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Beneficial influences of dietary *Aspergillus awamori* fermented soybean meal on oxidative homoeostasis and inflammatory response in turbot (*Scophthalmus maximus* L.)

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ABSTRACT

High levels of soybean meal (SBM) in aquafeed leads to detrimental inflammatory response and oxidative stress in fish. In the present study, fermentation with *Aspergillus awamori* was conducted to explore the potential effects on improving the nutritional quality of soybean meal and the health status of turbot. A 63-day feeding trial (initial weight 8.53 ± 0.11 g) was carried out to evaluate the utilization of fermented soybean meal (FSM) by juvenile turbot. 0% (FM, control), 30% (S30, F30), 45% (S45, F45), and 60% (S60, F60) of fish meal were replaced with SBM or FSM, respectively. As the results showed, fermentation significantly reduced the contents of anti-nutritional factors in SBM, including raffinose (−98.8%), glycinin (−98.5%), β-conglycinin (−97.4%), trypsin inhibitors (−80%) and stachyose (−80%). A depression of fish growth performance and activities of superoxide dismutase and lysozyme were observed in S45 and S60 groups, while these inferiorities were only observed in F60 group. Meanwhile, fermentation also improved the heights of enterocytes and microvillus significantly in the F45 and F60 groups compared with those in SBM. An induced expression of anti-inflammatory cytokine *transforming growth factor-β* and depression of pro-inflammatory cytokines *tumor necrosis factor-α* and *interleukin-1β* in the distal intestine were observed in the F45 and F60 groups. Taken together, this study indicated that fermentation with *Aspergillus awamori* could improve the replacement level with soybean meal from 30% to 45% in turbot.

1. Introduction

Soybean meal (SBM) has been showed as a promising alternative protein source to replace fish meal (FM) in aquafeed due to its relatively high protein content (about 43–48%), stable supply and low cost [1]. However, the replacement should be limited in rather low levels in carnivorous fishes, such as orange-spotted grouper (18.36%) [2], European seabass (25%) [3] and turbot (30%) [4]. High levels of SBM in diets led to adverse effects on growth, nutrition utilization and health status [5–7]. Meanwhile, suppressed immune function, detrimental inflammatory response and oxidative stress were also widely reported in aquaculture animals fed high-level SBM [4,8].

The anti-nutritional factors (ANFs) in SBM have much influence on the oxidative homoeostasis and immune responses in fish. Glycinin and β-conglycinin in SBM impaired the antioxidant system, such as superoxide dismutase (SOD), catalase (CAT) and glutathione-related enzymes, and induced oxidative damage in Jian carp [9,10]. β-conglycinin also induced intestinal inflammation in Jian carp through inducing the expression of interleukin 8 (*il-8*), tumor necrosis factor α (*tnf-α*) and transforming growth factor β (*tgf-β*) [10]. In turbot, the soya-saponins induced enteritis was related with the depression of antioxidant enzymes activities and increased secretion of pro-inflammatory cytokines [11]. Meanwhile, soybean agglutinin induced similar pathological disruptions of the intestinal tract in Atlantic salmon with high levels of SBM [12]. Recently, the cellular and molecular impacts of soybean agglutinin in zebrafish were reported. It induced cell apoptosis, inhibited the activities of cell survival pathways and shifted the cellular metabolism [13].

Removing or inactivating the ANFs is critical to improve the nutritional quality of SBM and fish growth performance. Compared with

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the thermal, mechanical or zymologic treatments, fermentation with microorganism is a more promising and economical method to decrease the contents of ANFs [2]. Fermentation process can degrade glycinin and β-conglycinin in SBM thereby reducing the immunoreactivity and allergic reactions [14]. Furthermore, the levels of bioactive factors, such as vitamins A and B, calcium and bioactive peptides can be improved by fermentation [15,16].

Aspergillus has been widely used in fermentation due to its high capacity to degrade the large antigenic proteins [15]. *Aspergillus awamori* showed a wealthy capacity to produce enzymes like cellulase, xylanase, pectinase and protease [17,18]. *A. awamori* has been widely used in the diet to enhance the growth performance of broiler chicken [19] and weaned pigs [20]. However, few studies about its application in aquaculture were reported.

In the present study, the application of *Aspergillus awamori* in aquaculture was explored. The nutritional profile of fermentation soybean meal (FSM) was measured. Meanwhile, a feeding trial was conducted using FSM, as well as SBM, as a partial replacement for FM in diets of turbot, which required a high protein level and was sensitive to protein sources [21]. The effects of dietary FSM on growth performance, antioxidant capacity and inflammatory response in turbot were analyzed.

2. Materials and methods

2.1. Fermentation of SBM

The strains of *Aspergillus awamori* were obtained from Culture Collection Center of Zhejiang Academy of Agricultural Science. SBM was obtained from Great-Seven Biotech. Co., Ltd. (Shandong, China). The fermentation parameters were optimized by quantification of crude protein and trichloroacetic acid soluble nitrogen (TCA-N) production. SBM was soaked with 110% water (containing 2 g K_2HPO_4, 2 g NaCl, 2 g $(NH_4)_2SO_4$, 1 g glucose, 2 g urea in per liter water) for 60 min and autoclaved in a steam tank at 100 °C for 30 min. After cooling down, the SBM were inoculated with *Aspergillus awamori* (~2.5×10^6 counts per g dry SBM), mixed and fermented in a thermostatic chamber at 31 °C for 37 h. After the fermentation, the FSM was dried in an oven at 55 °C until the moisture content was below 10%. The proximate composition, amino acids profile as well as the anti-nutritional factors in FSM were detected.

2.2. Experimental diets

Seven isonitrogenous and isoenergetic experimental diets were formulated in which 0% (FM, control), 30% (S30, F30), 45% (S45, F45), 60% (S60, F60) of FM were replaced with SBM or FSM. FM, wheat gluten meal and SBM or FSM were used as the major protein sources. Fish oil was used as the major lipid source. Lysine and methionine were supplemented to meet the requirements of turbot. Y_2O_3 was supplemented for apparent digestibility coefficients determination. All ingredients were ground to pass an 80-mesh screen, mixed gradually and blended with oil and water. Pellets (3 mm × 4 mm) were made automatically by pellet-making machine and dried for 12 h in a ventilated oven at 50 °C. All diets were stored at −20 °C until used. The compositions of the experiment diets were shown in Table 1.

2.3. Feeding trial

All procedures performed in present study were in strict accordance with the recommendations in the Guide for the Use of Experimental Animals of Ocean University of China. Juvenile turbots were obtained from a local farm (Qingdao, China). Feeding trial was carried out in a flowing water system of YiHaifeng Aquatic Product CO. Ltd (Qingdao, China). Fish were acclimated to the system and fed with commercial diet for 2 weeks. After 24 h fasted, turbot (8.53 ± 0.11 g) were randomly distributed to 21 tanks (300 L) with 30 fish per tank. Fish were hand-fed to apparent satiation twice a day at 6:00 and 18:00 for 63 days. During the experimental period, the water temperature was ranged from 19 °C to 22 °C and salinity was from 27‰ to 29‰. Both the concentrations of ammonia-nitrogen and nitrite were less than 0.1 mg/L. The dissolved oxygen was approximately 7 mg/L.

Table 1
Formulation and chemical proximate composition of the experimental diets (%).

