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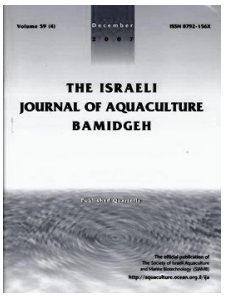

Effects of Replacing Fish Meal with Soybean Meal or Fermented and Phytase-Treated Soybean Meal Respectively, on Growth Performance, Feed Utilization, and Apparent Digestibility in Juvenile Turbot *(Scophthalmus maximus* L.*)*

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Keywords: ferment; phytase; soybean meal; growth; apparent digestibility; turbot

Abstract

The objective of this study was to evaluate the effect of fish meal (FM) substitution with gradient soybean meal (SBM) or fermented and phytase-treated soybean meal (PHSBM) in the diets of turbot (*Scophthalmus maximus*). A 9 week feeding trial was conducted using juvenile turbots (*Scophthalmus maxima*) fed seven experimental diets. The seven isonitrogenous (approximately 50% crude protein) and isoenergetic (approximately 21.0 kJ/g diet of gross energy) diets were formulated to include FM protein substitution with corresponding amounts of protein from soybean meal and phytase-treated soybean meal sources. Results showed that survival rate and feed intake did not differ significantly between the FM diet and any plant protein incorporated diets. Compared with the FM diet, final body weight and SGR were significantly reduced by the SBM2, SBM3, and PHSBM3 diets. Except for the PHSBM1 diet, feed efficiency ratio in the other SBM or PHSBM incorporated diets was much lower than in the FM diet. Body ash content was not affected by gradient PHSBM incorporated diets compared with the FM diet, while SBM incorporated diets (SBM2 and SBM3) showed a higher ash content than the FM diet (P<0.05). Body crude protein was significantly reduced when fishmeal protein was replaced by soybean meal up to 60% (SBM3). There was no significant difference in the crude lipid and moisture contents among different treatments. Fish meal replaced by gradient PHSBM did not affect the apparent digestibility coefficients (ADC) of dry matter and crude protein, while the ADC of dry matter was markedly reduced in all the soybean meal incorporated diets compared with the FM diet. These results show that 30% fish meal protein could be replaced by soybean meal in the diet of turbot, while PHSBM could be substituted for up to 45% dietary fish meal.

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Introduction

Turbot (*Scophthalmus maximus*), a carnivorous fish is widely cultured throughout Asia and Europe for its high economic value. Compared to other fish, turbot requires a dietary protein level as high as 50% to 60% (Lee *et al*., 2003). Fishmeal has been considered the most suitable protein source in aquafeeds due to its well-balanced amino acid composition, essential fatty acid content, good palatability, digestible energy, as well as vitamin and mineral contents (Reverter *et al*., 2014). However, with the depletion of marine fisheries, shortage of fishmeal has become a worldwide problem (FAO, 2014). Much attention is being directed to the replacement of fishmeal with other protein sources, especially plant protein sources (Kokou, *et al*., 2015). Soybean meal is considered one of the most promising substitutes for fishmeal because of its high protein content (43 to 48%), balanced amino acid components, stable supply, and low cost (Azarm & Lee, 2014). Soybean meal has been successfully used in the cultivation of snakehead (Hien, *et al*., 2015), rainbow turbot (Ávila, *et al*., 2015), Japanese seabass (Li, *et al*., 2014), etc. However, since soybean meal contains approximately 30% indigestible carbohydrates, the presence of indigestible non-starch polysaccharides (NSP) may affect osmotic conditions in the intestine and reduce absorptive capacity for nutrients. Anti-nutritional factors (ANFs), such as phytase, protease inhibitor, anti-vitamins, and lectin, may decrease the nutritional value of soybean (Chou, *et al*., 2004).

