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Replacement of fish meal with *Bacillus pumillus* SE5 and *Pseudozyma aphidis* ZR1 fermented soybean meal in diets for Japanese seabass (*Lateolabrax japonicus*)

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ABSTRACT

This study examined the effects of replacing fish meal (FM) with three different types of soybean meal (SM) including untreated SM, *Bacillus pumillus* SE5 (BP) fermented SM (BPFSM) and *Pseudozyma aphidis* ZR1 (PA) fermented SM (PAFSM) in diets for Japanese seabass (*Lateolabrax japonicus*). A basal diet was formulated using FM (FM diet), and six other diets were produced by substituting 40 or 80% of FM with SM, BPFSM or PAFSM (SM40, SM80, BPFSM40, BPFSM80, PAFSM40 and PAFSM80 diets). Each diet was fed to triplicate groups of fish (7.14 ± 0.05 g) twice daily for eight weeks. Replacing 40% of FM with SM sources did not significantly influence growth ($P > 0.05$), while increasing the substitution level to 80% led to reduced growth rates ($P < 0.05$). The groups received SM80 and PAFSM80 diets showed significantly higher feed conversion ratio and lower protein digestibility than FM group. Furthermore, notably lower dry matter digestibility was detected in SM80 group. Remarkably lower serum total antioxidant capacity was found in the SM80 group, and catalase activity did not significantly differ between FM and BPFSM40 groups. Serum malondialdehyde concentration was enhanced by increasing FM replacement level and the highest value was observed in the SM80 fed fish. FM and PAFSM40 groups showed significantly higher lysozyme activity than the SM80 group. Fish fed the BPFSM40 diet exhibited the highest complement C3 activity and the lowest value was observed in the SM80 group. Expression of lysozyme gene in spleen was down-regulated in the SM80 group, and no significant difference in expression of C3 gene was found among FM, BPFSM40 and PAFSM40 groups. Digestive enzymes activity and gut morphology were significantly influenced by FM replacement. Expression of HSP70 and pro-inflammatory genes including TNF-α and IL-1β were up-regulated by FM replacement and relatively lower expression levels were found by using fermented SM. An opposite trend was observed for the anti-inflammatory TGF-β gene expression. Serum D-lactate concentration was significantly increased by replacing 80% of FM with any of the SM sources. These findings indicated that using fermented SM, particularly BPFSM, beneficially influences feed utilization, antioxidant capacity, innate immunity and gut health in juvenile Japanese seabass.

1. Introduction

Fish meal (FM) has long been used as a source of high-quality protein for aquafeed formulation. However, reliance on FM has been recognized as a significant constraint to sustainable development of the aquaculture industry [1] due to the steady increase in its price which stems from its limited supply and increased incorporation in livestock and aquaculture feed [2,3]. Accordingly, pursuing less expensive and sustainable alternative protein sources of both animal and plant origin to reduce FM level in aquafeed without impairing growth performance is becoming a major continuing global interest [4–10].

A wide range of protein ingredients have been explored as potential FM replacers over the recent decades and plant proteins have received the main focus as the most viable candidates due to their lower cost and abundant availability [11,12]. Among the plant proteins investigated so far, soybean meal (SM) has shown promise owing to its stable supply, low price, competitive nutritional composition and relatively high digestibility [11,13]. Nevertheless, despite high nutritional value high

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

	FM	SM40	SM80	BPFSM40	BPFSM80	PAFSM40	PAFSM80
Fish meal[a]	35	21	7	21	7	21	7
Soybean meal[b]	0	20.55	41.10	0	0	0	0
BPFSM[c]	0	0	0	17.71	35.41	0	0
PAFSM[d]	0	0	0	0	0	17.74	35.47
Wheat flour	43.05	31.20	19.34	34.74	26.33	34.71	26.27
Wheat gluten meal	10.70	12.30	14.00	11.60	12.70	11.60	12.70
Squid visceral paste	3	3	3	3	3	3	3
Fish oil	2.5	3.88	5.26	3.99	5.47	3.99	5.47
Soybean oil	2	1.86	1.74	1.76	1.53	1.76	1.53
Lecithin	2	2	2	2	2	2	2
L-Ascorbyl-2-polyphosphate	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Monocalcium phosphate	0	1.5	3	1.5	3	1.5	3
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix[e]	0.60	0.84	1.08	0.84	1.08	0.84	1.08
Vitamin premix[f]	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Ethoxyquin	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Y_2O_3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Taurine	0	0.1	0.2	0.1	0.2	0.1	0.2
Lysine	0	0.37	0.73	0.37	0.73	0.37	0.73
Methionine	0	0.25	0.39	0.25	0.39	0.25	0.39
Proximate composition							
Dry matter	89.7	88.7	89.5	88.1	87.9	88.6	89.0
Protein	41.6	42.2	41.8	41.9	41.1	39.4	39.1
Lipid	10.2	10.4	10.5	10.4	10.5	11.0	9.80
Ash	6.97	7.38	7.89	7.52	7.85	7.22	7.27

[a] Xiamen ITG group Corp., Ltd., Xiamen, China, imported from Peru (crude protein: 73.9%, crude lipid: 9.87%).

[b] Soybean meal, obtained from Quanzhou Fuhai cereals and oils industry Co., Ltd. (crude protein: 50.6%, crude lipid: 1.3%).

[c] *Bacillus pumillus* SE5 fermented soybean meal.

[d] *Pseudozyma aphidis* ZR1 fermented soybean meal.

[e] Mineral premix (mg or g kg^{-1}diet): NaF, 2 mg; KI, 0.8 mg; CoCl2·6H2O (1%), 50 mg; CuSO4·5H2O, 10 mg; FeSO4·H2O, 80 mg; ZnSO4·H2O, 50 mg; MnSO4·H2O, 25 mg; MgSO4·7H2O, 200 mg; zoelite, 4.582 g.

[f] Vitamin premix (mg or g kg^{-1}diet): thiamin, 10 mg; riboflavin, 8 mg; pyridoxine HCl, 10 mg; vitamin B12, 0.2 mg, vitamin K3, 10 mg; inositol, 100 mg; pantothenic acid, 20 mg; niacin acid, 50 mg; folic acid, 2 mg; biotin, 2 mg; retinol acetate, 400 mg; cholecalciferol, 5 mg; alpha-tocopherol, 100 mg; ethoxyquin, 150 mg; wheat middling, 1.1328 g.

inclusion level of SM in diets for carnivorous fish is coupled with adverse impacts on growth, metabolism, and health status due to its several drawbacks such as deficiency of some essential amino acids, poor palatability and occurrence of anti-nutritional factors (ANFs) [14–16]. Additionally, a suppressed immune function following administration of high-SM diets has been demonstrated in several fish species [17,18]. Therefore, in order to increase the incorporation level of SM in aquafeed, it would be necessary to remove or inactivate its ANFs content [19].