Ingredients	Amount (% dry diet) in each treatment						
	FM	S30	S45	S60	F30	F45	F60
White fish meal[a]	60.00	42.00	33.00	24.00	42.00	33.00	24.00
Soybean meal[a]	0.00	24.80	37.21	49.61	0.00	0.00	0.00
Fermented soybean meal[b]	0.00	0.00	0.00	0.00	21.96	32.94	43.92
Wheat flour[a]	25.12	13.74	8.19	3.15	17.45	13.72	10.33
Wheat gluten meal[a]	1.88	3.85	4.66	5.36	3.04	3.47	4.17
Fish oil	4.00	5.30	6.40	7.10	5.30	6.40	7.10
Vitamin premix[c]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[d]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Attractants[e]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Amino acid	0.00	0.30	0.55	0.78	0.25	0.47	0.48
Cholesterol	0.00	1.00	1.00	1.00	1.00	1.00	1.00
Others[f]	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Proximate analysis (dry matter, %)							
Gross energy (kJ g^{-1})	19.92	20.30	20.46	20.53	20.27	20.41	20.51
Crude protein	50.31	50.26	50.21	50.15	50.18	50.11	50.11
Crude lipid	11.80	12.81	13.25	13.32	12.80	13.25	13.32
Crude ash	12.30	11.55	10.93	10.20	9.41	11.45	10.80

Note: FM, diet fish meal; S30, replacement of 30% fish meal protein by soybean meal protein; S45, replacement of 45% fish meal protein by soybean meal protein; S60, replacement of 60% fish meal protein by soybean meal protein; F30, replacement of 30% fish meal protein by fermented soybean meal protein; F45, replacement of 45% fish meal protein by fermented soybean meal protein; F60, replacement of 60% fish meal protein by fermented soybean meal protein.

[a] Supplied by Great-Seven Biotech. Co., Ltd. (Shandong, China); white fish meal, crude protein, 73.91%, crude lipid, 8.81%; soybean meal, crude protein, 53.64%, crude lipid 2.11%; wheat gluten meal, crude protein, 83.31%, crude lipid 1.75%; wheat flour, crude protein, 17.50%, crude lipid 2.22%.

[b] Fermented soybean meal got from soybean meal fermented with *Aspergillus awamori*, crude protein, 60.58%, crude lipid, 2.07%.

[c] Vitamin premix (mg kg^{-1} diet): thiamin, 25; riboflavin, 45; pyridoxine HCl, 20; vitamin B12, 10; vitamin K, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, 60; retinol acetate, 32; cholecalciferol, 5; alpha-tocopherol, 240; ascorbic acid, 2000; microcrystalline cellulose, 1473.

[d] Mineral premix (mg kg^{-1} diet): $CoCl_2$ (1%), 50; $CuSO_4{\cdot}5H_2O$, 10; $FeSO_4{\cdot}H_2O$, 80; $ZnSO_4{\cdot}H_2O$, 50; $MnSO_4{\cdot}H_2O$, 45; $MgSO_4{\cdot}7H_2O$, 1200; H_2NaOSe (1%), 20; H_2CaIO_4 (1%), 60; Zeolite powder, 8485.

[e] Attractants: betaine: DMPT: glycine: alanine: inosine-5′-diphosphate trisodium salt = 4: 2: 2: 1: 1.

[f] Others (10 g kg^{-1} diet): soy lecithin, 2.00; monocalcium phosphate, 1.00; choline chloride (99%), 0.30; taurine, 1.00; Y_2O_3, 0.10; calcium propionic acid, 0.05; ethoxyquin, 0.05; sodium alginate, 0.50.

2.4. Sample collection

Fecal samples, for determining the apparent digestibility coefficients (ADC), were collected from each tank by siphoning at about 5 h after feeding. The pooled feces were dried at 50 °C for 12 h and stored at −20 °C. At the end of the experiment, fish were fasted for 24 h, anaesthetized with eugenol (1:10,000) and group weighed. The intestine tissue for histological evaluation were sampled and immersed in Bouin's solution. Blood samples were obtained from the caudal veins using disposable sterile syringes and centrifuged at 4000 g for 10 min at 4 °C. The serum samples were stored at −80 °C prior to analysis. The distal intestine samples for gene expression analysis were collected, frozen in liquid nitrogen and then stored at −80 °C.

2.5. Analytical methods

2.5.1. Biochemical analysis

Moisture, crude protein, crude lipid, crude ash and energy of the ingredients, diets and fish samples were analyzed using standard Association of Official Analytical Chemist methods [22]. Moisture was analyzed by drying the samples to constant weight at 105 °C. Crude protein was determined by using the Kjeldahl method (Kjeltec TM 8400, FOSS, Hoganas, Sweden). Crude lipid was measured after diethyl ether extraction using Soxhlet method (Buchi 36680, Switzerland). Ash was examined after combustion in a muffle furnace at 550 °C for 20 h. Gross energy was determined with Parr1281 Automatic Bomb Calorimeter (Parr, Moline, IL, USA). Y_2O_3 level in diets and feces were determined according to the method of previous study, using inductively coupled plasma-atomic emission spectrophotometer (ICP-OES, Varian, CA, USA) [23].

2.5.2. ANFs determination

Trypsin inhibitors activity was measured using benzoyl-DL-arginine-p-nitroanilide (BAPA) method [24]. The trypsin inhibitor was extracted in 0.01 M NaOH and collected with centrifugation for 10 min at 10000 rpm. The supernatant was diluted with 0.05 M Tris-HCl and trypsin solution plus BAPA solution (10 mg/mL in 97% dimethyl sulphoxide plus H_2O, 1:3 v/v). After incubated at 37 °C for 45 min, the reaction was terminated by adding 30% (v/v) acetic acid. The absorbance at 410 nm was measured and the trypsin inhibitors activity was expressed as mg per g sample.

The contents of glycinin and β-conglycinin were determined using the competitive enzyme-linked immunosorbent assay [25]. The samples were dissolved in the extracting solution (0.03 mol/L Tris–HCl, containing 0.01 mol/L β-mercaptoethanol, pH 8.0) for 16 h at 25 °C. After centrifugation and dilution, sample was injected into 96-well microtiterplates, which were coated with purified glycinin and β-conglycinin. The monoclonal antibodies were added and the plates were incubated for 30 min at 37 °C. The secondary antibody was added. Chromogenic reaction was developed by adding o-phenylenediamine substrate solution. The absorbance at 450 nm was measured. The contents of glycinin and β-conglycinin were expressed as mg per g sample.

The oligosaccharide assay was carried out according to the method described by Medeiros et al. [26]. Briefly, 70% (v/v) ethanol was used to extract the stachyose and raffinose with the assistance of microwave. The supernatant was concentrated and reconstituted to 25 ml. 20 μl of the sample was injected into the HPLC system for determination of stachyose and raffinose concentrations. 70% acetonitrile was used as the mobile phase with the flow rate controlled at 1 ml/min.

2.5.3. Amino acids analysis

The amino acids composition of ingredients was determined according to the method by Gao et al. [27]. The samples were hydrolyzed with 6 N HCl at 110 °C for 22 h and then diluted to 5 ml with ultrapure water. The solvent solution was evaporated under nitrogen gas and then added 0.02 N HCl. The hydrolyzate was filtered through a 0.22 μm nylon syringe filter and then analyzed by the L-8900 amino acid analyzer (Hitachi, Japan).

2.5.4. Enzyme activities assay

Total anti-oxidant capacity (T-AOC), superoxide dismutase (SOD) and lysozyme (LSZ) activities in serum were measured by enzymatic colorimetric methods using the commercially kits (Jiancheng Bioengineering Institute, Nanjing, China). The malondialdehyde (MDA) content was measured using the thiobarbituric acid (TBA) method. The samples were mixed with sodium dodecyl sulfate, acetate buffer (pH 3.5) and TBA solution. The mixture was heated at 95 °C for 40 min and centrifuged at 4000 g for 10 min. The absorbance of the supernatant at 523 nm was measured. The MDA content was expressed as nmol per ml serum.

