Journal of the Persian Gulf
(Marine Science)/Vol. 5/No. 17/September 2014/7/65-76

Evaluating Growth Performance, Amino Acid Profile and Biochemical Changes in Juvenile Olive Flounder, *Paralichthys olivaceus* under Partial Feeding with Fermented Soybean Meal

Mohammadiazarm, Hamid[1*]; Sang-Min lee

1- Department of Fisheries, Faculty of Marine Natural Resources, Khorramshahr University of Marine Science and Technology, Khorramshahr, IR Iran
2- Department of Marine Bioscience and Technology, Gangneung-Wonju National University, Gangneung, South Korea

Received: December 2013 *Accepted: May 2014*

© 2014 Journal of the Persian Gulf. All rights reserved.

Abstract

This study was carried out to investigate the effect of replacing fish meal protein with fermented soybean meal (FSM) in diets on growth performance, feed utilization, morphological parameters, body composition, activities of antioxidant and digestive enzymes and also blood biochemistry of juvenile olive flounder (*Paralichthys olivaceus*). Five isonitrogenic and isolipidic diets were prepared to contain 0 (control), 80, 160, 240 and 320 g kg^{-1} FSM in diets. Triplicate groups (30 fish for each tank) of juvenile olive flounder averaging 11.9±0.19 g were fed each diet to visual satiation at two meals per day for 8 weeks. The fish fed diets containing up to 240 g kg^{-1} FSM had no significant differences in the survival, final growth, specific growth rate, feed efficiency and protein efficiency ratio compared with control group. Fish fed diet containing 320 g kg^{-1} FSM showed significantly lower survival, growth, specific growth rate, feed efficiency and protein efficiency ratio than in control group. Triglyceride and total protein contents of plasma in fish fed with the FSM diets as well as hepatosomatic indices were greater in fish fed with diets containing 240 and 320 g kg^{-1} FSM than in control. Proximate composition, amino acid profile of dorsal muscle, activity of digestive enzymes in intestine and activity of antioxidant enzymes in liver and plasma were not affected by different levels of FSM compared with control group. This study showed that FSM has potential to replace fish meal up to 240 g kg^{-1} diet for growth of juvenile olive flounder but better growth performance obtained with 80 g kg^{-1} diet.

Keywords: *Replacement, Fermented soybean meal, Paralichthys olivaceus, Growth*

1. Introduction

One of the important economic factors in success of aquaculture production is feed. Carnivorous species need a high fish meal, the inclusion level of which is above 50% in commercial diets (Lim and Lee., 2009). On the other hand, the cost of fish meal has increased during the last few years. Therefore, it is necessary to find alternative protein sources that

* *E-mail: azarmhamid@gmail.com*

can yield a profit on the price of fish diet (Tidwell et al., 2005).

Many researches try to find out alternative protein sources, both from plant and animal origin. Previous studies reported the reduction in growth and feed utilization by soybean meal protein through imbalanced dietary amino acid, reduced mineral content, increased fiber, reduced palatability and presence of anti-nutrients factors (Lim and Lee., 2009). Fibers and anti-nutrients are related to reduce digestibility in fish (Francis et al., 2001). In salmonids, reduced nutrient digestibility with soy non-starch carbohydrates or heat-stable anti-nutrients has been reported as important factors responsible for decreased growth performance (Harpez et al., 2006). Also, some researchers reported on the effect of soybean meal as an alternative protein source to fish meal in diet of flounder, but in relatively low quantity substitution of fish meal with soybean meal because of anti-nutritional factors (Kim et al., 2000; Ye et al., 2011).

It has been reported fermentation is a suitable technique for drying wet products with minimal nutrient loss (Lee et al., 2010; Yamamoto et al., 2004). Fermentation is a process which microorganisms such as *Bacillus subtilis* degrade protein macromolecules to a large extent water-soluble low molecular weight compounds (Shimeno et al., 1993; Kiers et al., 2000). Fermenting of plant protein in removal or inactivation of anti-nutrients (Reddy and Pierson., 1994) is beneficial and improves nutritional quality (Canella et al., 1984), increases digestibility (Kiers et al., 2000) and shelf life of the processed food (Skrede and Nes., 1988). For example, growth performance of Indian major carp, *Labeo rohita* was significantly improved by fermented sesame, black gram and duckweed leaf meal compared with non-fermented meal (Mukhopadhyay and Ray., 1999; Bairagi et al., 2002; Ramachandran and Ray., 2007). Similarly, fermented fish silage and soybean meal were found as a suitable protein sources in the diets of catfish, *Clarias gariepinus* and Nile tilapia *Oreochromis niloticus* (Fagbenro et al., 1994).

So far, limited information is available on the effects of FSM by *Bacillus subtilis* on the growth performance and feed utilization of olive flounder. Also, the relationship between dietary plant protein inclusion and fish physiological status remains neglected (Olsen et al., 2007). Therefore, this study was conducted to investigate the effects of partial substitution of dietary fish meal by FSM on growth performance and some biochemical parameters of juvenile olive flounder in an attempt to determine adequate proportion of FSM in diet for juvenile olive flounder.