Various processing approaches have been implemented for elimination of ANFs content of SM and improving its nutrients bioavailability and nutritional value which include thermal and mechanical processes, soaking, germination/malting and fermentation [20]. Among these procedures, fermentation has been suggested as the most cost effective means that improves the nutritional quality of SM not only through biodegradation of ANFs (such as trypsin inhibitors, oligosaccharides and phytic acid), proteins and fibers but also through production of probiotics and prebiotics that might subsequently boost palatability, nutrients digestibility and immune function [21–24]. Furthermore, it has been noted that fermentation of SM increases its protein content [25], facilitates antimicrobial and antioxidant activity [26–28], and reduces immunoglobulin E immunoreactivity [29].

To date, a large number of microorganisms have been tested for fermentation of SM and it has been realized that nutritional value of the produced fermented SM (FSM) varies depending on the type of microorganism. Plant proteins are rich in cellulose which is poorly digested by monogastric animals including fish [30–34]. It has been reported that microbes isolated from gastrointestinal tract of various fish species exert cellulolytic and amylolytic activities [35–38], and are able to reduce crude fiber, cellulose, hemicellulose and ANFs contents of plant proteins [39–41]. In the present study, *Bacillus pumillus* SE5 (BP) and *Pseudozyma aphidis* ZR1 (PA) isolated from gut microbiota of grouper (*Epinephelus coioides*) and bullfrog *Rana* (*Lithobates) catesbeiana*, respectively, were used for fermentation of SM. Then, the effects of replacing 40 or 80% of FM with SM, BP fermented SM (BPFSM) and PA fermented SM (PAFSM) were investigated on growth, feed utilization, blood biochemistry, innate immunity, digestive enzymes activity, gut morphology and immune-related genes expression in Japanese seabass (*Lateolabrax japonicus*).

2. Materials and methods

2.1. Fermentation of SM

A commercial defatted SM (crude protein: 50.6%, crude lipid: 1.3%) was obtained from Quanzhou Fuhai cereals and oils industry Co., Ltd. (China) and ground into powder with a mesh size of 250-μm prior to fermentation. The bacterial species used for fermentation in this study was isolated from gut microbiota of juvenile grouper and identified as *B. pumilus* SE5 based on the results of biochemical tests and 16S rRNA gene sequencing (GenBank Accession Number EU520331) [42]. Fifty microlitre of storage solution was inoculated in 20 ml nutrient broth. After 24 h of incubation, 5 ml was inoculated into 500 mL fresh nutrient broth and incubated for another 48 h at 28 °C. After incubation, the cells were harvested and re-suspended in phosphate buffered saline (PBS). The yeast species was isolated from gut microbiota of bullfrog and identified as *P. aphidis* ZR1 based on 18S rRNA gene sequencing (GenBank Accession Number KP269028.1). It was incubated at 30 °C for 30 h (pH 6.5) in the following medium: calcium phytic acid 0.1, glucose 3.0, NH_4NO_3 0.5, KCl 0.05, $MgSO_4{\cdot}7HO_2$ 0.05, $MnSO_4{\cdot}7HO_2$

0.003, $FeSO_4{\cdot}7HO_2$ 0.003, agar 1.8. A microbial concentration of 1×10^5 cell g^{-1} meal was used for solid-state fermentation (50% moisture) of SM at 30 °C for 48 h. Fermentation was performed in 5 L glass jars covered with aluminum foil, and the mixture was stirred every 12 h to release heat. The resultant product was dried at 60 °C to constant weight in a dry oven, ground to fine powder and kept in freezer until used. Glycinin and β-conglycinin contents of SM and FSM were analyzed using commercial assay kits (Beijing Longkefangzhou Bio-Engineering Technology Co., Ltd., China).

2.2. Experimental diets

Formulation and proximate composition of the experimental diets are shown in Table 1. The test diets were formulated to contain 42% crude protein and 10% lipid to support optimal growth of Japanese seabass [43]. A basal diet was formulated using brown FM and used as a FM-based diet, then six other diets were prepared by replacing 40 or 80% of FM with SM, BPFSM or PAFSM referred to as FM, SM40, SM80, BPFSM40, BPFSM80, PAFSM40 and PAFSM80 diets, respectively. A mixture of menhaden oil and soybean oil (Xiamen Jiakang Feed Co. LTD., Xiamen, China) were used as the lipid sources in the experimental diets. Squid visceral paste was used as a palatability enhancer in all diets. The coarse dry ingredients were finely ground using a hammer mill and passed through a 250-μm mesh. All the dry ingredients were thoroughly mixed, and a mash was produced after adding fish oil, soybean oil, lecithin and deionized water. Then, the pellets were produced by passing the mash through a 1.5- and 2.5- mm die using multifunctional spiral extrusion machinery (CD4XITS, South China University of Technology, Guangzhou, China). The pellets were dried overnight in a ventilated dry oven at 35 °C, sealed in airtight polyethylene bags and stored at −20 °C until used.

2.3. Feeding trial

Japanese seabass juveniles were obtained from Zhangpu Huifeng farm (Xiamen, China), transported to the Fisheries laboratory of Jimei University and stocked into two circular tanks of 1000-L capacity supplied with filtered seawater in a recirculating system. After two weeks of acclimation period, 20 randomly captured fish (7.14 ± 0.05 g) were stocked into each fiberglass circular tanks of 150 L capacity and supplied with filtered seawater at a flow rate of 3 L min^{-1} and aeration to maintain enough dissolved oxygen. Triplicate groups of fish were fed the seven test diets to apparent satiation twice daily (08:30 and 17:30) for 8 weeks. Uneaten food, if any, was siphoned out 30 min after feeding and weighed for determination of feed intake. The recirculating system consisted of a reservoir with a biological filter, a circulation pump and an automatic temperature control device. Feeding was stopped 24 h prior to weighing or blood sampling to minimize handling stress on fish. The water temperature during the feeding trial was 29 ± 2 °C, and a natural photoperiod (12:12 light:-dark) was implemented.

2.4. Sample collection

At the end of the feeding trial, all the fish in each tank were bulk-weighed and counted for calculation of growth parameters and survival. Nine fish per tank (27 fish per dietary treatment) were randomly captured, anesthetized (eugenol 1: 10 000), and blood samples were collected from the caudal vein with sterilized 1-mL syringes without anticoagulant. Blood from three fish per tank was pooled in 1.5-mL eppendorfs and kept at 4 °C overnight. Then serum was separated following centrifugation at 4000 × *g* at 4 °C for 10 min and kept at −80 °C for subsequent analysis of serum biochemical, antioxidant and innate immune parameters, and D-lactate concentration. Following blood sampling, the complete intestine was dissected from three fish per tank and fixed in Bouin's solution for subsequent histological observations. Two other sets of intestine samples were collected under sterile conditions from three fish per tank, flash frozen in liquid nitrogen, and subsequently stored at −80 °C for analysis of digestive enzymes activity and expression of pro-inflammatory (TNF-α, IL-1β), anti-inflammatory (TGF-β) and HSP70 genes. Also, spleen samples were collected from three fish per tank for analysis of immune-related genes (lysozyme and complement C3) expression. All the analyses were performed in three replicates. Three intact fish per tank were used for analysis of whole-body composition.

