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Doctor Forum

Effects of Brown Fish Meal Replacement with Fermented Soybean Meal on Growth Performance, Feed Efficiency and Enzyme Activities of Chinese Soft-shelled Turtle, *Pelodiscus sinensis*

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Abstract A 120-day feeding experiment was conducted to investigate the effects of partial replacement of brown fish meal (BFM) by fermented soybean meal (FSBM) in diets of Chinese soft-shelled turtle (*Pelodiscus sinensis*). The turtles (initial mean body weight, (115.52±1.05) g) were fed with three experimental diets, in which 0%, 4.72% and 9.44% BFM protein was replaced by 0%, 3% and 6% FSBM, respectively. Results showed that the feeding rate (FR), specific growth rate (SGR) and feed efficiency ratio (FER) of turtles fed with the diet containing 3% FSBM were not significantly different from the control group (0% FSBM) ($P>0.05$). However, FR, SGR and FER of turtles fed with the diet containing 6% FSBM were significantly lower than those of the control group ($P<0.05$). No significant differences were observed in the activities of serum glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase among dietary treatments ($P>0.05$). However, the uric acid concentration in turtles fed with the diet containing 3% or 6% FSBM was significantly lower than that in the control group ($P<0.05$). There were no significant differences in the activities of lysozyme, alkaline phosphatase and total superoxide dismutase among dietary treatments ($P>0.05$). The results suggested that FSBM could replace 4.72% BFM protein in turtle diets without exerting adverse effects on turtle growth, feed utilization and measured immune parameters.

Keywords Chinese soft-shelled turtle; *Pelodiscus sinensis*; brown fish meal; replacement; fermented soybean meal

1 Introduction

In the last decade, increased demand and fluctuating price of fish meal (FM) have emphasized the need to seek for alternative protein sources in aquafeeds. Many studies have focused on soybean meal (SBM), which are considered to be the most promising candidate for partial replacement of FM in diets of Chinese soft-shelled turtle (Qian, 1995; Ren *et al*., 1997) and other carnivorous aquatic animals (Refstie *et al*., 1998, 2001; Hernández *et al*., 2007). However, a number of antinutritional factors (ANFs) such as trypsin inhibitors, lectins and soybean globulins, have limited the application of SBM in aquafeed, especially for diets of juveniles (Peres *et al*., 2003; Refstie *et al*., 2005). Fermentation has been proven useful to improve the nutritional level of soybean by reducing ANFs and increasing the bioavailability of nutrients (Hachmeister and Fung, 1993; Egounlety and Aworh, 2003). The fermented soybean meal (FSBM) is generated *via* fermentation of soybean meal with *Aspergillus oryzae*, *Lactobacills plcillarum, Saccharomyces cereviceae*, or a mixture of yeast, bacterial and fungal strains (Hachmeister and Fung, 1993; Egounlety and Aworh, 2003; Hong *et al*., 2004; Hotz and Gibson, 2007). Kiers *et al*. (2003) and Feng *et al*. (2007) found that replacing SBM with FSBM in the diet significantly improved the growth and feed efficiency of pig and broiler.

Chinese soft-shelled turtle (*Pelodiscus sinensis,* Wiegmann), as a high-valued fresh water aquaculture species, has been cultured in many Asian countries such as China, Japan and Korea in the last two decades (Yin *et al*., 2005). Recently, approximately 200,000 tons of this species have been harvested every year in China (Li *et al*., 2008). However, diets for Chinese soft-shelled turtle, which have been based on high levels of FM (> 50%), contribute to most costs in culture of turtles. Hence, replacement of FM with alternative low-cost protein such as FSBM is needed for practical production of diets for Chinese soft-shelled turtle. Replacement of FM with FSBM has been shown to be positive in term of the

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growth performance of aquatic animals such as orange-spotted grouper *Epinephelus coioide* (Luo *et al*., 2004), Japanese flounder *Paralichthys Olivaceus* (Chen, 2006), hybrid tilapia *Oreochromis mossambicus* × *O. niloticus* (Hou, 2009), rainbow trout *Oncorhynchus mykiss* (Matsunari *et al*., 2010; Yamamoto *et al*., 2010), ovate pompano *Trachinotus ovatus* (Liu *et al*., 2010b), black sea bream *Acanthopagrus schlegelii* (Zhou *et al*., 2011), Pacific white shrimp *Litopenaeus vannamei* (Leng *et al*., 2006) and tiger prawn *Penaeus monodo* (Meunpol *et al*., 2009). Nevertheless, replacement of FM by FSBM in the diets of soft-shelled turtles has not previously been reported. Also, no information is available regarding the effects of FSBM on the metabolism and immunity in turtle. Therefore, the present study was designed to evaluate the effects of replacement of brown fish meal (BFM) by dietary FSBM (fermented by a mixture of bacterial and fungal strains) on the growth performance, feed utilization, transaminase activity and immunity of Chinese soft-shelled turtles under a high-intensive condition. The results obtained with fresh water species in the present work may also be of relevance to marine species.