2. Materials and Methods

2.1. Diet Preparation

The FSM used in this study was supplied by CJ CheilJedang Co. (Seoul, Korea). The FSM was fermented using *Bacillus subtilis* as described by Lim et al., 2010. Briefly, soybean meal was steam cooked in an autoclave at 100°C for 20 min (pH 5-6). After cooling, the steamed soybean meal was inoculated by evenly spraying spore suspension of *B. subtilis*. After a thorough mixing, the inoculated soybean meal substrate was incubated at 37°C for 24 h (pH 8.35). The products of fermentation were dried in a vacuum drying oven at below 60°C for 15 h (pH 7-8). Finally, FSM was ground to size of less than 400 μ.

Five isonitrogenus and isolipidic diets were formulated to contain 0, 80, 160, 240 and 320 g kg^{-1} FSM designated as a CON, FSM8, FSM16, FSM24 and FSM32. Ingredients and nutrient contents of the experimental diets and also amino acid profile of fish meal and FSM are presented in Tables 1 and 2. Pollack fish meal was used as the primary protein source. Cod liver oil and soybean oil were used as lipid sources. All ingredients were thoroughly mixed

with 300 g kg^{-1} distilled water, and pellets with size of 3 ml were prepared using a meat grinder. The pellets were dried at room temperature for 24 h and stored at -30°C until used.

Table 1. Ingredients and proximate composition of experimental diets

	Diets				
Ingredients (g $100g^{-1}$)	CON	FSM8	FSM16	FSM24	FSM32
Pollack fish meal	60.0	54.0	48.0	42.0	36.0
Fermented soybean meal	0	8.0	16.0	24.0	32.0
Wheat flour	23.8	21.2	18.6	16.0	13.4
Corn gluten meal	5.0	5.0	5.0	5.0	5.0
α-potato-starch	5.0	5.0	5.0	5.0	5.0
Cod liver oil	2.0	2.5	3.0	3.5	4.0
Soybean oil	0.4	0.3	0.2	0.1	0
Vitamin premix[1]	1.0	1.0	1.0	1.0	1.0
Mineral premix[2]	1.0	1.0	1.0	1.0	1.0
Stay-C (50%)	0.5	0.5	0.5	0.5	0.5
Choline salt (50%)	0.3	0.3	0.3	0.3	0.3
DL-Methione	0	0.1	0.2	0.3	0.4
L-Lysine	0	0.06	0.12	0.18	0.24
Taurine	1.0	1.05	1.1	1.15	1.2
Chromic oxide[3]	0.5	0.5	0.5	0.5	0.5
Nutrient content (dry matter basis)					
Crude protein (g $100g^{-1}$)	51.6	50.0	50.4	51.5	51.5
Crude lipid (g $100g^{-1}$)	7.3	7.2	7.2	7.2	7.2
Ash (g $100g^{-1}$)	14.0	12.8	12.6	12.0	11.0
EPA	0.72	0.74	0.75	0.77	0.79
DHA	0.99	0.99	0.98	0.98	0.97
Lys	8.3	8.1	8.6	7.5	7.8
Met + Cys	3.2	3.5	3.0	3.6	3.1

[1.] Vitamin premix contained the following amount which were diluted in cellulose (g/kg premix): DL-α-tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

[2.] Mineral premixcontained the following ingredients (g/kg premix); $MgSO_4.7\ H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 365.5; CuCl, 0.2; $AlCl_3.6H2O$, 0.15;KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[3.] Replaced 5 g/kg wheat flour when nutrient digestibility was determined.

Table 2. Essential amino acids in fish meal and fermented soybean meal

	Fish meal	Fermented soybean meal
Essential Amino acids(%) in protein		
Arginine	6.7	6.8
Histidine	2.3	2.7
Isoleucine	4.5	4.4
Leucine	8.3	7.9
Lysine	8.8	5.9
Methionine + Cysteine	5.1	3
Phenylalanine + Tyrptophan	8.1	8.2
Threonine	4.8	4.2
Valine	4.2	4.2

2.2. Fish Rearing

Juvenile flounder were obtained from a local farm (Namhae, Korea). The fish were acclimated to laboratory condition for 2 weeks before starting the feeding trial. Juvenile fish (initial mean weight, 11.9±0.19 g) were allocated randomly into 300 L cylindrical plastic tanks with 30 fish per tank for the feeding trial after being collectively weighed. Three replicate groups of fish were hand-fed to apparent satiation twice a day (9:00 and 17:00) for 8 weeks. Filtered sea water was supplied at a flow rate of 4 $Lmin^{-1}$ in each tank and the mean water temperature was 16.8±0.39°C. The photoperiod was left under natural conditions during the feeding trail. At the end of experiment, juvenile flounders in each tank were collectively weighed after anesthetizing with tricaine methanesulfonate (MS222, Sigma, St. Louis, MO, USA) at a concentration of 100 mg L^{-1} after starvation for 24 h.