2.5. Analytical methods

2.5.1. Chemical composition

Analyses of moisture, crude protein, crude lipid and ash contents of the experimental diets and whole-body samples were performed based on the standard procedures [44]. Moisture was determined by drying the samples in an oven at 105 °C to constant weight; crude protein was analyzed by the Kjeldahl method (N × 6.25) with a FOSS Kjeltec 8400 analyser (Tecator, Höganäs, Sweden) after acid digestion in an auto-digester (FOSS; Tecator); crude lipid was determined by Soxhlet extraction in ether; ash content was measured by the combustion method in a muffle furnace at 550 °C for 8 h. Yttrium oxide in the diets and feces samples were determined by inductively coupled plasma-atomic emission spectrophotometer (ICP-OES, Prodigy7, Leeman Labs, USA). The apparent digestibility coefficients (ADCs) of dry matter and protein for the test diets were calculated using the following formula [45]:

ADC of dry matter (%) = $(1 - Y_2O_3 \text{ in diet}/Y_2O_3 \text{ in feces}) \times 100\%$

ADC of nutrient (%) = $[1 - (Y_2O_3 \text{ in diet}/Y_2O_3 \text{ in feces}) \times (\text{nutrient in feces}/\text{nutrient in diet})] \times 100\%$

2.5.2. Serum biochemical, immune and antioxidant capacity parameters

Serum biochemical (total protein, total cholesterol, glucose and triglyceride), antioxidant capacity (total antioxidant capacity [T-AOC], superoxide dismutase [SOD] and catalase [CAT] activities, and malondialdehyde [MDA] concentration) and innate immunity parameters (myeloperoxidase and complement C3 activities) were analyzed spectrophotometrically using commercial diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China). T-AOC was the amount of activity in 1 ml min^{-1} serum for the absorbance value to increase 0.01 units at 37 °C. CAT activity was measured following hydrogen peroxide reduction at 405 nm, and one activity unit was defined as 1 μmol hydrogen peroxide degraded per min. SOD activity was measured by the ability of the sample to inhibit superoxide anions generated by xanthine and the xanthine oxidase reaction system, and one activity unit was defined as the amount of enzyme necessary to inhibit 50% of the colour formation measured at 550 nm. Lipid peroxidation level was determined based on the MDA level generated by oxidizing fatty acids. MDA was measured by the method of thiobarbituric acid (TBA) according to Ohkawa et al. [46] and expressed as nmol ml^{-1}. Serum lysozyme activity was measured according to methods described by Ellis [47]. Briefly, 50 ml of individual serum was mixed with 950 ml *Micrococcus lysodeikticus* suspension at 200 mg ml^{-1} in 0.05 M sodium phosphate buffer (pH6.2). The mixture was incubated at 25 °C, and its OD was measured after 0.5 and 6 min at 530 nm using a spectrophotometer (UV−2802S, Shimadzu, Kyoto, Japan). One unit of lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} ml^{-1} serum. MPO activity was measured based on the change in absorbance at 420 nm resulting from reaction of hydrogen peroxide catalysed by MPO. Analysis of C3 level included measurement of the increase in turbidity after immunity response of C3 and its increased antibody [48,49]. Results of C3 were presented as C3 mg ml^{-1}.

2.5.3. Digestive enzymes activity

Amylase and lipase activities were analyzed using commercial assay kits (Nanjing Jiancheng Institute, Nanjing, China). The protein content of the homogenates was measured using Folin-phenol reagent [50]. Protease activity was measured using casein as substrate as described by Ref. [51]. A typical assay was performed as follows: 2 ml of 0.5% casein, 0.1 ml of 0.04 mol l^{-1} EDTA-Na2, 0.4 ml of appropriate buffer, 0.2 ml of enzymatic extract and 0.8 ml distilled water were mixed and incubated for 15 min in specific conditions of pH, temperature and NaCl concentration. The reaction was terminated by adding 1 ml of 30% chilled trichloroacetic acid (TCA). The mixture was centrifuged at 2000 × *g* for 15–20 min. One milliliter of clear supernatant, 5 ml of 0.55 mol l^{-1} Na_2CO_3 and 1 ml of Folin reagent were mixed and stayed for 15 min, then OD was measured in a spectrophotometer at 680 nm against blanks in distilled water. Controls were made in which the enzymatic extracts were added at the end of the incubation period and just before the centrifugation. Enzymatic extracts were diluted if required. One unit of the activity was defined as the amount of the hydrolysis of casein that liberated 1 μg of tyrosine per min.

2.5.4. Gut morphology

The fixed foregut samples were dehydrated in a graded series of ethyl alcohol and embedded in paraffin. Three sections (7 μm thick) were cut from each sample and then stained with hematoxylin/eosin. The villus height and thickness of each slice were measured using the image analysis software Image-Pro Plus 6.0 (Media Cybernetics, Inc.). Histological alterations in intestinal epithelia were evaluated based on the degree of changes in villi and the absorptive epithelial cell area was examined using a light microscope Olympus BX53 (Olympus, Japan).

2.5.5. Serum D-lactate concentration

Serum D-lactate concentration was determined using Beckman CX4 Chemistry Analyser (Beckman Coulter, Brea, CA) with a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The quantification of D-lactate requires two enzyme reactions. In the first reaction catalysed by D-lactate dehydrogenase, D-lactate is oxidised to pyruvate and nicotinic acid dehydrogenase (NADH) in the presence of nicotinamide-adenine dinucleotide (NAD^+). However, since the equilibrium of reaction lies firmly in the favour of D-lactate and NAD^+, a further reaction is required to trap the pyruvate product. This is achieved by the conversion of pyruvate to D-alanine and 2-oxoglutarate, with the enzyme D-glutamate-pyruvate transaminase in the presence of a large excess of D-glutamate. The amount of NADH formed in the above coupled reaction is stoichiometric with the amount of D-lactate. It is the NADH which is measured by the increase in absorbance at 340 nm.

2.5.6. RNA extraction and real-time quantitative PCR (qPCR)

RNA was isolated from foregut and spleen samples (approximately 80 mg) using TRIzol Reagent (Invitrogen, USA). The purity and concentration of RNA were measured using a ND-2000 spectrophotometer (NanoDrop 2000, Wilmington, DE, USA). RNA integrity was confirmed by running 1 μg RNA on an ethidium-bromide stained 1.5% agarose gel with 1 × Tris Acetate EDTA (TAE) buffer. Gels were then observed under ultraviolet light and photographed in a GS-800 Ultraviolet Transilluminator (UVP, Upland, CA, USA).