2 Materials and Methods

2.1 Fermented Soybean Meal and Experimental Diets

The SBM (Golden Food/Oilseeds Co. Ltd., Zhejiang, China) and a commercial FSBM (Jiangsu Nangtong Babig Feed Co. Ltd., Jiangshu, China) were used in the present study. The FSBM was produced *via* solid-state fermentation with a mixture of yeast, bacterial and fungal strains including *Saccharomyces cereviceae*, *Lactobacillus plantarum*, *Bifidobacterium lactis* and *Aspergillus oryzae*. Nutrient composition and antinutritional factors of SBM and FSBM are provided in Table 1.

Table 1 Nutrient composition and antinutritional factors of soybean meal (SBM) and fermented soybean meal (FSBM)

Items	SBM	FSBM
Dry matter (%)	88.56	89.37
Crude protein (%)	43.93	49.72
Crude ash (%)	5.62	5.46
Lysine (%)	2.65	2.69
Methionine + Cystine (%)	1.21	1.22
Protein hydrolysis (%)	4.25	10.56
Phytate (mg g^{-1})	8.57	1.31
Trypsin inhibitor (mg g^{-1})	2.59	0.12
Oligosaccharides (mg g^{-1})	15.3	<1.0
Saponin (mg g^{-1})	0.56	<0.01
Soy glycinin (mg g^{-1})	6.4	<0.01
β-conglycinin (mg g^{-1})	2.6	<0.01

The ingredients and chemical composition of the experimental diets are presented in Table 2. Based on previous studies (Nuangsaeng and Boonyaratapalin, 2001; Huang and Lin, 2002; Huang *et al*., 2003, 2005), three experimental diets were formulated to meet nutrient requirements of Chinese soft-shelled turtle. Graded levels of BFM protein (0%, 4.72% and 9.44%) were replaced by those of FSBM (0%, 3% and 6%, Diets 1–3, respectively). Diet 1 with 0% FSBM was used as the control. Ingredients were separately ground into fine powder to pass through 320 μm mesh, then thoroughly dry mixed before the addition of approximately 350 L distilled water and 150 kg saturated vapor per 1 000 kg diet. The mixture was

Table 2 Ingredients and chemical composition of the experimental diets (%)

Items	Diet No. (Substitution levels of BFM protein)		
	Diet 1 (0%, control)	Diet 2 (4.72%)	Diet 3 (9.44%)
Ingredient composition	100.00	100.00	100.00
White fish meal[1]	8.00	8.00	8.00
White fish meal[2]	8.00	8.00	8.00
Brown fish meal (BFM)[3]	47.00	44.00	41.00
Soybean meal (SBM)[4]	5.00	5.00	5.00
Fermented soybean meal (FSBM)[5]	0.00	3.00	6.00
Wheat meal	21.00	21.00	21.00
Vitamin premix[6]	1.20	1.20	1.20
Mineral premix[7]	0.50	0.50	0.50
Other ingredients[8]	9.30	9.30	9.30
Proximate composition (dry matter basis)			
Crude protein	50.02	49.68	49.46
Crude lipid	6.23	5.92	5.54
Crude ash	14.63	14.11	13.50
Gross energy (kJ g^{-1})	20.48	20.45	20.41
Lysine	3.68	3.64	3.61
Methionine	1.06	1.02	0.98
Cystine	0.52	0.51	0.51