2.3. Digestibility Measurements

At the end of feeding trial, flounder were fed with their respective experimental diets containing 5 g kg^{-1} chromic oxide as an indicator to visual satiation at two times per day. Two hours after feeding, the rearing tanks were brushed to remove uneaten and fecal residues, and faces in each tank were collected for two consecutive weeks. Fecal samples were prepared for analysis as described by Lee, 2002. Chromic oxide was determined by a wet-acid digestion method (Furukawa and Tsukahara., 1966). Apparent digestibility coefficients (ADC) for dry matter, nutrient and energy of the diets were determined using the following equations:

ADC of dry matter (%) = (100 - (dietary Cr_2O_3/fecal Cr_2O_3) * 100)

ADC of nutrients or energy (%) = (1- (dietary Cr_2O_3 /fecal Cr_2O_3)* (fecal nutrient or energy / dietary nutrient or energy)) *100

2.4. Fish Sampling and Chemical Analysis

Five fish at the end of the feeding trial from each tank were randomly sampled and stored at -80°C in freezer for proximate composition and amino acid profile (AOAC, 1995). At the end of the feeding experiment, blood was drawn from caudal vessel with 1 ml heparinized syringes from three fish in each tank and transferred to micro centrifuge tubes. Also, five fish from each tank were sampled and stored in liquid nitrogen for digestive and antioxidant enzymes analyses.

Proximate analysis of the diets and dorsal muscle of fish were determined according to the method of AOAC (1995). Crude protein content was determined using the Kjeldahl method using an Auto Kjeldahl System (Buchi, Flawil, Switzerland). Crude lipid was analyzed by ether extraction, moisture content by a dry oven drying (105°C for 24 h) and ash by a furnace muffler (550°C for 4 hours).

Amino acid composition in the experimental diets and dorsal muscle of fish was performed with acid hydrolysis with 6 N HCL (reflux for 23 h at 110°C) followed by analysis in an automatic amino acids analyzer (Hitachi, Tokyo, Japan).

The collected blood was centrifuged at 7500 g for 10 min, the plasma separated and stored in -80°C freezer. Plasma glucose, total protein, triglyceride and cholesterol concentration were determined using a clinical investigation commercial kit (Asan Pharmaceutical Co., Seoul, Korea).

2.5. Digestive Enzymes Activity

At the end of feeding trial (after one day starvation), samples (5 fish per tank) were washed in cold distilled water, stored in liquid nitrogen and dissected on a glass maintained on ice. Samples of intestine were homogenized immediately in 50 mM Tris-HCl containing 20 mM $CaCl^2$ and 50 mM KCl (pH 7.5) by a homogenizer (Wiggenhauser, Berlin,

Germany), followed by centrifugation (15000 g for 40 min at 4°C). We used 100 mg tissue mL^{-1} buffer for homogenization and then homogenates were kept frozen in -80°C to determine biochemical analysis. All the assay techniques were based on photometric procedures and disappearance of the substrate for product formation was measured.

The activity of α-amylase, lipase and trypsin was assayed and reported as specific activity of U mg^{-1} protein. The specific activity of α-amylase was performed by the enzymatic photometric method using amylase kit (Abcam, Cambridge, UK). The formation rate of p-nitrophenyl (pNP) is proportional to the α-amylase activity present in the sample which is measured by the increase of the wavelength absorbance at 405 nm (405-420 nm). One unit amylase is defined as the amount of amylase which hydrolyses Ethylidene-pNP-G7 to generate 1 µmol of p-nitrophenol per minute at 25°C. Briefly, in this method, 50 µl assay buffer and 50 µl substrate were mixed to 50 µl sample and after completing 15 min incubation at room temperature; the mixture read at the wavelength of 405 nm by Tecan Sunrise (Mannedorf, Zurich, Switzerland) Elisa reader. The Specific activity of lipase was measured by the enzymatic photometric method using lipase kit (Abcam, Cambridge, UK).

Lipase hydrolyses specific substrate to generate TNB which is reacted with 5, 5-dithiobis (2-nitro benzoic acid) DTNB prob to generate color at the wavelength of 405 nm. Therefore, one unit lipase is defined as the amount of lipase which hydrolyses the substrate and generated 1 µmol of TNB per minute at 37°C. Briefly, in this method, 100 µl reaction mixture containing assay buffer, DTNB probe and lipase substrate was added to 50 µl sample and OD1 read at the wavelength of 412 nm. After completing 3 min incubation in 37°C, OD2 read at the wavelength of 412 nm by Tecan Sunrise (Mannedorf, Zurich, Switzerland) Elisa reader. Specific activity of trypsin was measured by the enzymatic photometric method using tripsin kit (Abcam, Cambridge, UK). It is based on cleaves a substrate to generate p-nitroaniline (p-NA) which is detected at the wavelength of 405 nm. One unit is defined as an amount of trypsin cleaves the substrate, yielding 1 µmol of p-NA per minute at 25°C. In this method briefly, 50 µl reaction mixture containing assay buffer and trypsin substrate was added to 50 µl sample and OD1 read at the wavelength of 405 nm. After completing 5 min incubation in 37°C, OD2 read at the wavelength of 405 nm by Tecan Sunrise (Mannedorf, Zurich, Switzerland) Elisa reader. Also, protein content in the supernatants was measured using the Bradford (1976) method.

