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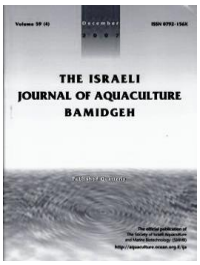

Effect of Partial Substitution of Fish Meal with Sunflower Meal on Feed Utilization, Intestinal Digestive Enzyme, Hematological Indexes, Intestinal, and Liver Morphology on Juvenile Turbot (*Scophthal musmaximus* L.)

Huihui Zhou[#], Chaoqun Li[#], Fuyun Bian, Mingsan Man, Kangsen Mai, Wei Xu, Gen He*

Key Laboratory of Aquaculture Nutrition and Feed, Ministry of Agriculture, and the Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, P. R. China

[#] These authors contributed equally to this study and share first authorship

Keywords: turbot; sunflower meal; replacement of fish meal; growth performance; hematological antioxidant defense system; intestinal and liver morphology

Abstract

A 70-day feeding trial was conducted to evaluate effects of partial substitution of fish meal (FM) by sunflower meal (SFM) on juvenile turbot (*Scophthal musmaximus* L.). Five isonitrogenous and isoenergetic diets were formulated with 0%, 15%, 25%, 35%, and 45% replacement of FM protein with protein from SFM. Triplicate groups of juvenile turbot (30 fish per group), were hand-fed twice daily to apparent satiation. Final body weight (FBW), specific growth rate (SGR), and weight gain rate (WGR), were not significantly influenced by type of plant protein at the 15% level ($P>0.05$), while higher levels showed significant reduction of FBW, SGR, WGR. Feed efficiency ratio (FER) and feed intake (FI) were significantly influenced when FM protein was replaced up to 45% ($P<0.05$). Body composition parameters were not affected by SFM substitution but body crude lipid was lowest and ash was highest at 45% ($P<0.05$). Trypsin and diastase values did not vary with experimental diets but lipase activity was significantly reduced ($P<0.05$). Catalase (CAT) values were significantly lower than the control ($P<0.05$) when substitution level reached or exceeded 35%; no significant differences were observed in total antioxidant capacity (T-AOC) and malondialdehyde (MDA) values ($P>0.05$). In the SFM diet groups, all superoxide dismutase (SOD) values were significantly higher than the control ($P<0.05$); villi length and enterocytes were significantly reduced ($P<0.05$), but there was no significant difference ($P>0.05$) in microvilli height between diets; parenchyma structure of liver was severely damaged; smaller hepatocyte areas and areas with high levels of hepatocyte vacuolization and disorganization were present. All results indicated that SFM protein can partially replace FM protein in juvenile turbot diets without adverse effects.

*Corresponding author: Gen He, Tel/Fax: +8653282031589, email: hegen@ouc.edu.cn

Introduction

Turbot, a marine, benthic, carnivorous fish, is widely cultured for its high economic value in Europe and East Asia, especially the northern area of China (Miao et al., 2016). However, dietary protein requirement for turbot is high (Danielssen and Hjertnes, 1993; Regost *et al*., 1999). Aquaculture of turbot is still heavily reliant upon high-quality fish meal.

Fish meal has been widely used in the aquafeed industry as a high-quality feed ingredient because of its well-balanced amino acid composition, good palatability, and low carbohydrate content. Along with decreasing natural fishery resources and increasing demands for fishery products, FM resources have been greatly depleted, resulting in rising prices (Shiu *et al*., 2015) and supply instability. Therefore, the search for new protein sources, which are cheaper and more abundant, has become imperative. Studies have been undertaken to examine the replacement of FM in diets for turbot by plant proteins such as soybean meal (Yigit *et al*., 2010, Zhang *et al*., 2016), corn gluten meal (Regost *et al*., 1999), rapeseed meal (Burel *et al*., 2000) and sunflower meal (SFM) (Nogales Merida *et al*., 2010).

A low-cost, locally available, and highly palatable plant protein source, SFM has produced good results as an alternative plant protein to fish meal. Based on previous studies, FM can be replaced by up to 30% SFM in sea bream juvenile diets (Nogales Merida *et al*., 2010), 33% in Atlantic salmon (Gill *et al*., 2006) and 22% in rainbow trout (*Onchorynchus mykiss*) (Martínez, 1984) without any adverse effects on growth performance of fish. Although SFM has high fibre and lignin content (and a low level of lysine), it is rich in methionine with low levels of anti-nutrition factors (Olvera-Novoa *et al*., 2002, Nogales Merida *et al*., 2010). Consequently SFM has been identified as a promising ingredient for fish feed, but little is known about the use of SFM in turbot diets. This study investigated the potential of SFM for use as an alternative plant protein source to FM in a carnivorous fish, the juvenile turbot (*Scophthal musmaximus* L.), by studying the effects of substituting FM with SFM on growth, feed utilisation, intestinal digestive enzymes, hematological antioxidant indices, and intestinal and liver morphology. This experiment also provides a reference for further application of plant protein sources in aquaculture and provides more comprehensive information about the effects on flatfish fed diets containing graded levels of SFM.

