compared to other dietary treatments. Replacement of animal meal with ESBM slightly increased the glucose level and addition of small amounts of squid products did not reduce the glucose level. In Florida pompano, the effect of soy source protein on glucose homeostasis has been poorly studied. Lovell (1989) suggests that fish have poorer control over the blood glucose and once it rises, it takes many hours to decrease. Considering the absorption of glucose as the major product of carbohydrate digestion is very efficient (National Research Council, 2011; Singh and Nose, 1967) and blood samples were taken 12 h after the last feeding, high glucose level in fish fed with carbohydrate-containing feedstuffs should not come as a surprise.

Various studies have investigated whether the use of dietary SBM or other plant-based protein source reduce the body pools of cholesterol and bile acid levels (Kaushik et al., 1995; Krogdahl et al., 2003; Sitjà-Bobadilla et al., 2005), due to the presence of hypocholesteromic effect (De Schrijver, 1990). Likewise, the use of SPC as an advanced product of SBM, to completely replace FM in diet formulation for European seabass (*Dicentrarchus labrax*) had lower total plasma triacylglycerols (TAG) and cholesterol (CHOL) levels compared to fish fed with FM (Kaushik et al., 2004). In contrast, our observations here with pompano do not confirm such relationship, where fish fed with PBM had lower cholesterol level compared to fish fed with basal and soy source protein supplemented with squid products. Data from Ye et al. (2011) also provide evidence that the cholesterol and triglyceride level in Japanese flounder *Paralichthys olivaceus* increased with the elevation of dietary SBM level. It is clear from Table 7, since bile salts have a role to emulsify cholesterols and aid them to be excreted from liver (Yamamoto et al., 2007), the lower value of bile salts in this study might be related to the lower value of cholesterol in fish fed PBM. However, given that all dietary treatments here contained similar amount of SBM, it is difficult to distinguish whether this effect are due to the inclusion of ESBM and squid products into the basal diet or the cholesterol level contain in PBM. In any case, the significance effect of ESBM and squid products to lower the cholesterol and their effects on cholesterol synthesis and metabolism effect in Florida pompano should be studied in more detail.

In the present study, determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was also performed to monitor the health status of liver in fish (Maita et al., 1985). Additionally, total alkaline phosphatase (ALP) activity was measured as a good indicator for liver and bone diseases diagnosis (Garnero and Delmas, 1993; Price et al., 1976), especially due to the dietary treatment (Salze et al., 2016). Since ALT and AST are released into blood during organ damage (Tietz and Andresen, 1986), increased levels of these enzymes gives information on organ dysfunction, specifically to the liver cells (Yuangsoi et al., 2014). In our study, there is no significant effect of dietary treatments to the ALT and AST activity, indicating that the dietary treatments did not cause any hepatocellular damage. However, the ALP activity in fish fed with PBM was significantly lower compared to fish fed with basal and soy source protein supplemented with squid products, which indicated a possible failure to maintain the skeletal growth (Salze et al., 2016). Similarly, a study from Xu et al. (2012) reported that the increase of dietary soy protein isolate over 50% to replace dietary FM significantly increased the ALP activity of juvenile Amur sturgeon *Acipenser schrenckii*. According to Salze et al. (2016) low ALP activity related to the decrease in serum protein. Our data confirm this finding, as they clearly show the decrease of serum protein level in fish fed PBM. However, the value of ALP activities might not be associated with dietary treatment and did not cause serious effect to pompano fed with PBM, since the growth performance of this fish was relatively better compared to other dietary treatments.

The intestinal and hepatic structure of fish can be used to evaluate the effect of dietary changes (Boglino et al., 2012) and can be negatively influenced by the inclusion of plant proteins in the diet (Rodiles et al., 2015). In this study, no severe histopathological damage was observed in the liver of fish. The examination of the liver showed a normal pattern in fish fed with PBM and slight increase of vacuolization and glycogen deposition in fish fed with basal diet. The inclusion of 4% SH in the basal diet seems to partially restore the liver condition to that of fish fed PBM. Knowledge on the effects of high inclusion level of soy protein source in Florida pompano liver is limited. The histopathological change and a metabolic dysfunction in fish liver could be caused by lack of dietary taurine (Salze et al., 2016) or long-term intake of plant-based protein (Martínez-Llorens et al., 2012). It is also possible if that the hepatic vacuolization is evidence of metabolic dysfunction with high inclusion of SBM in the diet, thus affecting the nutrient utilization and growth performance of fish. However, further study is needed to confirm if this occurs in Florida pompano.

The inclusion of a high amount of SBM in formulated diets has been reported to induce inflammation in the distal intestine of some species of fish (Nordrum et al., 2000; Refstie et al., 2000; Urán et al., 2009). The symptoms are characterized by increased amount of connective tissue; a profound infiltration of inflammatory cells in the lamina propria (Krogdahl et al., 2000; Refstie et al., 2000), a reduced number of supranuclear vacuoles in the absorptive epithelium (Trushenski, 2015); and increase in the number of goblet cells (Ng et al., 2005; Oehlers et al., 2011). In the current study, higher GC number was found in fish fed with basal and 4% SM in comparison to fish fed with PBM and 4% SH. The increase of GC could be due to the mechanical irritation and unsatisfactory process of protein digestion (Baeza-Ariño et al., 2016; Franco et al., 2015). The addition of 4% SH into the basal diet significantly reduced the presence of GC and showed a similar score with fish fed PBM. This suggests that the high concentration of peptides produced from enzymatic hydrolysis contained in SH lead to the greater absorption rate (Hou et al., 2017; Hou et al., 2015). Furthermore, in all sections of the intestine, a decrease in LP thickness with an increase of cellular infiltration was observed in fish fed with basal diet and 4% SM. Study from Baeza-Ariño et al. (2016) showed that as the mixture of vegetable protein concentrate increase to replace FM from 30 to 90%, a decrease in the LP thickness was observed in Sea bream *Sparus aurata* fed with higher FM substitution. In addition, Kokou et al. (2015) noted that the leukocyte infiltration became more obvious as bioprocessed soy inclusion level increase to replace FM in the diet of Gilthead sea bream. In this study, with the inclusion of 4% SH into the basal diet, LP and cellular infiltration did not showed any differences compared to fish fed with PBM. From the point of view of digestibility, the level of peptides and free amino acid generated from hydrolysis process may afford more sufficient digestible protein which may be beneficial to the distal intestine condition of fish.

5. Conclusion

In the present study, the growth performance of Florida pompano fed PBM diets was highest compared to the basal diet. However, the addition of 4% SH into the basal diet improved the nutritional value of plant-based diet and partly prevented the decrease in growth performance, serum biochemistry parameters and the alteration of liver and intestine of pompano. By contrast, under the conditions tested, the results have identified that the inclusion of SM resulted in limited improvements in growth performance and physiological conditions of pompano than the reference diet. The lack of significant differences in terms of growth performance between fish fed PBM and basal diet supplemented with 4% SH indicated that the combination of ESBM and SH has the potential to serve as an alternative protein source and attractant. Nevertheless, further investigation to determine the proper inclusion level of squid product or other attractants in plant-based diets are needed for pompano.

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