2.6. Antioxidant Enzymes Activity

To determine the activity of hepatic superoxide dismutase (SOD) and glutathione peroxides (GPx), 0.1 g of fish liver were homogenized in 9 volumes of 5 mM Tris and 35 mM glycine (pH 7.6). The homogenates were centrifuged at 10,000 g for 30 min to remove debris. The resultant supernatants were used for SOD and GPx assay. The inhibition rate of SOD in liver was assayed using kit (Sigma Aldrich Inc. Saint-Louis, Switzerland). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Therefore, the IC50 (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method. In this method 20 µl sample was added to 220 µl substrate solution and then inhibition activity determined by a kinetic method up to 20 min at the wavelength of 410 nm by Tecan Sunrise (Mannedorf, Zurich, Switzerland), Elisa reader. Also, the inhibition activity of SOD in plasma of juvenile fish was assayed in a similar approach with measurement inhibition activity of SOD in liver. Activity of GPx in fish liver was assayed using kit manufactured by (BioVision, Inc. California, USA). In this assay, cumene

hydroperoxide as a peroxide substrate (ROOH), glutathione reductase (GSSG-R) and NADPH (b−Nicotinamide Adenine Dinucleotide Phosphate, Reduced) were mixed by each other. Then the changing in the wavelength of 340 nm due to NADPH oxidation was monitored as an indicative of GPx activity. Briefly, 50 µl sample was added to 40 µl reaction mixture and incubated for 15 minutes. Then 10 µl Cumene Hydroperoxide was added and OD1 read at the wavelength of 340 nm. After completing 5 min incubation OD2 read in 340 nm by Tecan Sunrise (Mannedorf, Zurich, Switzerland) Elisa reader. Activity of glutathione peroxidase was calculated as a U mg $^{-1}$ protein.

2.7. Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) and if significant (P<0.05) differences were found, Duncan's multiple range test was used to rank the groups. The data are presented as mean ± SE of three replicate groups. All statically analyses were carried out using the SPSS version 19 (SPSS Inc., Michigan Avenue, Chicago, Illinois, USA).

3. Results

The results of growth performance and feed utilization are shown in Table 3. The fish fed diets containing up to 240 g kg^{-1} FSM had no significant difference of survival, final growth, weight gain (WG), feed efficiency (FE) and protein efficiency ratio (PER) compared with control group (P > 0.05). Survival, final growth, WG, FE and PER of fish fed the diet containing 320 g kg^{-1} FSM were significantly lower than in control group (P < 0.05). DFI, CF and VSI were not significantly affected by dietary FSM level (P > 0.05). Morphological parameters of fish are shown in Table 4. HSI of fish fed diets containing 80 and 160 g kg^{-1} FSM did not, but in fish fed diets containing 240 and 320 g kg^{-1} FSM differ significantly compared with control group (P > 0.05).

Table 3. Growth performances and feed utilization of juvenile olive flounder fed with the experimental diets for 8 weeks

	Diets				
	Con	FSM8	FSM16	FSM24	FSM32
Initial body weight (g fish^{-1})	12.0±0.1^{ns}	11.8±0.2	11.8±0.1	12.0±0.1	12.0±0.1
Final body weight (g fish $^{-1}$)	34.9±0.6^{bc}	36.5±1.3^{c}	32.7±1.8^{ab}	31.6±0.8^{ab}	29.8±0.1^{a}
Weight gain (%)	191.5^{bc}	207.90^{c}	177.50^{b}	165.00^{ab}	147.83^{a}
Specific growth rate[1]	1.9±0.0^{bc}	2.0±0.0^{c}	1.8±0.1^{b}	1.7±0.1^{ab}	1.6±0.0^{a}
Survival	81±1.1^{b}	71±5.5^{ab}	69±5.8^{ab}	68.7±8.0^{ab}	63±4.8^{a}
Feed efficiency[2]	118±5.7^{b}	107±5.6^{b}	104±3.9^{b}	104±3.7 ^{b}	84±0.5 ^{a}
Daily feed intake[3]	1.2±0.1^{ns}	1.2±0.1	1. 3±0.1	1.3±0.1	1.4±0.1
Protein efficiency ratio[4]	2.3±0.1^{b}	2.1±0.1^{b}	2.0±0.6^{b}	2.0±0.1^{b}	1.6±0.0^{a}

Values (mean ± SE of three replication) in the same row not sharing a common superscript are significantly different (P<0.05). ns = values are not significant (P>0.05).

[1]Specific growth rate = [ln (final fish wt.) - ln (initial fish wt.)] × 100/days of feeding.

[2]Feed efficiency = wet weight gain × 100/feed intake.

[3]Daily feed intake = feed intake × 100/ [(initial fish wt. + final fish wt. + dead fish wt.) × days reared/2].

[4]Protein efficiency ratio = (wet weight gain / protein intake) × 100.

Table 4. Morphological parameters of juvenile olive flounder fed with the experimental diets for 8 weeks

	Diets				
	Con	FSM8	FSM16	FSM24	FSM32
Condition factor (%)[1]	1.1±0.06^{ns}	1.1±0.12	1.2±0.00	1.1±0.01	1.2±0.04
Hepatosomatic index(%)[2]	1.5±0.26^{ab}	1.3±0.07^{a}	2.0±0.1^{bc}	2.3±0.11^{c}	2.2±0.17^{c}
Visceral somatic index(%)[3]	2.6±0.35^{ns}	2.0±0.10	2.4±0.09	2.6±0.04	2.5±0.00

Values (mean ± SE of three replication) in the same row not sharing a common superscript are significantly different ($P < 0.05$). ns = values are not significant ($P > 0.05$).