Fermentation is a traditional technique used to improve the quality of plant protein sources. By the end of the fermentation period, protein macromolecules can be degraded into low molecular weight, and water-soluble, compounds (Hong & Kim, 2004). The ANFs in soybean meal could be removed or inactivated by fermentation (Egounlety & Aworh, 2003). Soybean meal fermented by *Aspergillus oryzae* or Eurotium, improves the digestibility of protein and carbohydrate in yellowtail (Shimeno, *et al*., 1993). In addition, fermentation of soybean meal with *Lactobacillus brevis* also could improve the digestibility of lipids in Atlantic salmon (Refstie, *et al*., 2005). On the other hand, the addition of phytase is an alternate method of improving the quality of protein. Phytase, interacts with protein and minerals, reducing their availability; about two-thirds of the phosphorus in soybean meal is present as phytase. Studies have shown that phytase could release minerals and increase the absorption of minerals and protein. So far, it has been used in red sea bream (Biswas, 2007), milkfish (Hassan & Satyanarayana, 2009), rohu (Hussain, *et al*., 2015), and Nile tilapia (Liebert & Portz, 2005). Both the fermentation, and the addition, of phytase could improve both the digestibility of nutrients and fish growth performance. However, information of both fermentation and enzymatic processing technology on soybean meal is limited.

In the current study, a strain of *Aspergillus awamori*, which has significant enzymatic activity, was used for soybean meal fermentation. After being treated with phytase, the regenerated soybean meal was evaluated by replacing fish meal in turbot for growth performance, feed utilization, and apparent digestibility.

Materials and Methods

Diet preparation. Aspergillus awamori was obtained from a culture collection center, the Zhejiang Academy of Agricultural Science, Hangzhou, China. Soybean meal was soaked for 60 minutes with 110% distilled water which contained 2‰ K_2HPO_4, 2‰ NaCl, 2‰ $(NH_4)_2SO_4$, 1‰ glucose, 2‰ urea. Hydrated soybean meal was cooked in a steam tank (model HX14G-1, Shanghai, China) at 100-110°C for 1 hour. After being cooled to room temperature, the soybean meal was inoculated with *Aspergillus awamori* (2,500,000 counts/g of dry soybean meal), mixed, and fermented in a bed-packed thermostatic chamber at 31°C for 37 hours. Fermented soybean meal was then broken into small pieces. The treatment of fermented soybean meal by phytase (provided by Sukahan Bio-Technology Co. Weifang, China) was conducted as described previously (Cain and Garling, 1995). Two grams of phytase were dissolved in 1 kilogram citrate buffer at pH 5.0, and then mixed with one kilogram of fermented soybean meal. After constantly stirring by hand, the mixture was rapidly heated to 50-55℃ for 6 hours. The treated fermented soybean meal was then dried in an air drying oven for 24 hours. The soybean meal (SBM) and fermented and phytase treated soybean meal (PHSBM) were ground to be less than 300 μm mesh size.

Seven isonitrogenous (approximately 50% crude protein) and isoenergetic (approximately 21.0 KJ/g diet of gross energy) diets were formulated. The experimental diets were formulated to produce diets in which 0% (FM), 30% (SBM1/PHSBM1), 45% (SBM2/PHSBM2), and 60% (SBM3/PHSBM3) of protein from fishmeal was replaced with SBM or PHSBM respectively (Table 1). All diets were supplemented with lysine, and methionine to the levels similar to the control (FM) diet. Y_2O_3 (0.1%) was supplemented as the indicator for digestibility determination (Glencross& Allan, 2007).

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

	Amount (% dry diet) in each treatment						
Ingredients	*FM*	*SBM1*	*SBM2*	*SBM3*	*PHSBM*	*PHSBM*	*PHSBM*
Fish meal [a]	60.00	42.00	33.00	24.00	42.00	33.00	24.00
SBM [a]	-	24.80	37.21	49.61	-	-	-
PHSBM [a]	-	-	-	-	22.16	33.24	44.32
Wheat meal [a]	25.12	13.74	8.19	3.15	17.02	13.18	9.62
Wheat gluten meal	1.88	3.85	4.66	5.36	3.23	3.66	4.39
Fish oil	4.00	5.30	6.40	7.10	5.30	6.40	7.10
Lecithin	2.00	2.00	2.00	2.00	2.00	2.00	2.00
$CaH_2(PO)_4$	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lysine	-	0.14	0.26	0.39	0.14	0.26	0.25
Methionine	-	0.16	0.28	0.39	0.15	0.26	0.32
Choline chloride	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Taurine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin premix [b]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix [c]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mold inhibitor	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Antioxidant	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Attractant [d]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Sodium alginate	0.50	0.50	0.50	0.50	0.50	0.50	0.50
cholesterol	0.00	1.00	1.00	1.00	1.00	1.00	1.00
Yttrium oxide	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Proximate							
Gross energy/(KJ/g)	19.92	20.30	20.46	20.53	20.25	20.37	20.46
Crude protein	50.31	50.26	50.21	50.15	50.31	50.22	50.26
Crude lipid	11.80	12.81	13.25	13.32	12.74	13.15	13.18

