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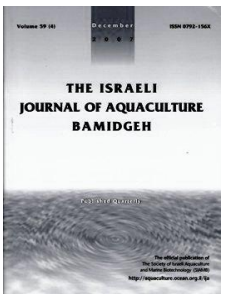

Effects of replacing fish meal with soybean meal or fermented and phytase-treated soybean meal respectively on growth performance, feed utilization, and apparent digestibility coefficients in juvenile turbot *(Scophthalmus maximus* L.*)*

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Key words:, 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 triplicate groups of turbot (mean initial mass of 8.53 ± 0.03 g) kept in fibreglass tanks (30 fish per tank). 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 proteins from fishmeal were replaced with that from SBM or PHSBM, respectively. The results showed that the survival rate (SR) and feed intake (FI) did not differ significantly between FM diet and any plant protein incorporated diets. Compared with the FM diet, the final body weight and SGR were significantly reduced by SBM2, SBM3, and PHSBM3 diets. Except for the PHSBM1 diet, the feed efficiency ratio (FER) in the other SBM or PHSBM incorporated diets were much lower than those in the FM diet. The 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 that of the FM diet ($P < 0.05$). The body crude protein was reduced significantly 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 was did not affect the apparent digestibility

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coefficients (ADC) of dry matter and crude protein, while all the soybean meal incorporated diets markedly reduced the ADC of dry matter compared with the FM diet. These results showed that 30% fish meal protein could be replaced by soybean meal in the diet of turbot, while the PHSBM could be substituted by up to 45% dietary fish meal.

Introduction

Turbot is a carnivorous fish and 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 as the most suitable protein source in aquafeeds due to its well-balanced amino acid composition, essential fatty acid content, better palatability, digestible energy, and vitamin and mineral contents (Reverter, *et al*., 2014). However, with the depletion of marine fisheries, shortage of fishmeal gradually becomes a challenge around the world (FAO, 2014). More attention has been paid to fishmeal replacement by other protein sources, especially plant protein sources (Kokou, *et al*., 2015). Therefore, soybean meal is considered as one of the most promising substitutes for fish meal because of its high protein content (43 to 48%), balanced amino acid content, stable supply, and low cost (Azarm & Lee, 2014). So far 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). Furthermore, the ANFs in soybean meal could be removed or inactivated by fermentation (Egounlety & Aworh, 2003). Soybean meal fermented by *Aspergillus oryzae* or Eurotium can improve 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 another alterative way 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 showed 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). It has been demonstrated that both the fermentation, and the addition, of phytase could improve 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 enzyme content, 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 culture collection centre, Zhejiang Academy of Agricutural Science, Hangzhou, China. Soybean meals were soaked with 110% distilled water which contained 2‰ K_2HPO_4, 2‰ NaCl, 2‰ $(NH_4)_2SO_4$, 1‰ glucose, 2‰ urea for 60 minutes. Hydrated soybean meal was cooked in a steam tank (model HX14G-1, Shanghai, China) at 100–110°C for 1 hour. After cooled to room temperature, the soybean meal were 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 were then broken to pieces.

Phytase was provided by SUKAHAN BIO-TECHNOLOGY CO. (Weifang, China).The treatment of fermented soybean meal by phytase was conducted as described previously (Cain and Garling, 1995)as follows; 2 grams of phytase were dissolved in 1 kilogram buffer which contained 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℃ and lasted for 6 hours. The treated fermented soybean meal was then dried in an air drying oven for 24 hours to reduce moisture. The soybean meal (SBM) and the fermented and phytase treated soybean meal (PHSBM) were ground to be below 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 proteins from fishmeal were replaced with SBM or PHSBM respectively (Table 1). All diets were supplemented with lysine, methionine to the levels similar to control (FM). Y_2O_3 (0.1%) was supplemented as the indicator for digestibility determination following previous description (Glencross& Allan, 2007).

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

	Amount (% dry diet) in each treatment						
Ingredients	*FM*	*SBM1*	*SBM2*	*SBM3*	*PHSBM1*	*PHSBM2*	*PHSBM3*
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 composition							
Gross energy/(KJ/g) [e]	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^{-1} diet): thiamin, 25; riboflavin, 45; pyridoxine HCl, 20; vitamin B12, 10; vitamin K, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid,20; biotin, 60; retinol acetate, 32; cholecalciferol, 5; alpha-tocopherol, 240; ascorbic acid, 2000; microcrystalline cellulose,1473.

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

[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 turbots (*Scophthalmus maxima*) (8.53±0.03g) were purchased from a fish rearing farm (Qingdao, China). Experiments were done 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 experiments. After being fasted for 24 h, fish were selected and randomly assigned to 21 experimental fiber glass tanks with 30 fish per tank. Each diet was randomly assigned to three replicate groups. Fish were manually fed to visual satiety twice daily at 6:00 and 18:00 with experimental diets and the feces waste was cleaned 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^{-1}, pH from 7.5 to 8.0. The feeding trial lasted for 9 weeks.

Fecal collection and chemical analysis. Fecal samples were collected from the fifth week from each tank, using an automatic fecal collector by siphoning 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 contents were measured by inductively coupled plasma-atomic emission spectrophotometer.