Materials and Methods

Feeding ingredients and diet formulations. The experimental diets were based on the nutritional requirement of juvenile turbot using FM and SFM as primary protein sources, fish oil and soy lecithin as main lipid sources, and wheat meal as carbohydrate source. SFM, the plant protein resource was supplied by BUNGE. Five isonitrogenous (approximately 50% crude protein) and isoenergetic (approximately 20.0 KJ/g diet of gross energy) diets were formulated to replace 0% (control), 15% (SFM15), 25% (SFM25), 35% (SFM35) and 45% (SFM45) of the FM proteins by SFM proteins. Crystal amino acids (lysine and methionine) were supplemented to meet the essential amino acid (EAA) requirements of juvenile turbot based on the amino acid composition of FM diet. Y_2O_3 (0.1%) was supplemented as the indicator for apparent digestibility determination according to Glencross (2007).

Diets and experimental ingredients were determined prior to diet formulation. All ingredients were ground into fine powder through 178 μm mesh and gradually mixed. The premix was blended with lipid source ingredients, then water which contained crystal amino acid and choline chloride was added. Pellets (3 mm × 4 mm) were made automatically using a pellet-making machine acquired from South China University of Technology and dried for 12h in a ventilated oven at 55°C. All diets were stored at -20°C until use. Composition of all diets is shown in Table 1.

Table 1. Formulas and proximate composition of the experimental diets (% dry matter)

	Treatments				
Ingredients	*FM*	*SFM15*	*SFM25*	*SFM35*	*SFM45*
Fish meal [a]	60.00	51.00	45.00	39.00	33.00
Sunflower meal [b]	0.00	12.90	21.50	30.10	38.70
Wheat meal [a]	27.00	20.18	15.82	11.29	6.92
Wheat gluten meal [a]	1.50	2.60	3.30	4.00	4.70
Fish oil	4.00	4.70	5.10	5.60	6.00
Soy lecithin	2.00	2.00	2.00	2.00	2.00
$Ca(H_2PO_4)_2$	0.00	0.80	1.30	1.80	2.30
Amino acid	0.00	0.32	0.48	0.72	0.88
Choline chloride	0.30	0.30	0.30	0.30	0.30
Taurine	1.00	1.00	1.00	1.00	1.00
Vitamin premix [c]	1.00	1.00	1.00	1.00	1.00
Mineral premix [d]	2.00	2.00	2.00	2.00	2.00
Calcium propionicacid	0.05	0.05	0.05	0.05	0.05
Ethoxyquinoline	0.05	0.05	0.05	0.05	0.05
Attractant [e]	0.50	0.50	0.50	0.50	0.50
Sodium alginate	0.50	0.50	0.50	0.50	0.50
Yttrium oxide	0.10	0.10	0.10	0.10	0.10
Total	100.00	100.00	100.00	100.00	100.00
Energy（KJ/g）	20.04	20.05	20.04	20.05	20.04
Crude protein	50.32	50.34	50.31	50.32	50.29
Crude lipid	11.10	11.32	11.41	11.59	11.67

Note: FM, diet fish meal; SFM15, replacement of 15% fish meal protein by sunflower meal protein; SFM25, replacement of 25% fish meal protein by sunflower meal protein; SFM35, replacement of 35% fish meal protein by sunflower meal protein; SFM45, replacement of 45% fish meal protein by sunflower meal protein.

[a] Supplied by Great seven Bio-Tech(Qingdao, China); fish meal, crude protein, 73.77%, crude lipid, 7.58%; wheat meal, crude protein, 17.82%, crude lipid, 2.24%; wheat gluten meal, crude protein, 83.31%, crude lipid, 1.75%.

[b] Supplied by BUNGE; sunflower meal, crude protein, 51.47%, crude lipid, 2.67%.

[c] Vitamin premix (mg kg^{-1} diet): retinal palmitate, 32; cholecalciferol, 5; DL- -tocopherol acetate, 240;menadione, 10; thiamin-HCl, 25; riboflavin, 45; pyridoxine-HCl, 20;cyanocobalamin, 10; D-calcium pantothenate, 60; amine nicotinic acid, 200; folic acid, 20;biotin, 60; mesoinositol, 800; ascorbyl polyphosphate (contained 35% ascorbic acid),2000; microcrystalline cellulose, 16473.