For each sample, 3-μg (0.15 μg μl^{-1}) RNA was reverse-transcribed into cDNA using a Revert Aid First-Strand Synthesis System (Thermo Scientific, Waltham, MA, USA) for quantitative reverse transcription PCR (RT-qPCR) with Oligo (dT) 18 primers according to the manufacturer's protocol. The reaction was incubated using a Peltier Thermal Cycler 200 (MJ Research, Watertown, MA, USA). cDNA integrity was confirmed by running 1-μg cDNA on an ethidium-bromide stained 1.5% agarose gel with 1 × TAE buffer. Gels were treated as reported before.

The PCR was performed in a total volume of 20 μL, containing 1 μL of each primer (10 μM), 9 μL of the diluted single strand cDNA product and 10 μL of AceQ® qPCR SYBR® Master Mix (Nanjing, China). The primers' sequence is presented in Table 2. The RT-qPCR program was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 s and an extension at °C for 60 s. At the end of each PCR reaction melting curve analysis was performed to confirm that only one PCR product was present in these reactions. Expression levels of the TNF-α, IL-1β, TGF-β, HSP70, lysozyme and complement C3 genes were normalized to β-actin using the $2-\Delta\Delta CT$ method [52]. Each sample was analyzed via RT-qPCR in triplicate. TNF-α, IL-1β, TGF-β, HSP70, lysozyme, complement C3 and β-actin genes were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) and primers were designed using primer Premier 5.0.

2.6. Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with Tukey's HSD multiple range test. Statistical significance was determined at $P < 0.05$. Data are presented as mean ± SE. Percentage data were arcsine transformed before statistical analysis.

3. Results

3.1. Growth, digestibility and body composition

The results of growth performance showed that 40% of FM could be substituted by SM and the FSM sources ($P > 0.05$), but significantly ($P < 0.05$) lower growth performance was achieved when substitution level increased to 80% irrespective of SM source (Table 3). Although, the groups fed BPFSM and PAFSM containing diets exhibited slightly higher growth rate than their respective SM groups, the observed differences were not statistically significant. The groups offered SM80 and PAFSM80 diets showed significantly higher feed conversion ratio (FCR) and lower apparent digestibility coefficient (ADC) of protein than the FM group. Moreover, significantly lower ADC of dry matter was found in the SM80 group. Feed intake (FI) and survival rate did not significantly differ among dietary treatments. Fish fed the SM80 diet had significantly lower whole-body lipid content than the FM group and this was inversely correlated with whole-body moisture content (Table 4).

3.2. ANFs content

Fermentation of SM with the selected microbial species resulted in enhancement of protein content of SM from 50.6% to 58.5% (BPFSM) and 58.4% (PAFSM) (Table 5). Also, glycinine content of SM decreased from 63.2 mg g^{-1} to 35.6 and 38.0 mg g^{-1} in BPFSM and PAFSM, respectively. Likewise, BPFSM (31.4 mg g^{-1}) and PAFSM (34.1 mg g^{-1}) had lower β-conglycinin contents than the untreated SM (73.9 mg g^{-1}). These results indicated that the two tested microbial species could be considered as potential candidates for improving nutritional value of SM via solid-state fermentation.

3.3. Blood biochemistry, antioxidant capacity and innate immunity

Replacing FM with either of the SM sources led to drastic reduction of serum total cholesterol concentration excluding the SM40 group (Table 6). However, serum total protein, glucose and triglyceride concentrations remained unchanged. Serum T-AOC significantly decreased in SM80 fed fish while the FSM fed groups showed comparable values to the FM group (Table 7). BPFSM40 fed fish showed a comparable catalase CAT activity to the FM group while significantly lower activity was found in the other groups. Serum MDA concentration was elevated by increasing FM replacement level and the SM80 fed fish exhibited the highest value. Significantly lower serum lysozyme activity was found in SM80 group whereas no significant difference could be found among FM group and those fed FSM. Likewise, significantly lower complement C3 activity was detected in SM80 group and the highest activity was

Table 2
Sequence of the primers used for real-time PCR.

Target genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temperature (°C)
β-actin	AACTGGGATGACATGGAGAAG	TTGGCTTTGGGGTTCAGG	60
TNF-α	GATCGTCATCCCACAAACCG	GCTTTGCTGCCTATGGAGTC	60
IL-1β	GTCAACTTACGTGCACCCTG	AAATCGTACCATGTCGCTGC	60
TGF-β	TGCACCTACATCTGGAACGC	GTGTTGCCTGCCCACATAGT	60
HSP70	ACTACGACCGTCTCCACAGA	CCTGGTCGTTGGCGATGATT	60
Lysozyme	AGCCAGGAAATTTGTTGCGG	TAATGCCTGGGGACTGATGC	60
C3	GGCGTCTACGTCCTCAACAA	AGCCCAGCATCGTAGAACAC	60

found in the BPFSM40 group. SOD and myeloperoxidase (MPO) activities were not significantly influenced by dietary treatments. SM80 group showed significantly lower expression of lysozyme gene while comparable values to that of the FM group were found for the other groups (Fig. 1a). No significant difference in expression of C3 gene was found among FM, BPFSM40 and PAFSM40 groups (Fig. 1b).

3.4. Digestive enzymes activity

Replacement of FM with either of the SM sources at both levels resulted in significant reduction of protease activity in foregut (Table 8). The groups fed PAFSM diets exhibited similar foregut lipase activity to the FM group while SM and BPFSM fed groups had significantly lower lipase activity. The SM40 group showed a comparable foregut amylase activity to the FM group while significantly lower activity was found in the remaining groups. In regards to the activity of digestive enzymes in midgut, only lipase activity was influenced by dietary treatments where FM replacement led to drastically reduced activity.

3.5. Gut morphology and pro-inflammatory genes expression

Replacing 80% of FM led to significant reduction of villus height irrespective of SM source (Table 9). Meanwhile, the SM40 and SM80 groups exhibited significantly lower villus thickness than FM group. The groups offered SM80, BPFSM80 and PAFSM40 diets exhibited significantly higher expression of TNF-α gene than the FM group (Fig. 2a), and expression of IL-1β remarkably increased in SM80 and BPFSM80 groups (Fig. 2b). An opposite trend to those of pro-inflammatory genes was observed for the anti-inflammatory TGF-β gene where replacing FM with either of the SM sources led to its down-regulation, and BPFSM40 group showed higher expression level than the other replacement groups (Fig. 2c). Also, expression of HSP70 gene was up-regulated in the groups received SM and PAFSM containing diets (Fig. 2d). Serum D-lactate concentration was significantly enhanced by increasing the substitution level to 80% and no beneficial effects of fermentation could be found on gut mucosal barrier function (Fig. 3).