Table 2
Sequences of primers used in this study for RT-qPCR.

Primer names	Primer sequence (5′ to 3′)	GenBank accession number
tnf α-F	CCCTTATCATTATGGCCCTT	FJ654645.1
tnf α-R	TCCGAGTACCGCCATATCCT	
il-1β-F	TACCTGTCGTGCCAACAGGAA	AJ295836.2
il-1β-R	TGATGTACCAGTTGGGGAA	
*tgf β*1-F	CTGCAGGACTGGCTCAAAGG	KU238187
*tgf β*1-R	CATGGTCAGGATGTATGGTGGT	
mhcII α-F	ACATTCCCTATCCCAACA	DQ094170
mhcII α-R	TCAGACCCAGTCCACAGA	
β-actin-F	GCTGTCTTCCCTTCTATCGTCG	AY008305.1
β-actin-R	TCCATGTCATCCCAGTTGGTC	

tnf α: tumor necrosis factor alpha; il-1β: interleukin1beta; tgf β1: transforming growth factor beta1; mhcII α: major histocompatibility complex II alpha.

2.5.5. Gut micromorphology

The distal intestinal samples were sliced transversely into 5 μm sections and stained with hematoxylin and eosin (H&E). The slides were examined under a light microscope (Olympus, DP72) equipped with a camera (Nikon E600) and CellSens Standard Software (Olympus). The height of villus, enterocytes and microvillus were measured and analyzed respectively.

2.5.6. Quantitative real-time PCR analysis

The relative expression level of *tumor necrosis factor alpha* (*tnf-α*), *interleukin-1 beta* (*il-1β*), *transforming growth factor beta1* (*tgf-β1*) and *major histocompatibility complex II alpha* (*mhc II α*) in distal intestines were examined by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from tissue (~50 mg) using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The integrity of RNA was determined by electrophoresis on a 1.2% agarose gel and the concentration was quantified using a Nano Drop®2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNA was reversely transcribed to cDNA by the Primer Script™ RT reagent Kit (Takara, Dalian, China). *β-actin* gene was chosen as the reference gene for sample normalization. All the primer sequences were listed in Table 2.

The qRT-PCR reactions were carried out in 25 μl reaction volume. The thermal profile was 95 °C for 20 s, followed by 39 cycles of 95 °C for 5 s, 57–58 °C for 30 s, and 72 °C for 30 s. Each sample was tested in triplicate. The relative expression level of target genes was calculated with the $2^{-\Delta\Delta Ct}$ method [28].

2.5.7. Calculations and statistical analysis

The following variables were calculated:

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

Specific growth rate (SGR) = (Ln final body weight - Ln initial body weight) × 100 / days

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

Protein efficiency ratio (PER) = wet weight gain (g) / protein ingested (g)

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

SPSS 19.0 was used to analyze the significant differences. All data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison method. $P < 0.05$ was considered to be statistically significant. Data were expressed as means ± standard error.

Table 3
Crude protein, crude lipid and anti-nutritional factors content in soybean meal and fermented soybean meal.

Ingredients	Crude protein (%)	Crude lipid (%)	Trypsin inhibitors (mg/g)	Glycinin (mg/g)	β-conglycinin (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)
SBM	50.84 ± 0.45[a]	2.11 ± 0.09	2.00 ± 0.02[a]	94.00 ± 1.15[a]	109.20 ± 0.29[a]	32.52 ± 0.01[a]	2.00 ± 0.12[a]
FSM	60.58 ± 0.36[b]	2.07 ± 0.11	0.40 ± 0.03[b]	1.40 ± 0.23[b]	2.80 ± 0.23[b]	0.39 ± 0.15[b]	0.40 ± 0.27[b]

Note: Values show mean ± standard error, n = 3; values in the same column with different superscripted small letters mean significant difference ($P < 0.05$). SBM: soybean meal; FSM: *Aspergillus awamori* fermented soybean meal.

Table 4
Amino acid composition (% dry matter basis) of soybean meal and fermented soybean meal.

Amino acids	SBM	FSM
Essential amino acids		
Arginine	2.80	2.86
Histidine	1.64	1.98
Isoleucine	1.94	2.39
Leucine	3.34	4.06
Methionine	0.35	0.66
Lysine	2.74	3.09
Phenylalanine	2.18	2.62
Threonine	1.78	2.20
Valine	2.01	2.50
Non-essential amino acids		
Tryptophan	0.63	0.86
Alanine	1.85	2.37
Aspartate	5.07	5.99
Cystine	0.69	0.71
Glutamate	8.04	9.30
Glycine	1.87	2.31
Serine	2.34	2.82
Tyrosine	1.38	1.42
Proline	2.21	2.62
Total	42.86	50.76

SBM: soybean meal; FSM: *Aspergillus awamori* fermented soybean meal.

3. Results

3.1. The nutritional profile of SBM and FSM

As shown in Table 3, the nutritional quality of SBM was significantly improved by *Aspergillus awamori* fermentation. Fermentation significantly improved the content of crude protein (from 50.84% to 60.58%), while no effects on the crude lipid level. Fermentation also significantly reduced the level of ANFs in SBM, such as raffinose (−98.8%), glycinin (−98.5%), β-conglycinin (−97.4%), trypsin inhibitors (−80%) and stachyose (−80%).

The amino acids composition of SBM and FSM were also determined (Table 4). The content of total amino acids in FSM was about 1.18 fold of that in SBM. For the essential amino acids, methionine showed a dramatic increase from 0.35% in SBM to 0.66% in FSM. The levels of histidine, isoleucine, leucine, phenylalanine, threonine and valine in FSM were about 20% higher than those in SBM. A mild increase was observed in the content of arginine and lysine. For non-essential amino acids, an increase was also observed in tryptophan (36.51%), alanine (28.11%), glycine (23.53%), serine (20.51%), proline (18.55%), aspartate (18.15%) and glutamate (15.67%). The contents of cystine and tyrosine in FSM were only 2.90% higher than those in SBM.

3.2. Growth performance and feed utilization

After the 63-day feeding trial, both SR and FI showed no significant difference among the groups ($P > 0.05$). Replacement 45% or more FM with SBM significantly impressed the FBW, SGR and PER of turbot. For the FSM groups, the inferior performance of FBW, SGR were only observed in F60 group ($P < 0.05$) and no significant difference of PER were observed ($P > 0.05$, Table 5).

As shown in Table 6, SBM replacement significantly reduced the ADC of dry matter ($P < 0.05$). The ADC of crude protein was significantly lower than FM group when the substitution level of SBM was over 45%. However, the ADC of dry matter and crude protein in FSM groups showed no significant differences with those in FM group ($P > 0.05$). Furthermore, the ADC of dry matter in F60 was significantly higher than that in S60. A significantly promotion of the ADC of crude protein by fermentation was observed when the substitution level was more than 45%.

3.3. Antioxidant activity and non-specific immunity

The activities of T-AOC in serum showed no significant differences among groups except a reduction in S60. The activities of SOD in SBM groups were significantly lower than the FM group ($P < 0.05$) when 45% or 60% FM protein was substituted. MDA content were significantly increased in S45, S60 and F60 groups compared to that in FM group ($P < 0.05$). Meanwhile, replacement FM significantly reduced the activities of LSZ in SBM45 and SBM60 groups. A significant improvement of LSZ activities by fermentation was observed in F60 when compared with the S60 group ($P < 0.05$, Fig. 1).