1 Condition factor = [fish weight (g)/fish length (cm) 3] × 100.

2 Hepatosomatic index = (liver weight/body weight) × 100.

3Visceral somatic index = (viscera weight/body weight) × 100.

Proximate composition and amino acid profile of fish was not affected ($P > 0.05$) by dietary FSM level compared with control group (Tables 5 and 6). Triglyceride and total protein contents of plasma in fish fed the diets containing 80-320 g kg^{-1} FSM were significantly greater ($P < 0.05$) than in control group (Table 7). Cholesterol concentration of plasma in fish fed 320 g kg^{-1} FSM diet was significantly less compared with other groups ($P < 0.05$). Total protein contents of plasma in FSM fed diet were significantly greater than in control groups ($P < 0.05$). Activities of digestive and antioxidant enzymes were not affected significantly by different levels of FSM (Table 8). Also Apparent digestibility coefficient for NFE and gross energy reduced with increased FSM levels (Table 9).

Table 5. Proximate composition (g kg^{-1} wet weight) of the whole body of juvenile olive flounder fed with the experimental diet for 8 weeks

	Diets				
	Con	FSM8	FSM16	FSM24	FSM32
Crude protein	174±3.2^{ns}	168±3.4	179±8.1	169±3.5	178±0.9
Crude lipid	17±1.7^{ns}	19±1.3	19±0.8	17±1.6	15±2.1
Moisture	748±3.2^{ns}	748±3.3	747±7.0	756±5.9	745±1.1
Ash	38±1.4^{ns}	39±1.3	40±1.9	39±1.9	40±0.9

Values are mean ± SE of three replication. ns = values are not significant ($P>0.05$).

Table 6. Amino acids composition (% of protein) of the dorsal muscle in juvenile olive flounder fed with the experimental diets for 8 weeks

	Diets				
	CON	FSM8	FSM16	FSM24	FSM32
Arginine	6.4±0.03^{ns}	6.2±0.06	6.7±0.1	6.3±0.05	6.5±0.07
Histidine	2.4±0.00^{ns}	2.5±0.02	2.2±0.05	2.3±0.08	2.7±0.03
Isoleucine	4.1±0.18^{ns}	4.3±0.07	4.2±0.23	3.9±0.13	3.8±0.29
Leucine	8.7±0.37^{ns}	8.9±0.03	9.1±0.03	9.5±0.07	9.3±0.03
Lysine	9.1±0.03^{ns}	9.3±0.07	9.5±0.05	9.2±0.07	9.6±0.12
Methionine + Cysteine	4.0±0.06^{ns}	4.4±0.04	4.1±0.10	4.2±0.07	4.3±0.01
Phenylalanine + Tyrptophan	7.9±0.03^{ns}	7.6±0.09	8±0.05	7.8±0.1	7.7±0.03
Threonine	4.8±0.07^{ns}	4.5±0.03	4.7±0.06	4.9±0.00	4.6±0.06
Valine	10.0±0.15^{ns}	10.5±0.09	10.1±0.07	10.7±0.08	10.3±0.13

Values are mean ± SE of three replication.

ns= values are not significant ($P>0.05$).

Table 7. Hematological values of the plasma in juvenile olive flounder fed with the experimental diets for 8 weeks

	Diets				
	Con	FSM8	FSM16	FSM24	FSM32
Total protein (g L^{-1})	34.0±5.0[a]	62.0±2.0[b]	70.0±1.0[c]	72.0±1.0[c]	70.0±1.0[c]
Glucose (m mol L^{-1})	1.1±0.2[ns]	1.2±0.1	1.2±0.0	1.2±0.1	1.1±0.1
Cholesterol (m mol L^{-1})	0.9±0.0[c]	0.8±0.1[b]	1.7±0.0[d]	1±0.0[c]	0.6±0.0[a]
Triglyceride (m mol L^{-1})	1.9±0.1[a]	3.9±0.4[b]	4.9±0.4[b]	7.9±0.9[c]	8.5±0.5[c]

Values (mean ± SE of three replication) in the same row not sharing a common superscript are significantly different (P <0.05). ns = values are not significant (P>0.05).

Table 8. Digestive and antioxidant enzyme activities in juvenile olive flounder fed with the experimental diets for 8 weeks

	Diets				
	Con	FSM8	FSM16	FSM24	FSM32
Amylase (U mg $^{-1}$ protein)	0.6±0.2[ns]	0.6±0.2	0.7±0.1	0.7±0.1	0.6±0.1
Lipase (U mg $^{-1}$ protein)	4.9±1.8[ns]	2.3±0.6	2.7±0.5	4.9±1.8	3.9±0.8
Trypsin (U mg $^{-1}$ protein)	56.9±0.3[ns]	57.4±2.3	61.5±7.5	59.26±7.4	57.2±9.9
Glutathione peroxidase of liver (U mg $^{-1}$ protein)	8.2±0.7[ns]	7.1±0.9	7.5±0.6	6.4±0.2	6.5±0.7
Superoxide dismutase of liver (%)	77±2.7[ns]	75±3.3	80±3.5	72±5.5	76±3.0
Super oxide dismutase of plasma (%)	67±0.3[ns]	62±5.3	58±4.9	61±2.5	62±3.4

Values are mean ± SE of three replication. ns = values are not significant (P>0.05).