Table 4
Whole-body composition of Japanese seabass fed the experimental diets for 8 weeks (% wet weight).

	Moisture	Protein	Lipid	Ash
Initial	74.3	23.9	4.18	5.78
FM	71.0 ± 0.31^{b}	15.9 ± 0.15	8.80 ± 0.33^{a}	4.35 ± 0.05
SM40	71.9 ± 0.40^{ab}	15.5 ± 0.13	8.10 ± 0.51^{ab}	4.55 ± 0.21
SM80	72.6 ± 0.20^{a}	15.3 ± 0.18	7.27 ± 0.76^{b}	4.82 ± 0.24
BPFSM40	71.5 ± 0.39^{ab}	15.6 ± 0.26	8.40 ± 0.70^{ab}	4.55 ± 0.13
BPFSM80	71.8 ± 0.16^{ab}	15.4 ± 0.09	7.97 ± 0.13^{ab}	4.80 ± 0.20
PAFSM40	71.2 ± 0.46^{ab}	15.6 ± 0.12	8.65 ± 0.41^{ab}	4.64 ± 0.08
PAFSM80	71.6 ± 0.10^{ab}	15.1 ± 0.56	8.14 ± 0.55^{ab}	4.88 ± 0.29

Values are mean of triplicate groups and presented as mean ± SE. Values in the same column having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

Table 5
Protein and anti-nutritional factors content of soybean meal and fermented soybean meals.

	Protein (%)	Glycinine (mg g^{-1})	β-conglycinin (mg g^{-1})
SM	50.6	63.2	73.9
BPFSM	58.5	35.6	31.4
PAFSM	58.4	38.0	34.1

SM: soybean meal, BPFSM: *Bacillus pumillus* SE5 fermented SM, PAFSM: *Pseudozyma aphidis* ZR1 fermented SM.

Table 3
Growth performance, feed utilization and apparent digestibility coefficients (ADC) of Japanese seabass (7.14 ± 0.05 g) fed the experimental diets for 8 weeks.

	FBW[a] (g)	WG[b] (%)	FI[c] (g/fish)	FCR[d]	ADCd[e] (%)	ADCp[f] (%)	Survival (%)
FM	51.5 ± 2.06^{a}	623 ± 28.5^{a}	48.5 ± 1.92	1.09 ± 0.01^{c}	75.4 ± 0.98^{a}	96.2 ± 0.21^{a}	100 ± 0.00
SM40	44.9 ± 0.67^{abc}	527 ± 10.1^{abc}	49.4 ± 0.09	1.31 ± 0.04^{abc}	71.3 ± 0.15^{ab}	95.7 ± 0.20^{ab}	98.3 ± 1.67
SM80	41.6 ± 1.57^{c}	485 ± 19.5^{c}	52.2 ± 3.79	1.51 ± 0.06^{a}	70.7 ± 0.39^{b}	94.5 ± 0.17^{b}	95.0 ± 2.89
BPFSM40	48.9 ± 1.86^{ab}	584 ± 28.9^{ab}	50.1 ± 1.78	1.21 ± 0.08^{bc}	71.6 ± 1.01^{ab}	95.1 ± 0.57^{ab}	98.3 ± 1.67
BPFSM80	43.4 ± 0.81^{bc}	506 ± 10.3^{bc}	44.8 ± 0.64	1.24 ± 0.01^{bc}	72.0 ± 0.04^{ab}	95.0 ± 0.29^{ab}	100 ± 0.00
PAFSM40	47.9 ± 0.60^{abc}	571 ± 11.3^{abc}	45.9 ± 1.53	1.31 ± 0.02^{bc}	73.7 ± 0.30^{ab}	95.3 ± 0.21^{ab}	98.3 ± 1.67
PAFSM80	43.6 ± 1.30^{bc}	513 ± 20.8^{bc}	48.4 ± 1.19	1.33 ± 0.04^{ab}	72.8 ± 1.32^{ab}	94.4 ± 0.27^{b}	98.3 ± 1.67

Values are mean of triplicate groups and presented as mean ± SE. Values in the same column having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[a] Final body weight.

[b] Weight gain = [(final body weight – initial body weight)/initial body weight × 100].

[c] Feed intake (g/fish) = dry feed consumed (g)/fish.

[d] Feed conversion ratio = dry feed fed/wet weight gain.

[e] Apparent digestibility coefficient of dry matter.

[f] Apparent digestibility coefficient of protein.

Table 6

Serum biochemical parameters of Japanese seabass fed the experimental diets for 8 weeks.

	TP[a]	T-CHO[b]	Glu[c]	TG[d]
FM	6.06 ± 0.52	6.17 ± 0.10^{a}	83.3 ± 3.17	0.61 ± 0.05
SM40	5.89 ± 0.17	4.47 ± 0.18^{ab}	87.9 ± 5.88	0.59 ± 0.02
SM80	5.27 ± 0.21	4.21 ± 0.35^{b}	81.4 ± 3.31	0.53 ± 0.02
BPFSM40	6.54 ± 0.73	4.14 ± 0.22^{b}	76.4 ± 6.01	0.66 ± 0.07
BPFSM80	6.62 ± 0.75	4.26 ± 0.20^{b}	73.7 ± 5.04	0.66 ± 0.08
PAFSM40	6.47 ± 0.72	4.08 ± 0.53^{b}	78.1 ± 8.69	0.65 ± 0.07
PAFSM80	5.56 ± 0.60	3.29 ± 0.58^{b}	68.2 ± 6.89	0.56 ± 0.06

Values are mean of triplicate groups and presented as mean ± SE. Values in the same column having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[a] Total protein (g dL^{-1}).

[b] Total cholesterol (mmol L^{-1}).

[c] Glucose (mg dL^{-1}).

[d] Triglyceride (mmol L^{-1}).