3.4. Gut micromorphology

The heights of villus (VH), enterocytes (EH) and microvillus (MH) in distal intestine were high affected by the diets. They were significantly lower in S45 and S60 than those in FM group ($P < 0.05$). However, for the FSM groups, only VH and EH in F60 were lower than those in FM groups. A significant improvement of MH and EH by fermentation were observed in the F45 and F60 when compared with the same substitution level by SBM ($P < 0.05$, Fig. 2).

3.5. Intestinal inflammatory response

Compared to the FM diet, no effect was found on the inflammatory gene expression when 30% FM replacement ($P > 0.05$). Replacement 45% or more FM with SBM significantly induced the expression of the pro-inflammatory genes (*tnf-α* and *il-1β*) and depressed the anti-inflammatory gene (*tgf-β1*). Fermentation significantly influenced the expression levels of *tnf-α*, *il-1β* and *tgf-β1* when substitution level reached 45% ($P < 0.05$). However, the *tnf-α*, *il-1β* and *tgf-β1* expression levels in F45 and F60 still had a significant difference with those in FM group. For the expression of *mhcII α*, a significant inducement was observed in S60 and F60 group. Meanwhile, the expression level of *mhcII α* in F60 group was significantly lower than that in S60 group (Fig. 3).

4. Discussion

4.1. Aspergillus awamori fermentation of SBM

Substitution of FM with high levels of SBM is still quite a challenging task for carnivorous fishes [8]. In the present study, *Aspergillus awamori* fermentation was conducted to improve the utilization of SBM. The results showed that fermentation significantly reduced the contents of ANFs in SBM. Previous studies reported that *Aspergillus awamori*

Table 5
Growth performance and feed utilization of turbot fed experimental diets.

Diet	IBW[a] (g)	FBW[b] (g)	SR[c] (%)	SGR[d] (%/d)	FI[e] (%/d)	PER[f] (g/g)
FM	8.54 ± 0.01	62.60 ± 0.96^{a}	100.00 ± 0.00	3.16 ± 0.02^{a}	1.76 ± 0.06	2.60 ± 0.04^{ab}
S30	8.53 ± 0.00	56.31 ± 1.66^{abc}	100.00 ± 0.00	2.99 ± 0.04^{abc}	1.95 ± 0.01	2.31 ± 0.02^{abc}
S45	8.53 ± 0.00	52.23 ± 1.37^{bc}	97.78 ± 2.22	2.88 ± 0.04^{bc}	2.09 ± 0.08	2.12 ± 0.09^{bc}
S60	8.52 ± 0.01	49.95 ± 1.82^{c}	98.89 ± 1.11	2.81 ± 0.06^{d}	2.17 ± 0.03	2.00 ± 0.07^{c}
F30	8.53 ± 0.01	63.41 ± 1.31^{a}	97.78 ± 2.22	3.18 ± 0.03^{a}	1.97 ± 0.00	2.40 ± 0.01^{abc}
F45	8.52 ± 0.01	59.67 ± 2.47^{ab}	100.00 ± 0.00	3.09 ± 0.07^{ab}	1.89 ± 0.05	2.40 ± 0.06^{abc}
F60	8.52 ± 0.01	52.09 ± 1.09^{bc}	100.00 ± 0.00	2.87 ± 0.03^{d}	1.99 ± 0.01	2.23 ± 0.01^{abc}

Note: Values show mean ± standard error, n = 3; values in the same column with different superscripted small letters mean significant difference ($P < 0.05$).
[a] IBW: initial body weight.
[b] FBW: final body weight.
[c] SR: survival rate = 100 × final fish number/initial fish number.
[d] SGR: Specific growth rate = (Ln final body weight – Ln initial body weight) × 100/days.
[e] FI: feed intake = 100 × dry feed intake (g)/[(FBW + IBW)/2]/days.
[f] PER: protein efficiency ratio = wet weight gain (g)/protein ingested (g).

Table 6
Apparent digestibility coefficients (%, ADC) for dry matter and crude protein of the experimental diets.

Diet	Dry matter	Crude protein
FM	90.85 ± 0.56^{a}	97.69 ± 0.15^{a}
S30	84.49 ± 1.59^{b}	96.59 ± 1.26^{a}
S45	82.10 ± 2.65^{b}	93.35 ± 0.79^{bc}
S60	80.42 ± 2.48^{c}	90.61 ± 0.88^{c}
F30	86.69 ± 0.77^{ab}	96.66 ± 0.23^{a}
F45	84.92 ± 0.48^{ab}	96.12 ± 0.77^{a}
F60	87.30 ± 0.81^{ab}	93.85 ± 0.69^{ab}

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

could produce various enzymes, such as amylases, cellulases, xylanases, tannase and proteases [17,29]. The exogenous enzymes produced by *Aspergillus awamori* during fermentation process may account for the degradation of ANFs.

The content of crude protein in SBM was remarkably improved after fermentation which was paralleled with the results of Jannathulla et al. [30], who found that *Aspergillus niger* fermentation increased the SBM crude protein content. The increases may be due in part to the decreased carbohydrate content [15] and increased homologous and heterologous protein produced by *Aspergillus awamori* during fermentation process [31,32]. Accompanied by the increase of crude protein, both the essential amino acids and non-essential amino acids contents in SBM were increased after fermentation. Hong et al. [15] reported that the essential amino acid profile was unchanged after fermentation by *Aspergillus oryzae* GB-107. It might be a result of the differential