Digestibility determinations and statistical analyses. 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 using 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, the survival rate (SR) and feed intake (FI) of turbot showed no difference ($P > 0.05$) among all testing groups (Table 2). It was interesting to note that the highest SGR was observed in the group fed with a 15% PHSBM diet. In fact, the FBW and SGR in groups of turbot fed with SBM1and PHSBM2 diets showed no significant difference to the fishmeal control (FM). In contrast, 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. For the feed efficiency ratio (FER), replacement of 30% of the fishmeal protein by PHSBM (PHSBM1) did not show any difference to the FM control; however, the decreasing trend in FER was observed when 30% of the total fish protein content was replaced by SBM and 45% by PHSBM.

The body composition of turbots fed each of the testing diets is as listed in Table 3. No significant differences were found in the crude lipid, and moisture, contents of fish among the groups tested. Body crude protein was reduced significantly when fishmeal protein was replaced by soybean meal at a level of up to 60% (SBM3). The body ash content of fish containing various levels of PHSBM showed no significant differences compared with those fed an FM diet, while SBM2 and SBM3 groups had higher ash contents ($P < 0.05$).

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

Treatments	*Initial body weight (g) /IBW*	*Final body weight (g) /FBW*	*Survival rate (%) /SR*	*Specific growth rate (%/d) /SGR*	*Feed intake (%/d) /FI*	*Feed efficiency ratio (g/g) /FER*
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±2.22	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±1.11	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 superscripted small letters mean significant difference ($P < 0.05$).

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 superscripted small letters mean significant difference (P < 0.05).

The apparent digestibility coefficients (ADC) of dry matter and crude protein are listed in Table 4. The ADC of all the PHSBM diets showed no significant difference compared to the FM diet. However, SBM diets showed significantly lower apparent digestibility coefficients 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 superscripted small letters mean significant difference (P < 0.05).

Discussion

Replacing fishmeal with PHSBM in the formulated feeds for turbot is a challenging task. As far as is known, this study is the first to report the fermentation, along with the enzyme treatment process, of soybean and its use as an alternative plant protein source in the diets of turbot. Some studies reported that fermented soybean meal had better results than soybean meal when replacing equal quantities of fishmeal. Luo, *et al*. (2004) indicated that diets containing 21% fermented soybean meal produced higher growth and PER than the diets containing 20% soybean meal in grouper. Zhou, *et al*. (2011) found that fish fed more than 30% fermented soybean meal as an FM replacement

adversely affected weight gain and specific growth rate in black sea bream. 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 (unpublished) as evinced by previous research. In the present study, the results indicated that PHSBM could replace 45% of the dietary fish meal protein in turbot without any obviously detrimental effects under the presented experimental conditions. This might be due to the addition of phytase. Pre-treatment of soybean meal with phytase therefore can serve as an alternative to improve the quality of protein. Studies showed that phytase could release minerals and increase the utilisation of minerals and then increase the growth performance of the fish (Wang, *et al*., 2009). Roys, *et al*. (2014) report that 30% sesame oilseed meal fermented by a phytase-producing strain significantly increased growth performance of rohu in comparison with fish fed raw sesame oilseed meal. 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 microorganism, particularly due to the 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 the whole-body protein content of turbot while there was a reduction in diets replacing more than 30% soybean meal. A similar observation is also reported in African bony-tongue and yellowtail (Shimeno, *et al*.,1993). The content of small protein molecules are more in fermented soybean meal than in SBM and diets with fermented soybean meal might have better induced protein anabolism than those with soybean meal. However, other studies found that body protein is not affected by dietary high plant protein (Martínez-Llorens, *et al*., 2007; Bonaldo, *et al*., 2008). This may be due to the difference between fish species and the alternative proteins. In this study, the whole-body ash content tended to increase when the substitution levels of SBM increased compared with the FM and PHSBM diets, and in particular, this was true of high-soybean meal content diets. That 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 fermented and phytase process can improve dry matter and crude protein of digestibility in turbot. The 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 utilise PHSBM at limited dietary levels when it was used as a replacement for FM. Based on growth performance and feed utilisation data, soybean meal could replace 30% of the protein of fish meal without any obviously detrimental effects. In contrast the fermented, and phytase-treated, soybean meal could replace 45% of the protein of fish meal. Dietary supplementation with essential amino acid, 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 that of an PHSBM-based diet. The fermented, and phytase-treated, soybean meal is a promising plant protein resource for turbot. From an economic perspective, the PHSBM could improve diet nutritional values, increase the growth performance of turbot, greatly reduce the utilisation of fishmeal, and be used as a cost-effective solution to overcome the problem posed by the global shortage of fishmeal. Currently, fermentation and enzyme treatments are the two efficient and cost effective methods for improving soybean meal utilization and have been widely used in feed industry (Azarm & Lee, 2014;Ávila, *et al.,* 2015). In the present study, we demonstrated that the combination of these two approaches provided superior performance. With high availability and low price, phytase has been widely used in feed industry. Therefore, the combination of fermentation and exogenous enzyme supplementation should be applicable in the aquafeed industry, and especially in diets for high valued fish species such as turbot.

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