Table 9. Apparent digestibility coefficient of dry matter, major nutrients and gross energy contents of the test diets with different levels of FSM (flounder)

Analysed Feed composition	FSM0	FSM8	FSM16	FSM24	FSM32	Regression line	R^2	P value
Dry matter	73.9	69.4	67.2	67.1	60.5	Y =73.44-0.364x	0.90	s
Protein	97.1	85.7	86.9	87.8	84.1	Y=89.85-0.164x	0.52	ns
Lipid	97.3	97.9	94.6	95.3	96.7	Y= 97.12-0.47x	0.19	ns
Carbohydrate[1]	60.8	62.8	56.5	51.4	47.2	Y=63.64-0.482x	0.88	s
energy	85.5	80.8	80.3	80.1	77.0	Y= 84.28-0.221x	0.84	s

Linear regression equation (where Y is the response and x the level of FSM in diet), R2 and P (significant when P < 0.05) are also given.
S: significant, ns: not significant.
[1]Carbohydrate = 100 - (crude protein + crude lipid + ash).

4. Discussion

Generally poor growth performance found in fish fed plant protein is related to palatability, amino acid imbalance, phosphorus availability, anti-nutritional factors (Gomes et al., 1995) and disorder in lipid metabolism (Ye et al., 2011). It has been reported that soybean meal could be used as a partial substitute for fish meal up to 100-159 g kg^{-1} diet for juvenile *flounder Paralichthys olivaceus* (Deng et al., 2006; Kim et al., 2000). Ye et al., (2011) reported that replacement of fish meal protein above 240 g kg^{-1} diet could adversely affect the growth and metabolism of protein and lipid of olive flounder. Similarly, it was observed that growth of turbot, *Scophthalmus masimus* was gradually decreased by increasing levels of soybean protein concentrate, even at the minimum level of 250 g kg^{-1} substitution (Day and Gonzalez., 2000).

Good result of growth performance of fish fed diet containing an ingredient is attributed to a number of factors including improved palatability, digestibility and reduced exposure to anti-nutritional factors. Several practical ways have been suggested to improve utilization of plant proteins including; blending (Jackson et al., 1982), feeding stimulants (Deng et al.,

2006) and fermentation (Kader et al., 2011; Lee et al., 2010). In the present study, survival, specific growth rate, feed efficiency and protein efficiency ratio of fish fed diets containing up to 240 g kg^{-1} FSM were not significantly different from those in control group. The different results may be due to improvement of digestibility and removing anti-nutritional factor of soybean meal, such as protease inhibitor by fermentation. The process of fermentation by microorganisms might help towards higher replacement of fish meal protein with alternative plant proteins by decreasing amount of anti-nutritional factors and increasing nutrient availability (Kader et al., 2011). It has been reported that fish meal protein could be replaced up to 500 g kg^{-1} by fermented fish offal, mustard oil cake and rice bran mixture in the diet for Indian major carp and catfish, *Heteropneustes fossilis* (Mondal et al., 2007; Mondal et al., 2008). It was shown FSM in the diet induced higher growth and feed efficiency compared to control diet for yellowtail (Shimeno et al., 1993). Also, Kader et al., (2011) reported on the beneficial effect of dietary FSM on the growth of flounder. Wee, (1991) suggested that nutrient value of plant ingredient improved during fermentation period by microbial activities.

In this investigation, it was observed that hepatosomatic index and plasma triglyceride content of juvenile olive flounder fed diet containing 240-320 g kg-1 FSM were greater than fish fed diet containing 0-80 g kg^{-1} FSM and 0-160 g kg^{-1} FSM, respectively. Recent evidence showed that the increasing hepatosomatic index of fish was related to liver lipid content (Ye et al., 2011). In addition, it was reported that liver lipid content was positively correlated with serum triglyceride content and negatively correlated with cholesterol content (Kotronen and Yki-Jarvinen., 2008). This can be related to over-expression of genes involved in liver lipid biosynthesis in connection with plant protein based diet which also is reported by Panserat et al., (2009) on rainbow trout fed the plant protein based diet. Also, it is shown the higher amount of soybean protein induced decrease in plasma and liver cholesterol concentrations in rat (Zhang and Beynen., 1993). Therefore, the increased hepatosomatic index and triglyceride content of fish fed high level of FSM may be due to some disorder in lipid metabolism leading to hyperlipidemia.