[d] Mineral premix (mg kg^{-1} diet): $MgSO_4 \cdot 7H_2O$, 1200; $CuSO_4 \cdot 5H_2O$, 10; $FeSO_4 \cdot H_2O$, 80; $ZnSO_4 \cdot H_2O$, 50; $MnSO_4 \cdot H_2O$, 45; $CoCl_2 \cdot 6H_2O$ (1%), 50; Na_2SeO_3 (1%), 20; calcium iodine, 60; zoelite, 8485.

[e] Attractant: betaine: dimethyl-propiothetin: glycine: alanine: 5-phosphate inosine = 4:2:2:1:1.

Experimental procedure. Experiments were conducted in Yi Haifeng Aquatic Product CO. Ltd (Qingdao, China). Juvenile turbot were obtained from a fish rearing farm (Yantai, China) and acclimated to the system for 2 weeks during which they were fed with commercial diets until onset of experiment. After being fasted for 24 h, healthy juvenile turbots (average initial weight 6.77± 0.02g) were selected and randomly assigned to 15 experimental fiber glass tanks (30 fish/tank). Seawater was continuously pumped and passed through sand filters into each tank at approximately 3 L/min. Each diet was randomly assigned to three replicate fiber glass tanks. The diets were hand-fed to the fish twice daily at 7:00 and 19:00 to apparent satiation for 70 days. Remaining feed and feces were cleaned immediately after feeding to ensure high water quality. During the experimental period, the water temperature ranged from 19°C-22°C, and salinity from 30‰-33‰, pH ranged from 7.5-8.0, and dissolved oxygen was approximately 7mg/L.

Sample collection. After approximately five-weeks of the feeding trial, fecal samples were collected from each tank by siphoning 3-4 hours after feeding. Collected fecal samples were stored at -20°C. At the end of the feeding trial, all the fish were starved for 24h. Total number and body weight of the fish in each tank were determined. Six fish from each tank were randomly sampled and stored at -20°C for whole body composition analysis. The entire intestine and liver were sampled and placed in Bouin's fixative solution and then transferred into 70% ethanol after 24 h for histological evaluation. After being fasted for 24 h, blood samples were taken from the caudal vasculature of six fish from each tank using disposable sterile syringes of 1.0 ml. The blood samples were then centrifuged at 4000g for 10min at 4°C to obtain serum samples which were stored

at -80°C prior to analysis. Intestines were collected and frozen in liquid nitrogen at -80°C for digestive enzyme analysis.

Chemical analyses:

Body composition. Moisture, crude protein, crude lipid and ash were analyzed for fish samples as well as for diets and experimental ingredients according to standard method (AOAC, 1995). Moisture was measured by drying the samples to constant weight at 105°C. Crude protein (N×6.25) was determined by using the Kjeldahl method (Kjeltec TM 8400, FOSS, Sweden) after acid digestion. Crude lipid was extracted with diethyl ether using Soxhlet method (Buchi 36680, Switzerland). Ash was measured after combustion in a muffle furnace at 550°C for 16 h.

Digestibility determination. Y_2O_3 (0.1%) was supplemented as the indicator for apparent digestibility determination of dry matter and crude protein (Glencross et al., 2007). Y_2O_3 (0.1%) content in feces and diets were determined by Inductively Coupled Plasma-atomic Emission SMectrophotometer (ICP-OES, VISTA-MPX) after acid digestion with perchloric acid.

Intestinal digestive enzyme and hematological antioxidant indexes determination. Protease activity was detected using Folin-Phenol method. Lipase and amylase activity was detected using kits from Nanjing Jiancheng Engineering Institute Co., Ltd., China. Catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) were detected using visible SMectrum, WST-1, and thiobarbituric acid (TBA) methods (kits were obtained from Nanjing Jiancheng Engineering Institute Co., Ltd., China) respectively. Total antioxidant capacity (T-AOC) was also detected using kits from Nanjing Jiancheng Engineering Institute Co., Ltd., China.

*Intestinal and liver histology. S*amples of distal intestine and liver tissue were fixed and transferred in 70% ethyl alcohol. Approximately 1cm length segments of fixed tissues were cut, routinely dehydrated in an alcohol series, and embedded in paraffin. Then approximately 7μm sections of the tissues were cut, mounted on albumin coated slides, and then stained with matoxylin and eosin (H&E). Morphological structure of these tissues was observed using an imaging microscope (Olympus, DP72, Nikon, Japan).