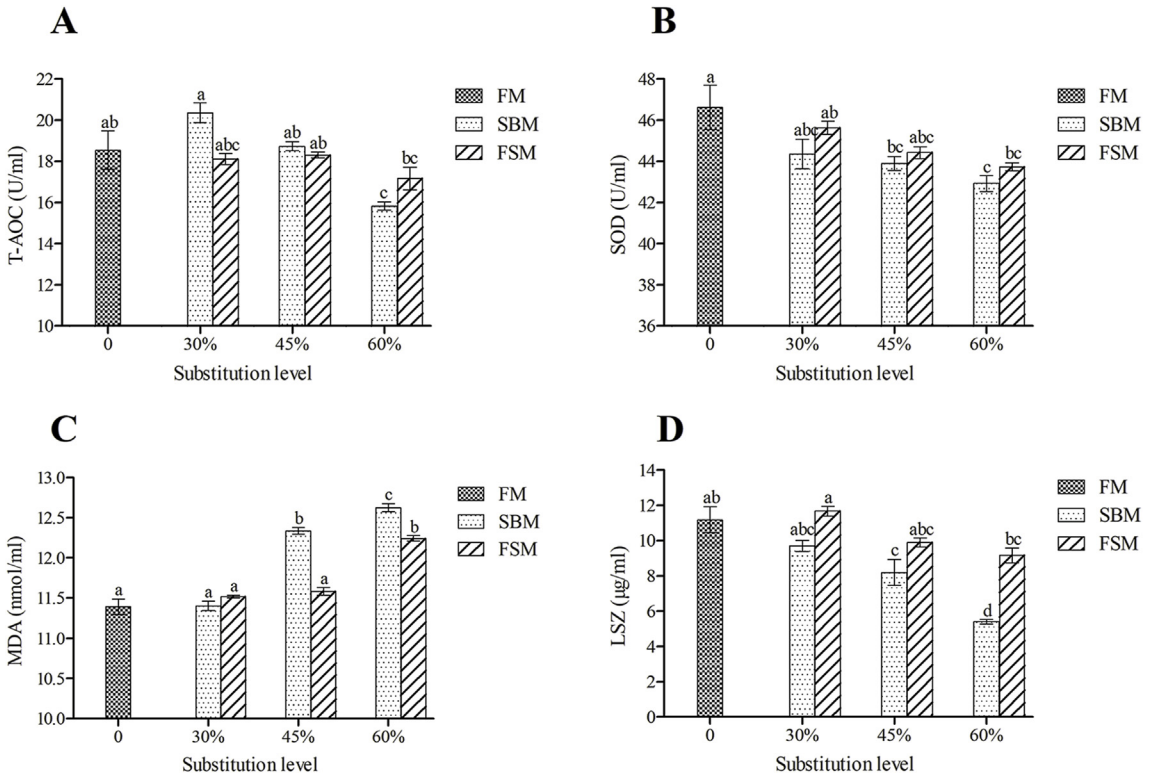

Fig. 1. The activities of T-AOC, SOD, LSZ and content of MDA in turbot serum. The values of T-AOC and SOD were expressed as U per ml serum. The MDA level was expressed as nmol per ml serum. The result of LSZ was expressed as μg per ml serum. The different superscript letters (a, b, c, d) among treatments indicate significance differences ($P < 0.05$). The results were showed as mean ± standard error (n = 6). T-AOC: the total antioxidant capacity; SOD: superoxide dismutase; MDA: malondialdehyde; LSZ: lysozyme.

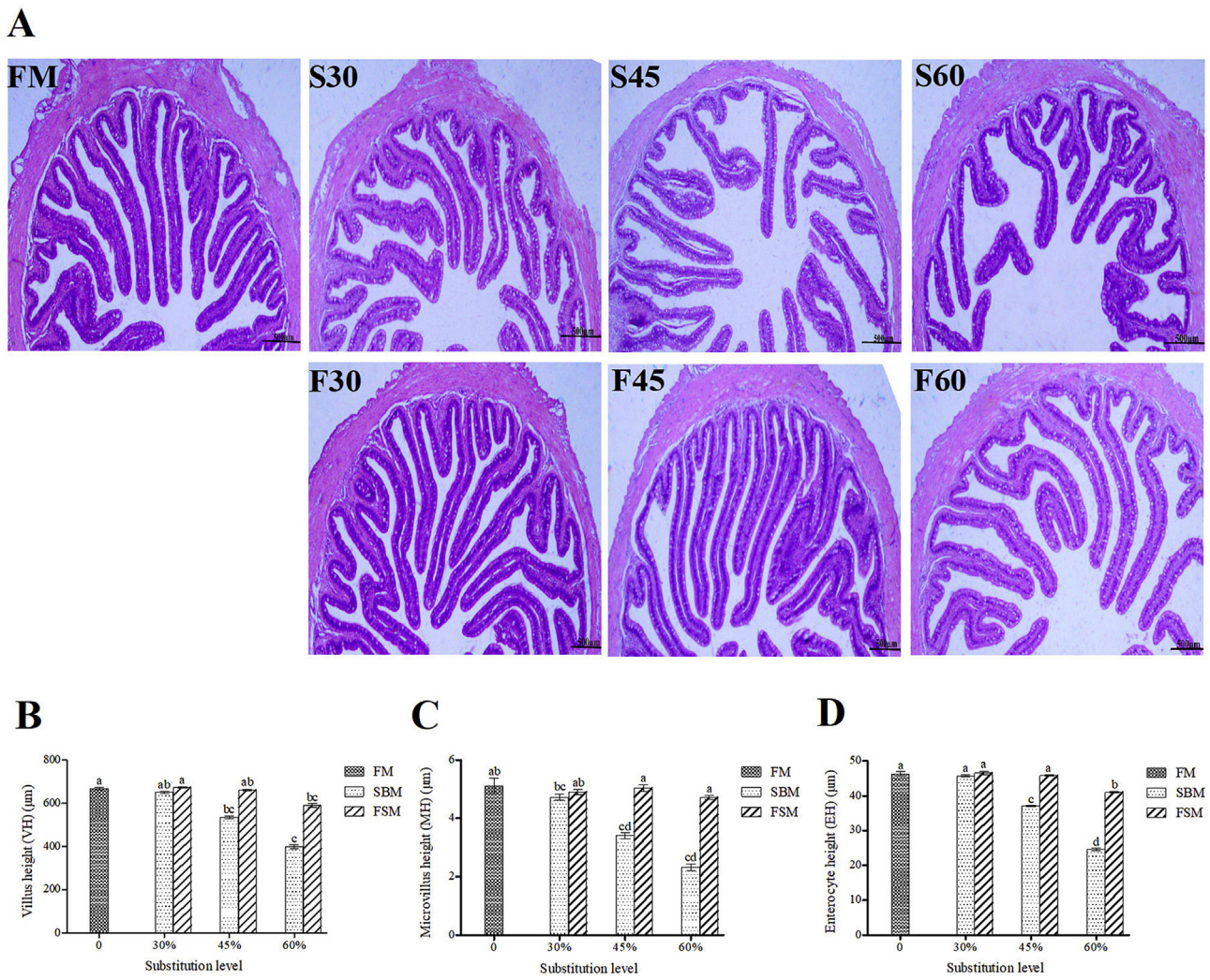

Fig. 2. The intestinal morphology of turbot fed with graded levels of SBM and FSM. Images of hematoxylin and eosin-stained sections (A) and the heights of villus (B), Microvillus (C) and Enterocyte (D) were showed. The different superscript letters (a, b, c, d) among treatments indicate significance differences ($P < 0.05$). The results were showed as mean ± standard error (n = 6).

strains of *Aspergillus* have different utilization pattern for essential amino acids. Methionine, the indispensable amino acid which plays unique roles in protein structure and metabolism [33], showed a dramatic increase in this study. It's possible that *Aspergillus awamori* fermentation promoted the conversion of certain amino acid to methionine. However, the precise mechanisms need to be further studied.

4.2. Growth performance and digestibility

In present study, the inferior growth performance was observed when the replacement level of SBM was up to 45%. FSM could substitute 45% of FM without causing any adverse effects. The suppression of feed intake (FI) might be the important factor for reducing growth performance when FM was replaced by SBM [4]. In present study, there were no significant differences among groups in FI and it did not account for the different growth performance of fish fed SBM or FSM diet. A remarkable promotion of ADC of dry matter and crude protein in FSM groups were observed, which could be partially responsible for the better growth performance. Wang et al. [4] reported the significant enhancement of ADC values in turbot fed *Lactobacillus plantarum* P8 fermented SBM. Previous studies in Japanese seabass [8] and Atlantic salmon [34] also indicated that microbial fermentation could significantly enhance the ADC values of SBM. This might be attributed to the degradation of macromolecules and ANFs in SBM during fermentation process.

The ANFs in SBM were reported to cause detrimental effects on feed utilization of animals. Trypsin inhibitor can inhibit the activity of pancreatic protease thereby affecting the efficiency of the digestive process [35,36]. The oligosaccharides, which cannot be utilized by monogastric animals [5], can cause discomfort in digestive tract and impede nutrient digestibility thus affecting the growth performance of animals [37,38]. In this study, fermentation process efficiently eliminated the trypsin inhibitor and indigestible oligosaccharides in SBM. However, the residual ANFs in F60 diets may still have negative effects on turbot.