Lower growth rate of flounder fed the diet containing 320 g kg^{-1} FSM could be related to non-starch polysaccharides content in FSM. It has been reported that non-starch polysaccharides fraction of soybean meal negatively affected growth performance of Atlantic salmon and European sea bass (Refstie et al., 1999; Harpaz et al., 2006) which could have resulted in reducing bioavailability of nutrients and energy through mechanisms involving a binding action with bile salts (Francis et al., 2001). Also, in this experiment it was observed that high level of FSM positively reduced apparent digestibility coefficient of dietary Carbohydrate ($r = 0.88$; $p < 0.05$) and digestible energy ($r = 0.84$; $p < 0.05$) maybe because of residues anti-nutritional factors in FSM.

It has been reported that using vegetable products from fermentation processing could enhance non-specific immune response, activity of antioxidant enzymes and disease resistance for fish (Ashida et al., 2002; Pham et al., 2007) however in this experiment, no significant effect of FSM on the activity of antioxidant enzymes in liver and plasma of olive flounder was observed.

It is concluded that using FSM produced by *B. subtilis* had beneficial effect on the nutritional quality of soybean meal. Therefore, fish meal protein could be replaced from 80 g kg^{-1} up to 240 g kg^{-1} by FSM without significantly different results than in control group. But, for higher growth performance we recommended 80 g kg^{-1} FSM in diet of fish.

Acknowledgements

This study was supported by Korea Sea Grant Program funded by the Ministry of Oceans and

Fisheries in Korea. We wish to thank the CJ Cheil Jedang Co. (Seoul, Korea) for supplying the fermented soybean meal.

References

AOAC (1995) Official Methods of Analysis of AOAC International, In: Cunniff, P. (Ed.), 16th edition. AOAC International, Arlington, Virginia, USA.1141p.

Ashida, T., Okimasu, E. and Amemura, A., 2002. Effects of a fermented vegetable product on hemolysis and lipid peroxidation of Japanese flounder erythrocytes. Fisheries Sciences. 68: 1324-1329

Bairagi, A., Ghosh, K.S., Sen, S.K. and Ray, A.K., 2002. Duckweed (*Lemna polyrhiza*) leaf meal as a source of feed stuff in formulated diets for rohu (*Labeo rohita Ham*.) fingerlings after fermentation with a fish intestinal bacterium. Bioresource Technology. 85: 17–24

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72: 248–254

Canella, M., Bernardi, A. and Marghinott, D., 1984. Improvement of germinated sunflower meal by fermentation. Food Science and Technology. 17: 314

Day, O.J., and Gonzalez, H.G., 2000. Soybean protein concentrate as a protein source for turbot (*Scophthalmus maximus L*). Aquaculture Nutrition. 6: 221-228

Deng, J., Mai, K., Zhang, W., Wang, X., Xu, W. and Liufu, Z., 2006. Effects of replacing fish meal with soy protein concentrate on feed intake and growth of juvenile Japanese flounder (*Paralichthys olivaceus*). Aquaculture. 258: 503-513

Fagbenro, O., Jauncey, K. and Haylor, G., 1994. Nutritive value of diets containing dried lactic acid fermented fish silage and soybean meal for juvenile (*Oreochromis niloticus*) and (Clarias gariepinus). Aquatic Living Resources. 7: 79–85

Francis, G., Makkar, H.P.S. and Becker, K., 2001. Anti-nutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 199: 197–227

Furukawa, A. and Tsukahara, H., 1966. On the acid digestion method for the determination of chromic oxide as an index substance in the study of digestibility of fish feed. Bulletin of the Japanese Society for the Science of Fish. 32: 502–506

Gomes, E.F., Rema, P. and Kaushik, S. J., 1995. Replacement of fish meal by plant proteins in the diet of rainbow trout (*Oncorhynchus mykiss*): digestibility and growth performance. Aquaculture. 130: 177–186

Harpez, S., Tibaldi, E., Hakim, Y., Uni, Z., Tulli, F., de Francesco, M. and Luzana, U., 2006. 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 (*Diacentrarchus labrax*) Aquaculture. 261: 182-193

Jackson, A. J., Capper, B.S. and Matty, A.J., 1982. Evaluation of some plant proteins in complete diets for the tilapia (*Sarotheroden mossambicus*). Aquaculture. 27: 97-109

Kader, M.A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M., Nguyen, B. T., Goa, J. and Laining, A., 2011. Can fermented soybean meal and squid by-product blend be used as fishmeal replacements for Japanese flounder (Paralichthys olivaceus)? Aquaculture Research. 43(10): 1427-1438

Kiers, J.L., Van Laeken, AE., Rombouts, F.M. and Nout, M.J., 2000. In vitro digestibility of bacillus fermented soya bean. International Journal of Food Microbiology. 60: 163-9

Kim, Y.S., Kim, B.S., Moon, T.S. and Lee, S.M., 2000. Utilization of defatted soybean meal as a substitute for fish meal in the diet of juvenile flounder (*Paralichthys olivaceus*). Journal of the Korean Fisheries Society. 33: 469-474

Kotronen, A. and Yki-Jarvinen, H., 2008. Fatty liver: a novel component of the metabolic syndrome. Arteriosclerosis, Thrombosis, and Vascular Biology. 28: 27–38

Lee, K.J., Kim. S., Pham, M.A., Kim, K.W. and Son, M.H., 2010. Effects of microbial fermentation of soybean on growth performances, phosphorus availability, and antioxidant activity in diets for juvenile olive flounder (*Paralichthys olivaceus*). Food Science and Biotechnology. 19: 1605-1610