Intestinal Images were analyzed to determine the ratio (R) between the villi height (VH) or enterocytes height (EH) or microvilli height (MH) and the lumen diameter (LD) of the gut [R1 = VH / LD, R2 = EH / LD, R3 = MH / LD, arbitrary units (AU)]. A high R value indicates high villi height or enterocyte height or microvilli height. Liver images were analyzed for hepatocyte area (HA). All data was able to reflect the possible alterations in intestine and liver tissue structure.

Calculation methods. The following variables were calculated:

Survival rate (SR, %) = (final fish number/initial fish number) × 100%

Weight gain rate (WGR, %) = (final body weight – initial body weight) /initial body weight × 100%

Specific growth rate (SGR, %)=(ln final body weight–ln initial body weight)/days × 100%

Feed intake (FI, %)=dry total feed intake/[(final total body weight + initial total body weight)/2]/days × 100%

Feed efficiency ratio (FER) = wet total weight gain (g) / dry total feed intake (g)

Apparent digestibility coefficients (ADC, %) = ($1-Y_2O_3$ in the diet / Y_2O_3 in feces × nutrient in feces / nutrient in diets) × 100%

Statistical analysis. All statistical evaluations were analyzed using SPSS 19.0 software. Data were obtained using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were regarded as significant when $P<0.05$. Data were expressed as means ± standard error.

Results

*Growth performance and feed utilization***.** No significant differences ($P>0.05$) were found in SR among diets (Table 2). There were no obvious differences ($P>0.05$) in FBW, SGR, and WGR of fish fed 15% SFM (SFM15) replacement of fish meal protein compared with fish fed the FM diet; higher levels of SFM replacement of FM protein significantly reduced ($P<0.05$) FBW, SGR, and WGR. No significant differences ($P>0.05$) were found in FER but there was a significant reduction at the 45% level ($P<0.05$); no significant differences

(P>0.05) were found in FI but a significant increase (P<0.05) was found at 45% (see Table 2).

Table 2. Growth parameters and feed utilization of juvenile turbot fed the experimental diets

Treatments	*Final body weight (g) /FBW*	*Weight gain rate (%) /WGR*	*Specific growth rate (%/d) /SGR*	*Survival rate (%) /SR*	*Feed efficiency ratio (g/g) /FER*	*Feed intake (%/d) /FI*
FM	59.38±1.76^{a}	776.16±25.96^{a}	3.1±0.04^{a}	100±0.00	1.36±0.02^{a}	1.81±0.03^{a}
SFM15	51.19±2.11^{ab}	656.31±32.05^{ab}	2.89±0.06^{ab}	100±0.00	1.32±0.02^{a}	1.79±0.01^{ab}
SFM25	46.87±2.24^{b}	593.92±34.68^{b}	2.76±0.07^{b}	98.89±1.11	1.24±0.04^{a}	1.86±0.03^{a}
SFM35	45.64±3.10^{bc}	572.5±45.94^{bc}	2.71±0.10^{b}	98.89±1.11	1.19±0.05^{ab}	1.79±0.05^{a}
SFM45	36.5±1.19^{c}	438.63±17.32^{c}	2.4±0.04^{c}	97.78±2.22	1.05±0.06^{b}	1.98±0.03^{b}

Note: Values show mean ± standard error, n = 3; values in the same column with different superscript letters indicate significant difference (P < 0.05).

Body composition. No significant differences were found in moisture and crude protein contents of fish body among treatments (P>0.05). Clear differences in whole body crude lipid and ash contents were observed in juvenile turbots fed 45% SFM (SFM45) replacement of fish meal protein. They also had the lowest body crude lipid and highest ash content (P<0.05) (see Table 3).

Table 3. Whole body composition of juvenile turbot fed the experimental diets

Treatments	*Moisture*	*Crude protein*	*Crude lipid*	*Ash*
FM	76.56±0.20	16.38±0.13	3.82±0.09 ^{a}	3.56±0.05 ^{a}
SFM15	77.54±0.52	15.68±0.45	3.36±0.24 ^{ab}	3.57±0.06 ^{a}
SFM25	77.27±0.10	16.16±0.19	3.3±0.18 ^{ab}	3.73±0.11 ^{ab}
SFM35	77.5±0.20	16.11±0.20	3.21±0.15 ^{ab}	3.75±0.08 ^{ab}
SFM45	77.5±0.17	16.18±0.18	2.87±0.27 ^{b}	4.03±0.16 ^{b}

Note: Values show mean ± standard error, n = 3; values in the same column with different superscript letters indicate significant difference (P < 0.05).