4. Discussion

4.1. Growth, digestibility and body composition

It has been well established that untreated SM can only replace comparatively low portion of FM in diets for carnivorous fish due to its damaging impacts on gut health [53,54]. Our previous research showed that although replacing 50% of FM by SM did not impair growth performance of Japanese seabass, it had detrimental impacts on feed utilization, digestive enzymes activity and gut health, and increasing the replacement level to 75% aggravated the damages [3]. Accordingly, in the present study 40 or 80% of FM was substituted with untreated SM or the two FSM sources in order to examine the efficacy of fermentation with the two microbial species on fish performance and severity of damages caused by high-SM diets. Likewise, in this study no significant alteration in growth performance was observed when 40% of FM was substituted by either untreated SM or FSM, but increasing the replacement level to 80% led to significant reduction of growth regardless of SM source. The groups received BPFSM and PAFSM containing diets exhibited numerically higher weight gain than their respective SM groups. Zhang et al. [55] compared the efficiency of a SM fermented with a blend of *Lactobacillus* spp., *Saccharomyces* spp. and *Bacillus* spp. with untreated SM at three different levels of 25, 50 and 75% in diets for Japanese seabass. In agreement to our findings, they could not find any significant difference in growth performance between SM and FSM groups at any of the replacement levels. Moreover, they found that replacing up to 50% of FM with SM or FSM does not influence growth performance while notably lower growth rates were recorded at 75% substitution. The results of a recent study by Liang et al. [56] on the same species showed the significant reduction of growth by replacing FM with a commercially available FSM at all the replacement levels ranging from 25 to 75%. Overall, literature review shows that the optimum replacement level of FM with untreated SM or FSM is highly variable and ranges from 25 to 60% [3,55–58]. This controversy may stem from the divergence in age, feeding strategy, rearing conditions, dietary composition, differences in ANFs content of SM and the discrepancy in efficiency of microbial species used for SM fermentation [3,59]. In the current study, the groups offered SM80 and PAFSM80 diets exhibited remarkably higher FCR than FM group. Likewise, Zhang et al. [55] reported the significant increase of FCR in Japanese seabass when 75% of FM was substituted with untreated SM or FSM. Also, Liang et al. [56] reported the significant enhancement of FCR in the same species when FSM replaced ≥50% of FM. The observed trend for FCR in this study could be associated with the reduced ADC of protein in both SM80 and PAFSM80 groups and lower ADC of dry matter in SM80 group.

The reduction of whole-body lipid content in the SM80 group in this study is consistent with previous findings in Japanese seabass [3,55,58], Asian seabass (*Lates calcarifer*) [60], cuneate drum (*Nibea miichthioides*) [61], spotted rose snapper (*Lutjanus guttatus*) [62], turbot (*Scophthalmus maximus* L.) [18] and marbled spinefoot (*Siganus rivulatus*) [63]. Reduced diet digestibility and digestive enzymes activity could be at least partially responsible for the reduction of whole-body lipid content in the SM80 group [3,55]. In regards to the application of FSM, similar to our results Wang et al. [18] reported no significant changes in whole-body composition of turbot when 15–60% of FM was substituted with SM fermented with *Lactobacillus plantarum* P8. This observation may indicate better digestibility and uptake of nutrients from the two FSM sources compared to untreated SM [18].

4.2. Blood biochemistry, antioxidant capacity and innate immunity

Hematological indices are used as key diagnostic tools for assessment of fish physiological and health conditions and are affected by season, temperature and nutritional changes [64–70]. The hypocholesterolemic effect of SM has been well established [71]. Likewise, in the current study replacement of FM with SM or FSM, with the exception of the SM40 group, resulted in significant reduction of serum total cholesterol level. This is consistent with the results of a rainbow trout study in which replacing FM with SM or FSM led to significant reduction of plasma total cholesterol concentration [72]. Similarly, Dossou et al. [12] reported the significant reduction of plasma total

Table 7

Serum antioxidant and innate immune parameters of Japanese seabass fed the experimental diets for 8 weeks.

	T-AOC[a]	SOD[b]	CAT[c]	MDA[d]	Lysozyme[e]	MPO[f]	C3[g]
FM	35.7 ± 2.04^{a}	130 ± 12.3	26.9 ± 6.54^{a}	62.6 ± 4.02^{de}	339 ± 6.99^{a}	13.9 ± 1.46	0.22 ± 0.01^{ab}
SM40	29.8 ± 6.93^{ab}	112 ± 10.5	15.5 ± 4.31^{bc}	109 ± 7.16^{bc}	252 ± 11.2^{ab}	8.55 ± 2.49	0.20 ± 0.01^{abc}
SM80	24.1 ± 3.16^{b}	102 ± 17.3	6.76 ± 1.17^{c}	153 ± 9.69^{a}	210 ± 31.3^{b}	10.6 ± 1.96	0.15 ± 0.02^{c}
BPFSM40	30.9 ± 6.11^{ab}	116 ± 7.29	18.4 ± 1.85^{ab}	76.4 ± 3.23^{cd}	268 ± 26.7^{ab}	11.1 ± 2.12	0.24 ± 0.02^{a}
BPFSM80	28.7 ± 2.44^{ab}	115 ± 6.97	14.4 ± 3.41^{bc}	136 ± 9.83^{ab}	258 ± 11.2^{ab}	9.92 ± 1.67	0.16 ± 0.01^{bc}
PAFSM40	25.5 ± 0.90^{ab}	105 ± 6.97	14.2 ± 4.38^{bc}	26.6 ± 4.69^{e}	321 ± 30.5^{a}	11.6 ± 3.54	0.22 ± 0.01^{abc}
PAFSM80	26.8 ± 3.83^{ab}	117 ± 4.71	14.3 ± 2.96^{bc}	77.7 ± 11.0^{cd}	240 ± 19.3^{ab}	8.34 ± 1.29	0.22 ± 0.01^{ab}

Values are mean of triplicate groups and presented as mean ± SE. Values in the same column having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[a] Total antioxidant capacity (U ml^{-1}).

[b] Superoxide dismutase (U ml^{-1}).

[c] Catalase activity (U ml^{-1}).

[d] Malondialdehyde concentration (nmol ml^{-1}).

[e] Lysozyme activity (U ml^{-1}).

[f] Myeloperoxidase (U ml^{-1}).

[g] Complement C3 (mg ml^{-1}).

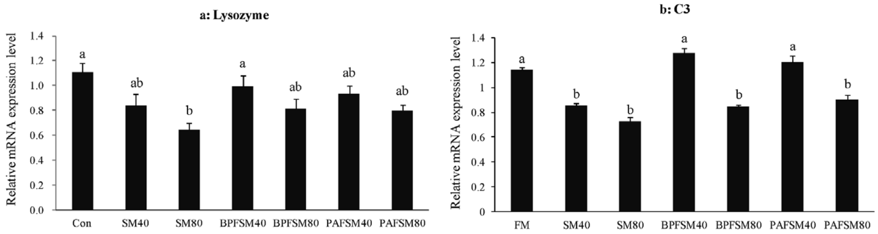

Fig. 1. Relative expression of lysozyme (a) and C3 (b) genes in spleen of Japanese seabass fed the experimental diets for 8 weeks.

cholesterol level in red sea bream (*Pagrus major*) when 75–100% of FM was substituted with *Aspergillus oryzae* fermented rapeseed meal. The cholesterol lowering impact of soy products has been ascribed to enhanced faecal excretion of bile acids, facilitated apolipoprotein B/E receptor activity and reduced hepatic lipoprotein secretion which are believed to result from imbalanced amino acid profile or occurrence of certain non-starch polysaccharides [73,74].