[a] Red fish meal (dry mater, %): protein 73.91, crude lipid 8.81; soybean meal (dry mater, %): crude protein 53.64, crude lipid 2.11; wheat gluten meal (dry mater, %): crude protein 83.31, crude lipid 1.75; wheat meal (dry mater, %): crude protein 17.50, crude lipid 2.22. These ingredients were obtained from Great seven Bio-Tech (Qingdao, China).

Fermented-phytase treated soybean meal (dry mater, %): crude protein 60.58, crude lipid 2.07. The ingredient was manufactured from xiaoguo biological technology co., LTD (HuZhou, Zhe Jiang, China).

[b] Vitamin premix (mg/kg 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.

[c] Mineral premix: (mg/kg 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.

[d] Attractants (% dry diet): betaine, 0.2; DMPT, 0.1; glycine, 0.1; alanine, 0.05; inosine-5′-diphosphate trisodium salt, 0.05.

[e] Gross energy of experimental diets was calculated according to gross energy values 23.64 KJ/g crude protein, 39.54 KJ/g crude fat, 17.57 KJ/g carbohydrate, respectively

Fish and experimental conditions. Juvenile turbot (*Scophthalmus maxima*) (8.53±0.03g) were purchased from a fish rearing farm (Qingdao, China). Experiments were conducted in Qingdao Yihaifeng Aquatic Product CO. Ltd (Qingdao, China). All fish were acclimated to laboratory conditions for 2 weeks by feeding the commercial diets before the experiment. After being fasted for 24 h, fish were selected and randomly assigned to 21 experimental fiberglass tanks with 30 fish per tank. Each diet was randomly assigned to three replicate groups. Fish were manually fed with the experimental diets to visual satiety twice daily at 6:00 and 18:00. Feces and uneaten food was siphoned out after feeding. During the experimental period, the water

temperature ranged from 20°C to 22°C, salinity from 27‰ to 29‰ and dissolved oxygen was approximately 7 mg/L, pH from 7.5 to 8.0. The feeding trial lasted 9 weeks.

Fecal collection and chemical analysis. Fecal samples were collected from the fifth week from each tank, siphoning with an automatic fecal collector after feeding 5 hours, and stored at -20°C. When the feeding trial was completed, all the fish were starved for 24h. Then total number and total body weight of fish in each tank were measured. Six fish were randomly sampled from each tank and stored at -20°C for whole body composition analysis. Crude protein, crude lipid, ash and moisture were analyzed using the method described by Liu, *et al*., 2014. Yttrium oxide content was measured by inductively coupled plasma-atomic emission spectrophotometer.

Digestibility determinations and statistical analysis. The following variables were calculated:

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

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

Feed intake (FI, %) = 100 × dry feed intake / [days × (final body weight + initial body weight)/2] Feed efficiency ratio (FER) = wet weight gain (g) / dry feed intake (g)

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

Statistical analysis. All statistical evaluations were analyzed using the software SPSS 19.0. Data were submitted to one-way analysis of variance (ANOVA) followed by Tukey's test. Homogeneity of variance test was conducted to ensure that variance is homogeneous. Differences were regarded as significant when $P<0.05$. Data are expressed as means ± standard error.

Results

In the current study, there was no difference ($P > 0.05$) in the survival rate (SR) and feed intake (FI) of turbot among all test groups (Table 2).