Apparent digestibility coefficients. When FM protein was replaced with up to 35% of SFM protein, apparent digestibility coefficients (ADC) of dry matter decreased significantly with increasing replacement levels of fish meal protein in diets, while ADC of crude protein of SFM45 was significantly lower (P < 0.05) than in an FM diet (Table 4).

Table 4. Apparent digestibility coefficients (%, ADC) for dry matter and crude protein of the experimental diets

Treatments	*Dry matter*	*Crude protein*
FM	54.22±1.90 ^{a}	85.55±1.07 ^{ab}
SFM15	54.78±0.63 ^{a}	89.28±0.19 ^{a}
SFM25	52.54±0.38 ^{a}	88.23±0.66 ^{a}
SFM35	38.68±2.80 ^{b}	86.2±1.21 ^{ab}
SFM45	36.09±0.89 ^{b}	83.64±0.81 ^{b}

Note: Values show mean ± standard error, n = 3; values in the same column with different superscript letters indicate significant difference (P < 0.05).

Intestinal digestive enzyme activity and hematological antioxidant indexes. Trypsin and diastase values showed no variation among experimental diets (P>0.05). There was a significant (P<0.05) reduction in lipase activity in the fish meal replacement groups compared with the control (see Table 5).

Table 5. Activity of intestinal digestive enzyme of juvenile turbot fed the experimental diets

Treatments	*trypsin (10U/mg)*	*diastase AMS(U/mg)*	*lipase LPS(U/mg)*
FM	11.81±0.89	0.58±0.11	70.10±2.45^{a}
SFM15	11.82±0.23	0.93±0.02	54.85±2.05^{bc}
SFM25	12.12±1.06	0.92±0.09	50.95±1.09^{c}
SFM35	12.03±0.12	0.65±0.14	51.21±0.97^{c}
SFM45	12.22±0.23	0.69±0.15	59.77±0.78^{b}

Note: Values show mean ± standard error, n = 3; values in the same column with different superscript letters indicate significant difference (P < 0.05).

T-AOC and CAT values were affected by treatment used: CAT values were significantly lower than the control when substitution level reached, or exceeded, 35%, while no significant differences were obtained in total antioxidant capacity (T-AOC) and malondialdehyde (MDA) values compared to the control (P > 0.05). All values of superoxide dismutase (SOD) in SFM treatments were significantly higher (P < 0.05) than the control. (see Table 6).

Table 6. Antioxidant indexes of juvenile turbot fed the experimental diets

Treatments	*T-AOC(U/ml)*	*CAT(U/ml)*	*SOD(U/ml)*	*MDA(U/ml)*
FM	8.36±0.41^{ab}	2.00±0.02^{ab}	15.17±0.66^{a}	8.57±0.22
SFM15	9.74±0.26^{a}	2.33±0.67^{a}	34.16±0.54^{d}	7.29±0.49
SFM25	8.14±0.50^{ab}	1.67±0.12^{bc}	26.15±0.26^{c}	8.90±0.33
SFM35	8.12±0.20^{ab}	1.48±0.14^{c}	24.21±0.26^{b}	8.90±0.41
SFM45	7.52±0.58^{b}	1.27±0.09^{c}	22.74±0.34^{b}	7.48±0.34

Note: Values show mean ± standard error, n = 3; values in the same column with different superscript letters indicate significant difference (P < 0.05).

Intestinal and liver histology. Intestinal and liver samples of fish from different treatments were compared to those of the control. SFM replacement diets significantly (P<0.05) reduced the length of villi and enterocytes, but microvilli height showed no significant differences (P>0.05) among diets (Table 7, Fig. 1). The parenchyma structure of liver from fish fed diets containing graded levels of SFM was significantly damaged. Smaller hepatocyte areas, and areas with high levels of hepatocyte vacuolisation and disorganisation, were present in some samples from fish fed SFM diets (Fig. 2).

Table 7. Distal intestinal and liver histology indexes of juvenile turbot fed the experimental diets

Treatments	R_1[a] (10^{-1})	R_2[b] (10^{-2})	R_3[c] (10^{-3})	*Hepatocyte area* (μm^2)
FM	3.48±0.04^{a}	1.97±0.08 ^{a}	1.79±0.06	377.7±22.14^{a}
SFM15	2.88±0.07^{b}	1.62±0.03 ^{b}	1.8±0.08	315.89±9.89^{b}
SFM25	2.77±0.06^{b}	1.62±0.05 ^{b}	1.8±0.09	311.07±9.66^{b}
SFM35	2.47±0.06^{bc}	1.52±0.06 ^{b}	1.57±0.05	326.28±10.41^{b}
SFM45	2.64±0.07^{c}	1.39±0.03 ^{b}	1.54±0.08	313.95±4.66^{b}

Note: Values show mean ± standard error, n = 3; values in the same column with different superscript letters indicate significant difference (P < 0.05).