Lee, S.M., 2002. Apparent digestibility coefficients of various feed ingredients for juvenile and grower rockfish (*Sebastes schlegeli*). Aquaculture. 207: 79-95

Lim, S.J. and Lee, K.J., 2009. Partial replacement of fish meal by cottonseed meal and soybean meal with iron and phytase supplementation for parrot fish Oplegnathus fasciatus. Aquaculture. 290: 283-289

Lim, S.J., Kim, S.S., Pham, M.A., Song, J.W., Cha, J.H., Kim, J.D., Kim, J.U. and Lee, K.J., 2010. Effects of fermented cottonseed and soybean meal with phytase supplementation on gossypol degradation, phosphorus availability, and growth performance of olive flounder (*Paralichthys olivaceus*). Fisheries and Aquatic Sciences. 13: 284-293

Mondal, K., Kaviraj, A., Mukhopadhyay, P.K., Datta, M. and Sengupta, C., 2007. Evaluation of fermented fish offalin formulated diet of the Indian major carp (rohu, *Labeo rohita* Hamilton). Acta Ichthyologica Et Piscatoria.37: 99–105

Mondal, K., Kaviraj, A. and Mukhopadhyay, P.K., 2008. Evaluation of fermented fish-offal in the formulated diet of the freshwater catfish (*Heteropneustes fossilis*). Aquaculture Research. 39: 1443–1449

Mukhopadhyay, N. and Ray, A.K., 1999. Effect of fermentation on the nutritive value of sesame seed meal in the diets for rohu (Labeo rohita Hamilton), fingerlings. Aquaculture Nutrition. 5: 229–236

Olsen, R.E., Hansen, A.C., Rosenlund, G., Hemre, G.I., Mayhew, T.M., Knudsen, D.L., Eroldogan, O.T., Myklebust, R. and Karlsen, O., 2007. Total replacement of fish meal with plant proteins in diets for Atlantic cod (*Gadus morhua L.*) II—Health aspects. Aquaculture. 272: 612–624

Panserat. S., Hortopan, G.A., Plagnes-Juan, E., Kolditz, C., Lansard, M., Skiba-Cassy, S., Esquerre, D., Geurden, I., Medale, F., Kaushik, S. and Corraze, G., 2009. Differential gene expression after total replacement of dietary fish meal and fish oil by plant products in rainbow trout (*Oncorhynchus mykiss*) liver. Aquaculture. 294:123–131.

Pham, M.A., Lee, K.J., Lim, S.J. and Park, K.H., 2007. Evaluation of cottonseed and soybean meal as partial replacement for fishmeal in diets for juvenile Japanese olive flounder (*Paralichthys olivaceus*). Fisheries Sciences. 73: 760-769

Ramachandran, S. and Ray, A.K., 2007. Nutritional evaluationof fermented black gram (Phaseolus mungo) seed meal in compound diets for rohu (*Labeo rohita Hamilton*), fingerlings. Journal of Applied Ichthyology. 23: 74–79

Reddy, N.R. and Pierson, M.D., 1994. Reduction in anti-nutritional and toxic components in plant foods by fermentation. Food Research International . 27: 281–290

Refstie, S., Svihus, B., Shearer, K.D. and Storebakken, T., 1999. Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch polysaccharide content in different soya bean products. Animal Feed Science and Technology .79: 331–345

Shimeno, S., Mima, T., Yamamoto, O. and Ando, Y., 1993. Effects of fermented defatted soybean meal in diet on growth, feed conversion and body composition of juvenile yellowtail. Nippon Suisan Gakkaishi. 59: 1883-1888

Skrede, A. and Nes, I.F., 1988. Slaughter house by-products preserved by (Lactobacillus plantarum)

fermentation as feed for mink and foxes. Animal Feed Science and Technology. 20: 287-298

Tidwell, J.H., Coyle, S.D., Bright, L.A. and Yasharian, D., 2005. Evaluation of plant and animal source proteins for replacement of fish meal in practical diets for the largemouth bass (*Micropterus salmoides*). Journal of the World Aquaculture Society. 36: 454–463

Wee, K.L., 1991. Use of nonconventional feedstuffs of plant origin as fish feeds is it practical and economically feasible In: Fish Nutrition Research in Asia.proc.4th Asian Fish Nutrition Workshop. (De Silva, S.S ed), pp.13-32. Asian Fisheries Society. Manila, The Philippines.

Yamamoto, M., Saleh, F. and Hayashi, K., 2004. A fermentation method to dry and convert Shochu distillery byproduct to a source of protein and enzymes. The Journal of Poultry Science. 41: 275–280

Ye, J., Liu, X., Wang, Z. and Wang, K., 2011. Effect of partial fish meal replacement by soybean meal on the growth performance and biochemical indices of juvenile Japanese flounder (Paralichthys olivaceus). Aquaculture International. 19: 143-153

Zhang, X., and Beynen, A., 1993. Influence of dietary fish proteins on plasma and liver cholesterol concentrations in rats. British Journal of Nutrition. 69: 767-777