The antioxidant system correlates with health status and immune system in fish [75]. A wide range of antioxidant enzymes are engaged in reduction of oxidative stress and protection of cells against free radicals [76–80]. T-AOC mirrors the total capacity of antioxidant systems in the body. SOD and catalase are important anti-oxidant enzymes that play key role in protection against damaging free radicals in organism and serve as valuable indices of oxidative stress and immune function in fish [81]. MDA is a final product of lipid peroxidation that acts as a valuable biomarker of endogenous oxidative damage. There are several reports indicating that plant feedstuff trigger oxidative stress in animals due to their ANFs content [82,83]. Wang et al. [18] found a significant reduction in T-AOC of turbot fed the diet in which 45% of FM was replaced with SM while replacing the equal amount of FM with FSM was along with the enhancement of T-AOC. Likewise, in this study the group received SM80 diet exhibited the lowest T-AOC whereas the BPFSM80 and PAFSM80 groups showed comparable values to that of the FM group. This result was consistent with the trend observed for serum MDA concentration where the highest value was found in SM80 group signifying an enhanced oxidative stress. Substitution of FM led to significant reduction of catalase activity in all groups, excluding the BPFSM40 group, and confirmed earlier findings in *Macrobrachium nipponense* [4] and red sea bream [12]. The results achieved for antioxidant capacity in this study provide further evidence for the notion that microbial fermentation of SM enhances its antioxidant activity which paralleled with studies on parrot fish [84], olive flounder [85], rockfish [86] and prawns [4]. It has been suggested by several authors that microbial fermentation of plant ingredients augments the bioavailability of potential antioxidant substances such as glucosinolates and phenolic compounds that enhance the anti-oxidative defense in fish [85,87,88].

It is well known that nutritional status is one of the major factors that influence the non-specific immune response in fish [89]. Literature review shows that administration of high plant protein diets suppresses immune function leading to lower disease resistance [90,91]. Suppression of immune function by high SM diets has been reported in several fish species such as red sea bream [92], turbot [18] and amberjack (*Seriola dumerili*) [93]. Likewise, in the present study replacing 80% of FM with SM led to dramatic decrease of lysozyme and complement C3 activities. Reduction of lysozyme activity by offering high SM diets has also been shown in tilapia [59], red sea bream [92] and turbot [18]. Also, Hossain et al. [93] reported the significant reduction of peroxidase and bactericidal activities in amberjack when 50–75% of FM was replaced by SM. However, our results showed no significant changes in MPO activity. It is believed that nutritional imbalances particularly deficiency of some essential amino acids resulting from high substitution levels of FM with plant proteins along with the occurrence of ANFs and antigens are the potential deleterious factors that damage the immune system in fish and crustaceans [53,91,94,95]. On the other hand, our results revealed that using FSM rather than SM beneficially impacts immune function as the groups received BPFSM and PAFSM exhibited comparable lysozyme activity to the FM group and even numerically higher complement C3 activity was detected in fish fed the BPFSM40 diet. This is consistent with the results of the study on turbot where the groups of fish received FSM exhibited significantly higher lysozyme activity than those fed SM containing diets [18]. Also, Lin and Mui [96] showed that fermentation of SM with *Lactobacillus* spp. prevents the suppression of immune function triggered by SM in white shrimp. Zhuo et al. [97] claimed that microbial fermentation results in degradation of SM protein into small bioactive

Table 9
Foregut morphology of Japanese seabass fed the experimental diets for 8 weeks.

	Villus height (μm)	Villus thickness (μm)
FM	274 ± 6.04^{b}	31.1 ± 2.05^{a}
SM40	247 ± 5.68^{b}	22.6 ± 0.77^{b}
SM80	196 ± 7.68^{c}	23.8 ± 2.46^{b}
BPFSM40	318 ± 18.3^{a}	32.1 ± 0.95^{a}
BPFSM80	188 ± 9.47^{c}	29.5 ± 1.36^{ab}
PAFSM40	260 ± 5.25^{b}	32.6 ± 0.63^{a}
PAFSM80	189 ± 5.27^{c}	31.6 ± 0.45^{a}

Values are mean of triplicate groups and presented as mean ± SE. Values in the same column having different superscript letters are significantly different ($P < 0.05$). Values are mean of triplicate groups and presented as mean ± SE.

Table 8
Digestive enzymes activity (U mg^{-1} prot) in foregut and midgut of Japanese seabass fed the experimental diets for 8 weeks.

Diets	Foregut			Midgut		
	Protease	Lipase	Amylase	Protease	Lipase	Amylase
FM	50.5 ± 1.34^{a}	212 ± 1.60^{a}	0.28 ± 0.02^{a}	29.3 ± 0.86	130 ± 6.65^{a}	0.23 ± 0.00
SM40	26.2 ± 3.00^{b}	152 ± 4.93^{b}	0.23 ± 0.02^{ab}	21.3 ± 0.97	105 ± 1.88^{b}	0.18 ± 0.00
SM80	19.9 ± 2.43^{b}	158 ± 4.19^{b}	0.18 ± 0.00^{c}	20.1 ± 1.22	104 ± 5.71^{b}	0.19 ± 0.02
BPFSM40	20.2 ± 3.49^{b}	153 ± 10.2^{b}	0.22 ± 0.01^{bc}	20.2 ± 1.64	109 ± 2.72^{b}	0.21 ± 0.01
BPFSM80	24.3 ± 2.43^{b}	149 ± 3.63^{b}	0.21 ± 0.02^{bc}	18.0 ± 4.66	110 ± 13.4^{b}	0.20 ± 0.02
PAFSM40	19.6 ± 1.72^{b}	214 ± 9.14^{a}	0.22 ± 0.01^{bc}	18.1 ± 3.82	107 ± 0.43^{b}	0.20 ± 0.01
PAFSM80	22.7 ± 3.51^{b}	200 ± 16.9^{a}	0.23 ± 0.00^{b}	23.2 ± 4.26	104 ± 1.98^{b}	0.19 ± 0.00

Values are mean of triplicate groups and presented as mean ± SE. Values in the same column having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