[a] R_1= villi height/ lumen diameter

[b] R_2= enterocytes height/ lumen diameter

[c] R_3= microvilli height/ lumen diameter

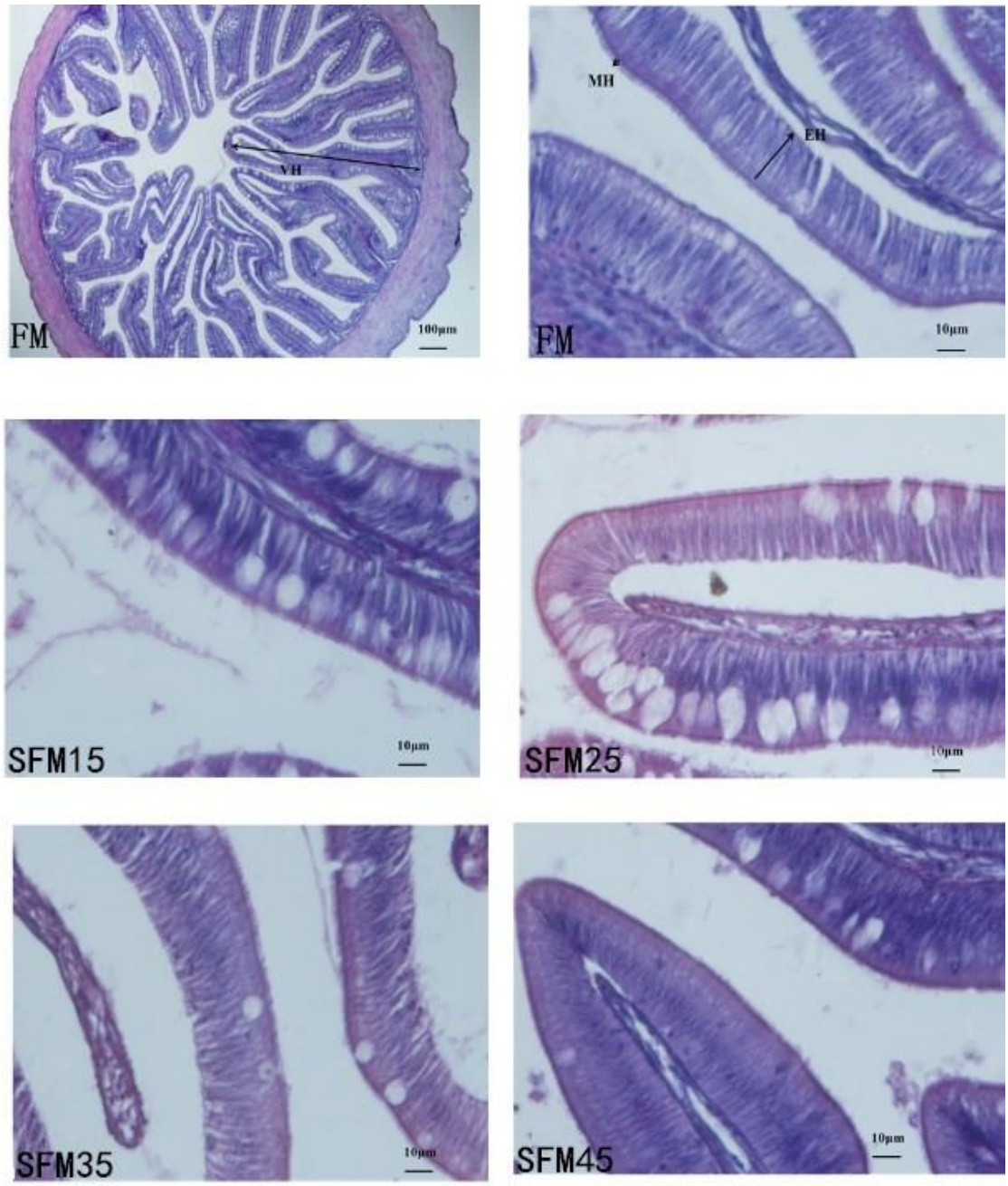

Fig.1 Transverse section photomicrographs of turbot's distal-intestine.
Villi height (VH) was analyzed at a magnification of ×40; (B) enterocytes height (EH) and microvilli height (MH) were analyzed at higher magnification of ×400.