4.3. Antioxidant capacity and non-specific immunity

Fish has a powerful antioxidant defense system which can protect organisms against oxidative stress or damage [39]. T-AOC, SOD and MDA play vital roles in the antioxidant defense system [40,41]. Several researches have indicated that the ANFs in plant protein sources can induce oxidative stress in fish [42,43]. In present study, significant SBM-induced oxidative stress was observed in S45 and S60 groups with reduced SOD activity and high content of MDA. The FSM groups showed a better performance and only F60 group induced the oxidative stress. It suggested that dietary FSM could enhance the antioxidant capacity of turbot. The reduction of ANFs level in SBM after *Aspergillus*

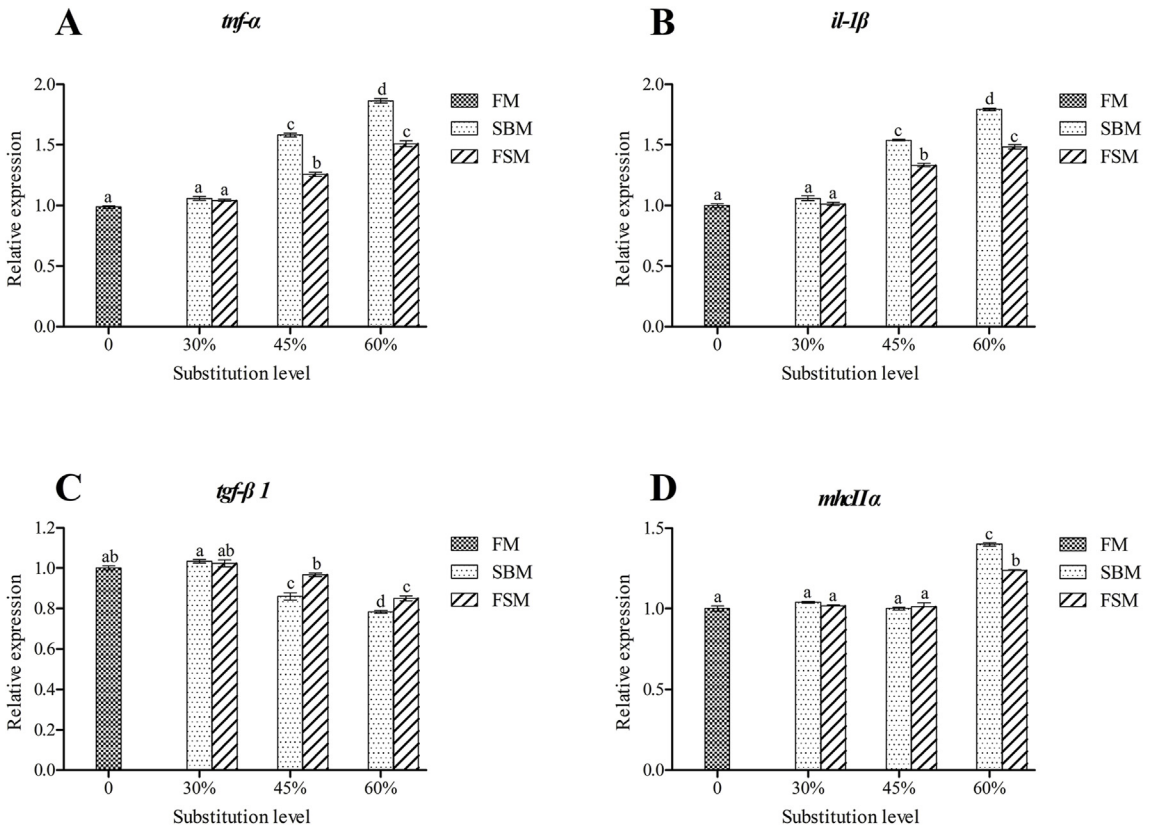

Fig. 3. The relative expression of *tnf-α*, *il-1β*, *tgf-β*1 and *mhcII α* in the distal intestines of turbot. The value of FM group was normalized to 1.0 and the rest groups were expressed as relative expression levels to the FM group. The different superscript letters (a, b, c, d) among treatments indicate significance differences ($P < 0.05$). The results were showed as mean ± standard error (n = 6).

awamori fermentation may partially account for the antioxidant capacity. It was reported that the bioavailability of flavone and isoflavones, potential antioxidant compounds in SBM, can be improved by *Aspergillus oryzae* fermentation [44,45]. Furthermore, the allergens in SBM can be degraded into bioactive peptides which exhibit strong anti-oxidative activity [46].

Lysozyme (LSZ) is an indispensable enzyme in the non-specific immune system that has the ability to disrupt cell walls by splitting peptidoglycans to resist bacteria [47]. It was reported that the non-specific immune capacity could be smothered by high-level dietary SBM [48,49]. In the current study, the serum LSZ activities of fish were significantly reduced when the dose of SBM was up to 45%. It indicated that the immune system of turbot was damaged by high content of SBM. However, FSM groups significantly alleviated the suppression of LSZ activities. Previous studies suggested *Aspergillus awamori* was a promising probiotic which can remarkably improve the growth performance of broiler chickens [50,51]. In present study, the increasing bioactive components in FSM and concomitantly ingested *Aspergillus awamori* may account for the improvement of immune status in fish.

4.4. Gut micromorphology and inflammatory response

High-level SBM in diets for carnivorous fish could induce gut pathological changes and detrimental inflammatory response [52,53]. The inclusion of 26–54% SBM in turbot diet caused severe pathological changes and remarkably up-regulated expression of inflammatory marker genes [54]. In Atlantic salmon, merely 5–10% SBM in the diet could cause detrimental inflammatory effects in the intestine [55]. In this study, gut pathological changes were observed when the replacement level of SBM was up to 45%. Dietary FSM in this study partially alleviated the SBM-induced gut pathological changes and detrimental inflammatory response with less abnormal structural changes, relatively lower gene expressions of *tnf-α, il-1β, mhcII α* and relatively higher gene expressions of *tgf-β*1. High contents of ANFs in SBM could be responsible for the damage of gut health [56]. Actually, the presence of antigenic components in SBM, such as glycinin and β-conglycinin, may be primarily responsible for inducing abnormal gut structural changes and activating the immune system leading to detrimental inflammatory response [57,58]. In present study, most of the glycinin (−98.5%) and β-conglycinin (−97.4%) were degraded by *Aspergillus awamori* fermentation, which could account for the improvement of gut health. Damage of the gut health in FSM60 group may be related to the residues of such ANFs, revealing that *Aspergillus awamori* fermentation cannot completely remove the ANFs of affecting gut health.

In conclusion, *Aspergillus awamori* fermentation can remarkably improve the nutrition quality of SBM by increasing crude protein, total amino acid and reducing ANFs. Dietary FSM could improve nutrient utilization, enhance antioxidant capacity, alleviate the SBM-induced gut injury and inflammatory response. Our results indicated that *Aspergillus awamori* is a promising fermentation strain to be applied in aquaculture and *Aspergillus awamori* fermentation is an effective method to improve the utilization of SBM.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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References

[1] H.M. Azarm, S.-M. Lee, Effects of partial substitution of dietary fish meal by fermented soybean meal on growth performance, amino acid and biochemical parameters of juvenile black sea bream *Acanthopagrus schlegeli*, Aquacult. Res. 45 (6) (2014) 994–1003.

[2] Y.-L. Shiu, S.-L. Hsieh, W.-C. Guei, Y.-T. Tsai, C.-H. Chiu, C.-H. Liu, Using *Bacillus subtilis* E20-fermented soybean meal as replacement for fish meal in the diet of orange-spotted grouper (*Epinephelus coioides*, Hamilton), Aquacult. Res. 46 (6) (2015) 1403–1416.