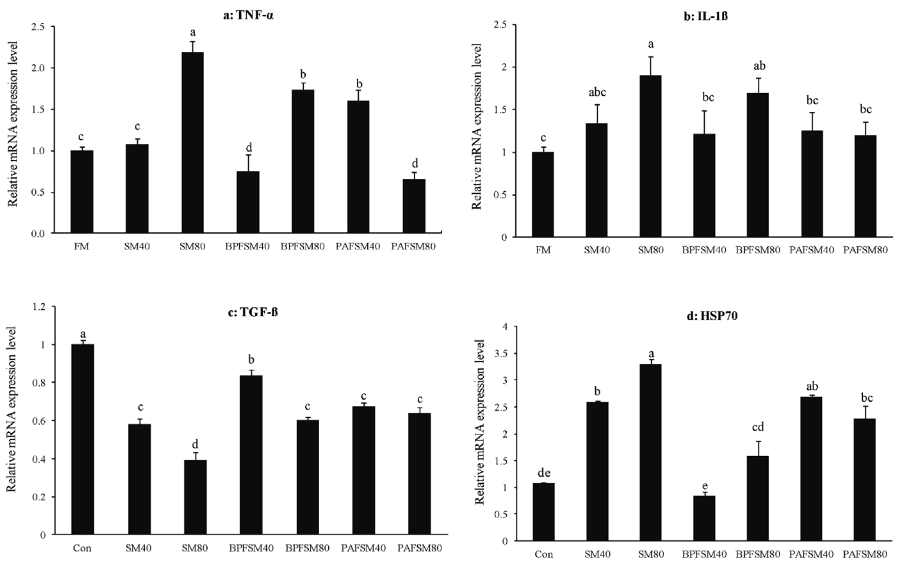

Fig. 2. Relative expression of TNF-α (a), IL-1β (b), TGF-β (c) and HSP70 (d) in foregut of Japanese seabass fed the experimental diets for 8 weeks.

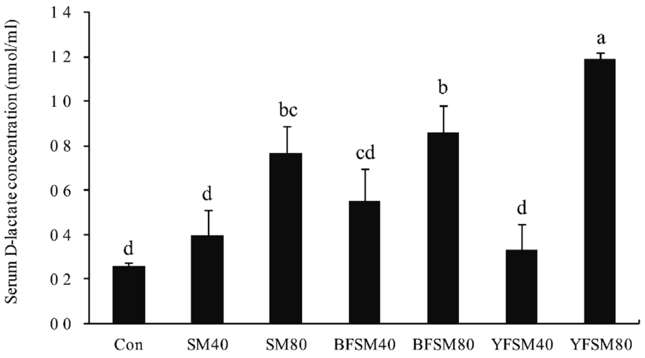

Fig. 3. Serum D-lactate concentration of Japanese seabass fed the experimental diets for 8 weeks.

peptides with numerous health benefits including anti-oxidative and immunomodulatory activities. In addition, presence of the bacterial and yeast species in FSM which may act as probiotics could be responsible for boosting immune response in FSM groups [98]. Results of immune-related genes expression including lysozyme and C3 genes further confirmed that fermentation of SM partially inhibits suppression of immune response.

4.3. Digestive enzymes activity

In the current study, replacing FM with SM or FSM led to reduced protease activity in foregut, and lipase activity decreased in SM and BPFSM fed groups. Also, a drastic decrease in amylase activity was observed in all replacement groups excluding the SM40 group. Similarly, the results of our previous research on the same species showed the significant reduction of protease, lipase and amylase activity in foregut when 50–75% of FM was substituted with SM [3]. Furthermore, in both studies it was only the lipase activity that was influenced by high SM diets in midgut which may result in lower uptake of dietary lipid leading to lower body lipid content. Reduction of digestive enzymes activity by SM has also been reported in several other fish species such as Atlantic salmon (*Salmo salar* L.) [99], hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) [59] and red seabream [100]. In regards to the application of fermented plant proteins, in agreement to our findings Dossou et al. [12] reported the significant reduction of protease activity in red seabream by replacing 75–100% of FM with *Aspergillus oryzae* fermented rapeseed meal. However, the results of a study by Das and Ghosh [101] showed that the groups of *Labeo rohita* fed *Bacillus subtilis* subsp fermented sesame oil cake (SOC) exhibited significantly higher digestive enzyme activities than those fed untreated SOC. Their results showed that fermentation of SOC leads to drastic decrease of ANFs such as phytate, trypsin inhibitor and tannins. It has been pointed out that digestive enzymes activity in fish is influenced by several factors such as feeding habits and preferences, dietary composition, and occurrence of ANFs [102,103]. There are several reports indicating that presence of various ANFs, particularly protease inhibitors, in SM interrupts digestive enzymes activity in fish [104–106]. Das and Ghosh [101] attributed the reduced protease activity of *L. rohita* at higher inclusion levels of untreated SOC to the presence of tannin and phytic acid. Meanwhile, a red seabream study showed the lower expression of digestive enzymes genes in SM fed fish compared to the group received FM [100]. The authors suggested that expression of these genes is induced by a water-soluble fraction of FM. In the current study, efficiency of the two microbial species in reducing the ANFs contents of SM such as phytate and trypsin inhibitor that may interrupt the digestive enzyme activity were not explored and remains to be elucidated in the future studies.

4.4. Gut histology and pro-inflammatory genes expression

It has been demonstrated that incorporating high levels of untreated SM in carnivorous fish feed has detrimental impacts on gut integrity [53,54] which stems from its ANFs content [107]. The results of our previous research indicated that replacing 50–75% of FM with standard SM leads to shortening of villus height, and decreased villus and muscular thickness in Japanese seabass, and the severity of damages increased by increasing SM inclusion level [3]. In the current study the highest villus height was observed in fish fed the BPFSM40 diet followed by FM and PAFSM40 groups, respectively. However, replacing 80% of FM resulted in remarkable reduction of villus height regardless of the of SM source. Also, SM40 and SM80 groups exhibited significantly lower villus thickness than the FM group whereas those fed BPFSM and PAFSM showed comparable values to the FM group. Similarly, Miao et al. [108] reported the significant reduction of microvillus height and fold height in distal intestine of northern snakehead (*Channa argus* Cantor, 1842) by replacing 50–75% of FM with SM. The results of a study on turbot showed that substituting 45–60% of FM with SM triggers the significant reduction of microvillus height, enterocyte height and fold height in distal intestine while replacing the equal amount of FM with FSM did not harm the gut structure [18]. The results of gut morphology in this study paralleled with the expression of gut pro-inflammatory genes as in most cases increasing FM replacement level was along with up-regulated expression of TNF-α and IL-1β and down-regulation of TGF-β expression. Meanwhile, SM groups showed relatively higher expression of TNF-α and HSP70 (which is a sign of increased stress response) than FSM groups. On the other hand, increasing FM substitution level led to increased serum D-lactate concentration which indicates the impairment of intestinal mucosal barrier function [109,110]. This is in agreement with our previous findings in the same species where serum D-lactate and diamine oxidase concentrations were enhanced by increasing FM replacement level with SM [3].

In conclusion, the findings in this study showed that using the FSM sources, particularly BPFSM, improves nutrients utilization, antioxidant activity and non-specific immune response, and alleviates the damaging impacts of SM on gut health. Complementary studies in the future are needed for determination of the optimum replacement level of FM with the two FSM sources.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.009.

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