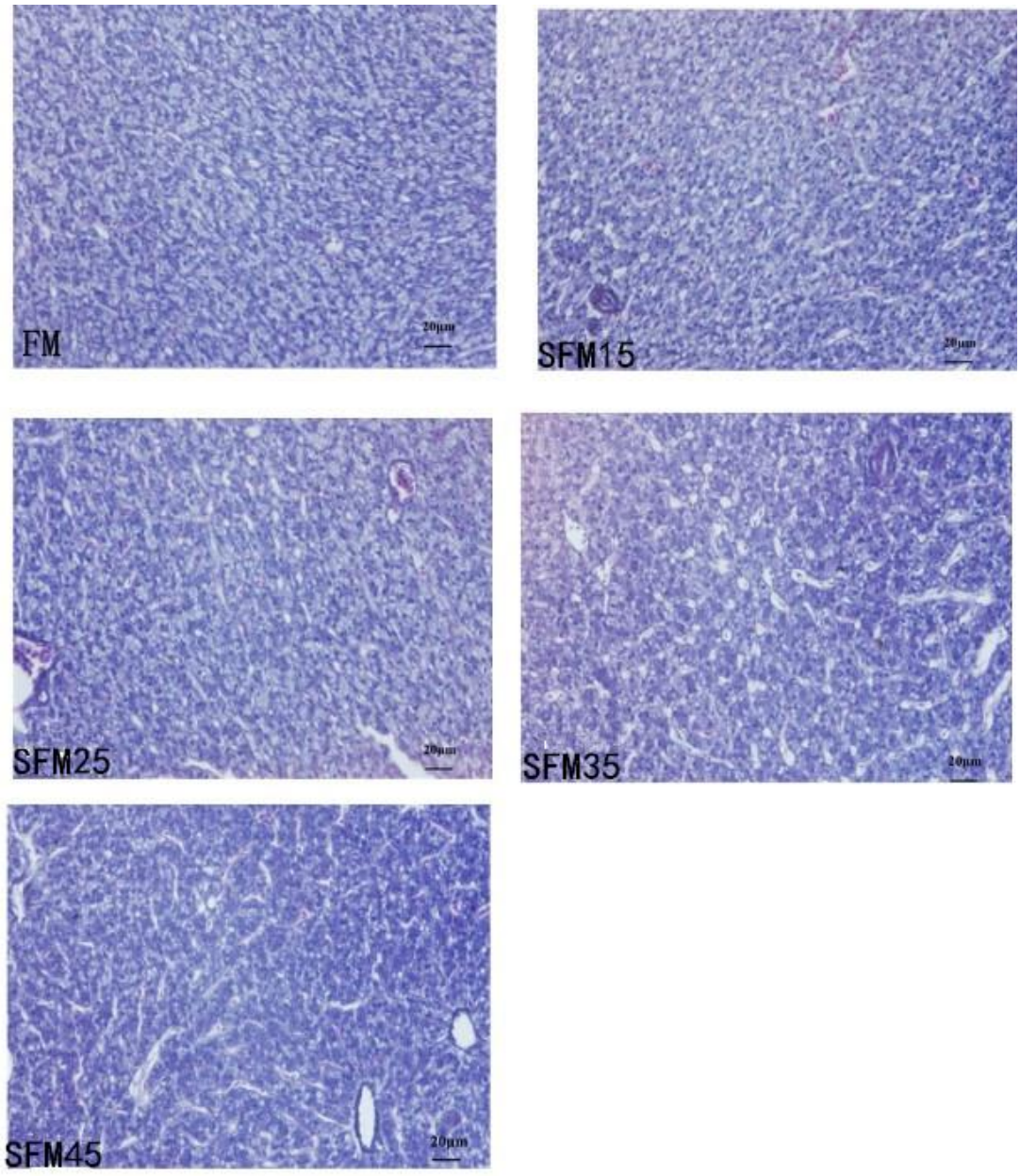

Fig. 2 Transverse section photomicrographs of turbot's liver (magnification ×200)

Discussion

The present study indicated that FM could be replaced by SFM at the level of 15% without any adverse effects on growth and feed utilisation of juvenile turbot. However, FBW, SGR, WGR and FER were significantly reduced with increasing levels of FM substitution. The acceptable replacement rate by SFM in this study was lower than that by soybean meal in the diet of turbot (Zhang et al., 2016). SFM substitution at 14% had no adverse effects on the growth performance on tilapia (Furuya *et al*. (2000). SFM substitution level of FM up to 33% had no adverse effects on growth performance in Atlantic salmon (Gill *et al*. 2006).