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

Treatments	*Initial body weight*	*Final body weight*	*Survival rate (%)*	*Specific growth rate*	*Feed intake (%/d) /FI*	*Feed efficiency*
FM	8.54±0.01	62.6±.96^{ab}	100±0.00	3.16±0.02^{ab}	1.76±0.06^{ab}	1.43±0.03^{a}
SBM1	8.53±0.00	56.31±1.66^{bcd}	100±0.00	2.99±0.04^{bc}	1.95±0.01^{ab}	1.26±0.01^{bcd}
SBM2	8.53±0.00	52.23±1.37^{cd}	97.78±2.2	2.88±0.04^{c}	2.09±0.08^{ab}	1.14±0.05^{de}
SBM3	8.52±0.01	49.95±1.82^{d}	98.89±1.1	2.81±0.06^{d}	2.17±0.03^{ab}	1.1±0.04^{e}
PHSBM1	8.53±0.00	66.86±3.14^{a}	100±0.00	3.27±0.07^{a}	1.63±0.19^{b}	1.42±0.02^{a}
PHSBM2	8.53±0.01	58.85±1.95^{abc}	100±0.00	3.06±0.05^{ab}	1.98±0.23^{ab}	1.28±0.03^{bc}
PHSBM3	8.55±0.01	51.52±0.86^{cd}	100±0.00	2.85±0.03^{cd}	2.34±0.22^{a}	1.08±0.02^{e}

Note: * Values show mean ± standard error, n = 3; values in the same column with different superscripts are significantly different ($P < 0.05$).

The highest SGR was observed in the group fed the 15% PHSBM diet. FBW and SGR in groups of turbot fed with SBM1and PHSBM2 diets showed no significant difference to the fishmeal control (FM). When soybean meal was substituted up to a level of 45% (SBM2) of the dietary fishmeal protein, or fermented soybean meal was substituted up to a level of 60% (PHSBM3) of the dietary fish meal protein, FBW and SGR were significantly reduced compared with the FM diet. There was no difference in feed efficiency ratio (FER) with replacement of 30% of the fishmeal protein by PHSBM (PHSBM1) compared to the control; when 30% of the total fish protein content was replaced by SBM and 45% by PHSBM, a decreasing trend in FER was observed. The body composition of turbots fed each of the test diets is listed in Table 3.

Table 3. Whole body composition of juvenile turbot fed the experimental diets (% wet weight)*

Treatments	*Crude lipid*	*Ash*	*Crude protein*	*Moisture*
FM	4.09±0.14	3.53±0.07^{a}	16.31±0.18^{a}	76.38±0.27
SBM1	3.82±0.17	3.87±0.03^{ab}	15.65±0.11^{ab}	76.54±0.39
SBM2	3.67±0.17	4.02±0.07^{bc}	15.84±0.2^{ab}	76.09±0.18
SBM3	3.62±0.03	4.18±0.1^{c}	15.39±0.14^{bc}	77.07±0.13
PHSBM1	4.05±0.02	3.8±0.05^{ab}	15.65±0.04^{ab}	76.11±0.41
PHSBM2	3.87±0.09	3.85±0.12^{ab}	15.79±0.22^{ab}	76.42±0.36
PHSBM3	3.95±0.08	3.77±0.12^{ab}	15.62±0.12^{ab}	76.41±0.19

Note: * Values show mean ± standard error, n = 3; values in the same column with different superscripts are significantly different ($P < 0.05$).

No significant differences were found in crude lipid, and moisture content of fish among the groups tested. Body crude protein was significantly reduced when fishmeal protein was replaced by soybean meal at a level of up to 60% (SBM3). There were no significant differences in body ash content of fish containing various levels of PHSBM compared with those fed a FM diet, while SBM2 and SBM3 groups had higher ash content ($P<0.05$).

The apparent digestibility coefficients (ADC) of dry matter and crude protein are listed in Table 4. There was no significant difference in the ADC among all the PHSBM diets compared to the FM diet. However apparent digestibility coefficients were significantly lower in groups fed the SBM diets than FM, and PHSBM, diets.

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

Treatments	*Dry matter*	*Crude protein*
FM	90.85±0.56^{a}	97.69±0.15^{a}
SBM1	84.49±1.59^{b}	96.59±1.26^{a}
SBM2	82.1±2.65^{bc}	93.35±0.79^{bc}
SBM3	80.42±2.48^{c}	90.61±0.88^{c}
PHSBM1	89.79±0.82^{a}	97.91±0.31^{a}
PHSBM2	89.11±0.81^{a}	96.28±0.97^{a}
PHSBM3	88.92±0.16^{ab}	94.22±0.33^{ab}

Note: * Values show mean ± standard error, n = 3; values in the same column with different superscripts are significantly different ($P < 0.05$).