[3] E. Tibaldi, Y. Hakim, Z. Uni, F. Tulli, M. de Francesco, U. Luzzana, S. Harpaz, Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (*Dicentrarchus labrax*), Aquaculture 261 (1) (2006) 182–193.

[4] L. Wang, H. Zhou, R. He, W. Xu, K. Mai, G. He, Effects of soybean meal fermentation by *Lactobacillus plantarum* P8 on growth, immune responses, and intestinal morphology in juvenile turbot (*Scophthalmus maximus* L.), Aquaculture 464 (2016) 87–94.

[5] R.L. Chou, B.Y. Her, M.S. Su, G. Hwang, Y.H. Wu, H.Y. Chen, Substituting fish meal with soybean meal in diets of juvenile cobia *Rachycentron canadum*, Aquaculture 229 (1–4) (2004) 325–333.

[6] S.-J. Lim, K.-J. Lee, Partial replacement of fish meal by cottonseed meal and soybean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus*, Aquaculture 290 (3–4) (2009) 283–289.

[7] W. Chen, Q. Ai, K. Mai, W. Xu, Z. Liufu, W. Zhang, Y. Cai, Effects of dietary soybean saponins on feed intake, growth performance, digestibility and intestinal structure in juvenile Japanese flounder (*Paralichthys olivaceus*), Aquaculture 318 (1–2) (2011) 95–100.

[8] S. Rahimnejad, K. Lu, L. Wang, K. Song, K. Mai, D.A. Davis, C. Zhang, Replacement of fish meal with *Bacillus pumillus* SE5 and *Pseudozyma aphidis* ZR1 fermented soybean meal in diets for Japanese seabass (*Lateolabrax japonicus*), Fish Shellfish Immunol. 84 (2018) 987–997.

[9] W.-D. Jiang, K. Hu, J.-X. Zhang, Y. Liu, J. Jiang, P. Wu, J. Zhao, S.-Y. Kuang, L. Tang, W.-N. Tang, Y.-A. Zhang, X.-Q. Zhou, L. Feng, Soyabean glycinin depresses intestinal growth and function in juvenile Jian carp (*Cyprinus carpio* var Jian): protective effects of glutamine, Br. J. Nutr. 114 (10) (2015) 1569–1583.

[10] J.-X. Zhang, L.-Y. Guo, L. Feng, W.-D. Jiang, S.-Y. Kuang, Y. Liu, K. Hu, J. Jiang, S.-H. Li, L. Tang, X.-Q. Zhou, Soybean beta-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish, PLoS One 8 (3) (2013).

[11] M. Gu, Q. Jia, Z. Zhang, N. Bai, X. Xu, B. Xu, Soya-saponins induce intestinal inflammation and barrier dysfunction in juvenile turbot (*Scophthalmus maximus*), Fish Shellfish Immunol. 77 (2018) 264–272.

[12] L.G. Buttle, A.C. Burrells, J.E. Good, P.D. Williams, P.J. Southgate, C. Burrells, The binding of soybean agglutinin (SBA) to the intestinal epithelium of Atlantic salmon, *Salmo salar* and Rainbow trout, *Oncorhynchus mykiss*, fed high levels of soybean meal, Vet. Immunol. Immunopathol. 80 (3–4) (2001) 237–244.

[13] K. Wang, C. Liu, Y. Hou, H. Zhou, X. Wang, K. Mai, G. He, Differential apoptotic and mitogenic effects of lectins in zebrafish, Front. Endocrinol. 10 (2019) 356.

[14] S.-H. Seo, S.-J. Cho, Changes in allergenic and antinutritional protein profiles of soybean meal during solid-state fermentation with *Bacillus subtilis*, LWT - Food Sci. Technol. 70 (2016) 208–212.

[15] K.J. Hong, C.H. Lee, S.W. Kim, *Aspergillus oryzae* GB-107 fermentation improves nutritional quality of food soybeans and feed soybean meals, J. Med. Food 7 (4) (2004) 430–435.

[16] B.P. Singh, S. Vij, S. Hati, Functional significance of bioactive peptides derived from soybean, Peptides 54 (2014) 171–179.

[17] A.M. de Castro, L.R. Castilho, D.M. Guimares Freire, Multivariate optimization and supplementation strategies for the simultaneous production of amylases, cellulases, xylanases, and proteases by *Aspergillus awamori* under solid-state fermentation conditions, Appl. Biochem. Biotechnol. 175 (3) (2015) 1588–1602.

[18] S.J. Cho, S.H. Oh, R.D. Pridmore, M.A. Juillerat, C.H. Lee, Purification and characterization of proteases from *Bacillus amyloliquefaciens* isolated from traditional soybean fermentation starter, J. Agric. Food Chem. 51 (26) (2003) 7664–7670.

[19] A.A. Saleh, K. Hayashi, D. Ijiri, A. Ohtsuka, Effect of feeding *Aspergillus awamori* and canola seed on the growth performance and muscle fatty acid profile in broiler chicken, Anim. Sci. J. 86 (3) (2015) 305–311.

[20] D.N. Lee, Y.S. Hung, T.S. Yang, J.H. Lin, C.F. Weng, *Aspergillus awamori*-fermented mung bean seed coats enhance the antioxidant and immune responses of weaned pigs, J. Anim. Physiol. Anim. Nutr. 101 (5) (2017) e342–e351.

[21] Y. Liu, G. He, Q. Wang, K. Mai, W. Xu, H. Zhou, Hydroxyproline supplementation on the performances of high plant protein source based diets in turbot (*Scophthalmus maximus* L.), Aquaculture 433 (2014) 476–480.

[22] P. Fox, Association of Official Analytical Chemists, Official Methods of Analysis, 3rd rev, AOAC, Arlington, VA AOAC, Arlington, VA, 1997.

[23] Z. Cheng, Q. Ai, K. Mai, W. Xu, H. Ma, Y. Li, J. Zhang, Effects of dietary canola meal on growth performance, digestion and metabolism of Japanese seabass, *Lateolabrax japonicus*, Aquaculture 305 (1–4) (2010) 102–108.

[24] C. Dai, H. Ma, R. He, L. Huang, S. Zhu, Q. Ding, L. Luo, Improvement of nutritional value and bioactivity of soybean meal by solid-state fermentation with *Bacillus subtilis*, LWT - Food Sci. Technol. 86 (2017) 1–7.

[25] Y. Wang, X.T. Liu, H.L. Wang, D.F. Li, X.S. Piao, W.Q. Lu, Optimization of processing conditions for solid-state fermented soybean meal and its effects on growth performance and nutrient digestibility of weanling pigs, Livest. Sci. 170 (2014) 91–99.

[26] S. Medeiros, J. Xie, P.W. Dyce, H.Y. Cai, K. DeLange, H. Zhang, J. Li, Isolation of bacteria from fermented food and grass carp intestine and their efficiencies in improving nutrient value of soybean meal in solid state fermentation, J. Anim. Sci. Biotechnol. 9 (2018).

[27] Z. Gao, X. Wang, C. Tan, H. Zhou, K. Mai, G. He, Effect of dietary methionine levels on growth performance, amino acid metabolism and intestinal homeostasis in turbot (*Scophthalmus maximus* L.), Aquaculture 498 (2019) 335–342.

[28] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (4) (2001) 402–408.

[29] R.C. Rajak, A. Singh, R. Banerjee, Biotransformation of hydrolysable tannin to ellagic acid by tannase from *Aspergillus awamori*, Biocatal. Biotransform. 35 (1) (2017) 27–34.