Many authors attributed lower growth with increasing levels of SFM substitution to essential amino acid (EAA) deficiency; improved growth was observed in rainbow trout fed diets containing SFM supplemented with Met, Lys, and Leu than diets without EAA supplementation (Sanz *et al*.,1994). EAA could be the first limiting factor for replacement of FM by high levels of SFM (Olvera-Novoa *et al*. 2002). In the present study, despite the supplemented lysine and methionine based on the EAA composition of an FM diet, other EAA such as arginine, associated with fish growth (Fournier *et al*., 2002), could cause

reduction in growth. Some authors regard the suppression of feed intake (FI) as a reason for unsatisfactory growth performance with plant protein replacement in diets for fish (Chen *et al*., 2011; Peng *et al*., 2013). Together with rising substitution levels of FM, FI fluctuation was observed in this study. It is possible that higher fibre content in SFM causes reduced growth of fish. Similar results were seen in sea bream when the fibre content in diets was increased (Sanchez-Lozano *et al*. 2007).

High fibre and lignin contents in SFM could affect the digestibility of protein or dry matter in carnivorous fish (Olvera-Novoa *et al*., 2002, Stickney *et al*., 1996). In the present study, no adverse effects on apparent digestibility coefficients (ADC) of dry matter were obtained up to 35% of FM protein replacement. Significant ADC reduction of crude protein was observed only at 45% level. Results confirmed that relatively low FM substitution levels by SFM did not affect the ADC of protein or dry matter in turbot.

In general, plant protein such as soybean meal containing anti-nutrition factors (ANFs) could affect digestive enzyme activity (Yu *et al*., 2013); however, in this study, values of trypsin and diastase content showed no variation among treatments but there was a significant reduction in lipase activity with increasing substitution levels of FM. The adverse influence on lipase activity could be due to patho-morphological changes in the distal intestine and the presence of ANFs in SFM. Reduction in lipase activity indicated that high substitution levels of FM impaired digestion and absorption of lipids.

Animals have a powerful antioxidant defence system, which can reduce the physiological damage to the body by removing oxygen free radicals. SOD, CAT, T-AOC, and MDA play extremely important roles in this antioxidant defence system. MDA is a final product of lipid peroxidation which can reflect the degree of endogenous oxidative damage (Ding *et al*., 2015). Hematological antioxidant parameters showed the lowest MDA level and highest T-AOC, SOD, and CAT activities in SFM15. This indicated that antioxidant defence mechanisms in the body had been developed to reduce oxidative stress and protect biological systems from free radical toxicity (Nordberg and Arner, 2001) when the substitution level reached 15%. An increasing trend in MDA and decreasing trend in T-AOC, SOD, and CAT were observed when the substitution level reached, or exceeded 25%. This indicated that higher SFM levels (above 15%) in diets could cause severe oxidative stress and free radical toxicity which could not be alleviated by the antioxidant defence system of the fish body, consistent with results seen in other fish fed diets containing plant proteins (Lin and Li, 2011; Peng *et al*. 2013). The anti-nutrition factors (ANFs) in SFM were considered to have been the principal cause of observed damage.

The parenchyma structure of the liver from fish fed SFM diet was significantly damaged. This differed from findings of Nogales Merida *et al*. (2010). Areas with high levels of hepatocyte vacuolisation and disorganisation were present and the volume of hepatocytes showed significant shrinkage in some samples from fish fed SFM diets. As the main site for toxin accumulation in the animal body, both the structure and function of the liver would be damaged by higher levels of ANFs from SFM. Further research is needed to establish the effects of prolonged feeding with SFM.

This study indicated that SFM substitution treatments significantly reduced the height of both villi and enterocytes. These could be signs of damage to the structure of the distal intestine at high substitution levels of FM. However the microvilli height in the present study showed no significant differences among treatments, which suggests that intestinal function was not significantly impaired by higher substitution levels of FM. This mirrored the result of Nogales Merida *et al*. (2010).

In conclusion, this study investigated the potential for SFM to be used as an alternative plant protein to FM in juvenile turbot (*Scophthal musmaximus* L.) diets. It was found that FM could be replaced by SFM at the 15% level with no significant adverse effects on growth and feed utilisation in turbot. Low growth associated with increased SFM substitution levels was considered to be due to the adverse effects on ADC of dry matter, intestinal digestive enzyme activity, the hematological antioxidant defence system, and the histology of intestine and liver.

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