Discussion

Replacing fishmeal with PHSBM in formulated feeds for turbot is challenging. We believe that this is the first study to report the use of fermentation, along with the enzyme treatment process, of soybean to produce an alternative plant protein source in the diets of turbot. Some studies have reported that fermented soybean meal produced better results than soybean meal when replacing equal quantities of fishmeal. Diets containing 21% fermented soybean meal produced higher growth and PER than the diets containing 20% soybean meal in grouper (Luo, *et al*. 2004). Weight gain and specific growth rate was adversely affected in black sea bream fed more than 30% fermented soybean meal as an FM replacement (Zhou, *et al*. 2011). In this study, a strain of *Aspergillus awamori* which produced high levels of extracellular hydrolysis was used to reduce the concentration of anti-nutritional factors as shown in previous (unpublished) research. Under the presented experimental conditions in this study, results indicated that PHSBM could replace 45% of dietary fish meal protein in turbot without any obviously detrimental effects. This might be due to the addition of phytase. Pre-treatment of soybean meal with phytase therefore can serve as an alternate method to improve the quality of protein. Studies have shown that phytase could release minerals and increase their utilization, and thereby increase growth performance (Wang, *et al*., 2009). 30% sesame oilseed meal fermented by a phytase-producing strain significantly increased growth performance of rohu in comparison with fish fed raw sesame oil seed meal (Roys *et al*. 2014). The mode of action of the quality improvement of raw plant protein by fermentation and phytase treatment was ascribed to the break-down of anti-nutritional factors (protease inhibitor, phytic acid) and non-digestible carbohydrates (crude fibre) by the activity of the fungi, particularly due to hydrolysis produced by those strains. Using fermentation and phytase treatment to improve the digestible quality of plant protein is a better way to improve its nutritional value.

Compared with the FM diet, PHSBM diets showed no significant differences in whole-body protein content of turbot while it was reduced when more than 30% soybean meal replaced the FM. A similar observation was also reported in African bony-tongue and yellowtail (Shimeno, *et al*.,1993). The content of small protein molecules is higher in fermented soybean meal than in SBM and diets with fermented soybean meal may induce protein anabolism more than those with soybean meal. However, other studies have found that body protein is not affected by high dietary plant protein (Martínez-Llorens, *et al*., 2007; Bonaldo, *et al*., 2008). This may be due to differences between fish species and alternative proteins. In this study, whole-body ash content tended to

increase when substitution levels of SBM increased compared with FM and PHSBM diets. This was especially true of diets with high-soybean meal content. This could have been caused by the positive effects generated during phytase treatment. Similar reports were found in tiger puffer (Lim, *et al*., 2011), parrot fish (Lim, & Lee, 2009).

The current results showed that the ADC of dry matter and crude protein was highest in fish meal, followed by PHSBM, and lowest in soybean meal for turbot. This suggests that the fermenting and phytase processes can improve digestibility of dry matter and crude protein in turbot. Trends were similar in apparent digestibility for rohu (Das & Ghosh, 2015.), Chinese sucker (Yuan, *et al*., 2010) and tilapia (Dong, *et al*. 2010).

In conclusion, turbot juveniles are able to utilize PHSBM at limited dietary levels when used as a replacement for FM. Based on growth performance and feed utilization data, soybean meal could replace 30% of FM protein without any obvious detrimental effects. In contrast the fermented, and phytase-treated, soybean meal could replace 45% of the fish meal protein. Dietary supplementation with essential amino acids, or non-starch polysaccharide enzymes, may allow a higher replacement level and this warrants further investigation. The apparent digestibility coefficients of SBM-based diets were much lower than from the PHSBM-based diet. The fermented, and phytase-treated, soybean meal is a promising plant protein resource for turbot. From an economic perspective, PHSBM could improve diet nutritional values, increase growth performance of turbot, greatly reduce utilization of fishmeal, and be a cost-effective solution to overcoming the problem of global shortage of fishmeal. Currently, fermentation and enzyme treatments are two efficient and cost effective methods for improving soybean meal utilization and have been widely used in the feed industry (Azarm & Lee, 2014; Ávila, *et al.,* 2015). In the present study, we demonstrated that performance was superior with the combination of these two methods. Phytase has been widely used in the feed industry as it is inexpensive and readily available. Therefore, the combination of fermentation and exogenous enzyme supplementation should be suitable for the aquafeed industry, especially in diets for valuable fish species such as turbot.

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