[30] R. Jannathulla, J.S. Dayal, K. Ambasankar, M. Muralidhar, Effect of *Aspergillus niger* fermented soybean meal and sunflower oil cake on growth, carcass composition and haemolymph indices in *Penaeus vannamei* boone, Aquaculture 486 (2018) 1–8 1931.

[31] R.J. Gouka, P.J. Punt, J.G.M. Hessing, C. vandenHondel, Analysis of heterologous protein production in defined recombinant *Aspergillus awamori* strains, Appl. Environ. Microbiol. 62 (6) (1996) 1951–1957.

[32] R.J. Gouka, P.J. Punt, C. vandenHondel, Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects, Appl. Microbiol. Biotechnol. 47 (1) (1997) 1–11.

[33] J.T. Brosnan, M.E. Brosnan, R.F.P. Bertolo, J.A. Brunton, Methionine: a metabolically unique amino acid, Livest. Sci. 112 (1–2) (2007) 2–7.

[34] S. Refstie, S. Sahlstrom, E. Brathen, G. Baeverfjord, P. Krogedal, Lactic acid fermentation eliminates indigestible carbohydrates and antinutritional factors in soybean meal for Atlantic salmon (*Salmo salar*), Aquaculture 246 (1–4) (2005) 331–345.

[35] C.-H. Liu, Y.-L. Shiu, J.-L. Hsu, Purification and characterization of trypsin from the pyloric ceca of orange-spotted grouper, *Epinephelus coioides*, Fish Physiol. Biochem. 38 (3) (2012) 837–848.

[36] J.J. Olli, A. Krogdahl, T. Vandeningh, L.E. Brattas, Nutritive value of four soybean products in diets for Atlantic salmon (*Salmo salar*, L), Acta Agric. Scand. Sect. A Anim. Sci. 44 (1) (1994) 50–60.

[37] L. Montagne, J.R. Pluske, D.J. Hampson, A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals, Anim. Feed Sci. Technol. 108 (1–4) (2003) 95–117.

[38] M. Choct, Y. Dersjant-Li, J. McLeish, M. Peisker, Soy Oligosaccharides and solublenon-starch polysaccharides: a review of digestion, nutritive and anti-nutritive effects in pigs and poultry, Asian-Australas. J. Anim. Sci. 23 (10) (2010) 1386–1398.

[39] R.M. Martinez-Alvarez, A.E. Morales, A. Sanz, Antioxidant defenses in fish: biotic and abiotic factors, Rev. Fish Biol. Fish. 15 (1–2) (2005) 75–88.

[40] Z. Ding, Y. Zhang, J. Ye, Z. Du, Y. Kong, An evaluation of replacing fish meal with fermented soybean meal in the diet of *Macrobrachium nipponense*: growth, non-specific immunity, and resistance to *Aeromonas hydrophila*, Fish Shellfish Immunol. 44 (1) (2015) 295–301.

[41] J.Q. Wu, T.R. Kosten, X.Y. Zhang, Free radicals, antioxidant defense systems, and schizophrenia, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 46 (2013) 200–206.

[42] A. Sitja-Bobadilla, S. Pena-Llopis, P. Gomez-Requeni, F. Medale, S. Kaushik, J. Perez-Sanehez, Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*), Aquaculture 249 (1–4) (2005) 387–400.

[43] Q. Zheng, X. Wen, C. Han, H. Li, X. Xie, Effect of replacing soybean meal with cottonseed meal on growth, hematology, antioxidant enzymes activity and expression for juvenile grass carp, *Ctenopharyngodon idellus*, Fish Physiol. Biochem. 38 (4) (2012) 1059–1069.

[44] S.-S. Kim, G. Bueno Galaz, M.A. Pham, J.-W. Jang, D.-H. Oh, I.-K. Yeo, K.-J. Lee, Effects of dietary supplementation of a meju, fermented soybean meal, and *Aspergillus oryzae* for juvenile parrot fish (*Oplegnathus fasciatus*), Asian-Australas. J. Anim. Sci. 22 (6) (2009) 849–856.

[45] S.-S. Kim, P. Minh Anh, K.-W. Kim, M.-H. Son, K.-J. Lee, Effects of microbial fermentation of soybean on growth performances, phosphorus availability, and antioxidant activity in diets for juvenile olive flounder (*Paralichthys olivaceus*), Food Sci. Biotechnol. 19 (6) (2010) 1605–1610.

[46] C.-H. Chi, S.-J. Cho, Improvement of bioactivity of soybean meal by solid-state fermentation with *Bacillus amyloliquefaciens* versus *Lactobacillus* spp. and *Saccharomyces cerevisiae*, LWT - Food Sci. Technol. 68 (2016) 619–625.

[47] S. Saurabh, P.K. Sahoo, Lysozyme: an important defence molecule of fish innate immune system, Aquacult. Res. 39 (3) (2008) 223–239.

[48] C. Burrells, P.D. Williams, P.J. Southgate, V.O. Crampton, Immunological, physiological and pathological responses of rainbow trout (*Oncorhynchus mykiss*) to increasing dietary concentrations of soybean proteins, Vet. Immunol. Immunopathol. 72 (3–4) (1999) 277–288.

[49] S. Lin, L. Luo, Effects of different levels of soybean meal inclusion in replacement for fish meal on growth, digestive enzymes and transaminase activities in practical diets for juvenile tilapia, *Oreochromis niloticus x O. aureus*, Anim. Feed Sci. Technol. 168 (1–2) (2011) 80–87.

[50] A.A. Saleh, Y.Z. Eid, T.A. Ebeid, T. Kamizono, A. Ohtsuka, K. Hayashi, Effects of feeding *Aspergillus Awamori* and *Aspergillus Niger* on growth performance and meat quality in broiler chickens, J. Poult. Sci. 48 (3) (2011) 201–206.

[51] A.A. Saleh, K. Hayashi, D. Ijiri, A. Ohtsuka, Beneficial effects of *Aspergillus awamori*

in broiler nutrition, World's Poult. Sci. J. 70 (4) (2014) 857–864.
[52] A. Krogdahl, A.M. Bakke-McKellep, G. Baeverfjord, Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.), Aquacult. Nutr. 9 (6) (2003) 361–371.
[53] A. Krogdahl, M. Penn, J. Thorsen, S. Refstie, A.M. Bakke, Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids, Aquacult. Res. 41 (3) (2010) 333–344.
[54] M. Gu, N. Bai, Y. Zhang, A. Krogdahl, Soybean meal induces enteritis in turbot *Scophthalmus maximus* at high supplementation levels, Aquaculture 464 (2016) 286–295.
[55] P.A. Uran, J.W. Schrama, J.H.W.M. Rombout, J.J. Taverne-Thiele, A. Obach, W. Koppe, J.A.J. Verreth, Time-related changes of the intestinal morphology of Atlantic salmon, *Salmo salar* L., at two different soybean meal inclusion levels, J. Fish Dis. 32 (9) (2009) 733–744.
[56] G. Francis, H.P.S. Makkar, K. Becker, Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish, Aquaculture 199 (3–4) (2001) 197–227.
[57] J. Feng, X. Liu, Z.R. Xu, Y.Y. Liu, Y.P. Lu, Effects of *Aspergillus oryzae* 3.042 fermented soybean meal on growth performance and plasma biochemical parameters in broilers, Anim. Feed Sci. Technol. 134 (3–4) (2007) 235–242.
[58] Y. Hao, Z. Zhan, P. Guo, X. Piao, D. Li, Soybean beta-conglycinin-induced gut hypersensitivity reaction in a piglet model, Arch. Anim. Nutr. 63 (3) (2009) 188–202.