Notes: [1] White fish meal, obtained from Fujian Gaolong Industrial Co. Ltd. (origin America), crude protein 67.95%, crude lipid 9.53%, crude ash 21.64%; [2] White fish meal, obtained from Fujian Gaolong Industrial Co. Ltd. (origin Russia), crude protein 65.14%, crude lipid 11.52%, crude ash 22.97%; [3] Brown fish meal, obtained from Zhejiang Wenling Nailuojiao Fish Meal Co. Ltd. (origin Wenling, Zhejiang, China), crude protein 67.23%, crude lipid 12.24%, crude ash 19.96%; [4] Soybean meal, obtained from Golden Food/Oilseeds Co. Ltd. (origin Ningbo, Zhejiang, China); [5] Fermented soybean meal, obtained from Jiangsu Nangtong Babig Feed Co. Ltd. (origin Nantong, Jiangsu, China); [6] Vitamin premix, according to Jia *et al*. (2005); [7] Mineral premix, according to Jia *et al.* (2005); [8] Other ingredients, composition of beer yeast, chicken powder and feeding attractant, etc.

then pelleted through a double-screw extruder with a 3.0 mm die (Beijing Jindi Sanfu Extruder Manufacture Co. Ltd., China). After pelleting, the diets were dried in a convection drier at < 35℃ and stored at −20℃ until use.

2.2 Feeding Experiment

Chinese soft-shelled turtles were obtained from and reared at Hangzhou Wensli Biology Science and Technology Stock Co. Ltd. (Hangzhou, Zhejiang, China). Prior to the feeding experiment, all turtles were fed with the control diet in an indoor culture system for two weeks to acclimate to the experimental diet and environment. Then, 4 800 turtles of homogeneous sizes (initial mean body weight (115.52 ± 1.05) g, n = 12 tanks) were randomly distributed into 12 tanks (3.6 m × 5.0 m × 0.8 m, water depth 60 – 70 cm) using a completely randomized design with 3 treatments and 4 replicate tanks per treatment. The turtles were hand-fed to apparent satiation three times daily (06:00, 12:00 and 20:00).

During the 120-day feeding experiment, each tank was individually aerated and approximately 10% water was exchanged weekly with fully aerated tap water. Water temperature and quality parameters were monitored during the experimental period: rearing water temperature, 29 – 32℃; dissolved oxygen > 4.0 mg L^{-1}; pH 7.0 – 8.5; NH_4^+-N < 3.50 mg L^{-1} and NO_2^--N < 0.15 mg L^{-1}. The light intensity was 0 lx – 20 lx at the water surface.

2.3 Sample Collection and Measurement

2.3.1 Sample collection

During the 120-day feeding experiment, 30 turtles were randomly sampled from each tank and weighed individually on days 0, 60 and 120. The survival rate of turtles on days 60 and 120 was determined by counting remaining individuals in each tank. Prior to sample collection, the turtles were subjected to starvation for 24 h. Then, eight turtles were selected from each tank and killed by a blow on the head. Blood samples were collected from the jugular vein using a tube (10 mL). The serum was separated by centrifugation (H-1850, Hunan, China) at 4 000 × g for 10 min at 4℃. The stomach, pancreas and intestinal tract contents were obtained, with chyme removed using distilled water. All samples were stored at –20℃ prior to analysis.

2.3.2 Analysis of feed ingredients and diets

The chemical composition of feed ingredients, diets and turtle whole body was determined following standard procedures (AOAC, 1995). Samples of feed ingredients, diets and turtle whole body were dried to a constant weight at 105℃ to determine moisture. Crude protein was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method; crude lipid by ether extraction using the Soxhlet method; crude ash by combustion at 550℃, and energy by an adiabatic bomb calorimeter (PARR 1281, USA). The contents of amino acids in feed ingredients and diets were determined by high performance liquid chromatography following the modified procedure of Gardner and Miller (1980). Samples were hydrolyzed with 6 mol L^{-1} HCl at 110℃ for 24 h and the chromatographic separation and analysis of amino acids were performed after orthophthaldehyde (OPA: Sigma) derivation using a reverse-phase high performance liquid chromatograph (HPLC, HP1100, USA). For methionie and cysteine analysis, samples were oxidized with performic acid at −10℃ for 3 h to obtain methionine sulfone and cysteic acid, and then freeze-dried twice with deionized water (Mai *et al*., 2006).

2.3.3 Antinutritional factor assays

The degree of protein hydrolysis in SBM and FSBM, defined as the percentage of peptide chain cleaved, was determined using an amino nitrogen analyzer with trinitrobenzenesulphonic acid (Adler-Nissen, 1979). Phytate content in SBM and FSBM was measured according to Ellis and Morris (1983). Trypsin inhibitor content was analyzed as previously described by Kakade *et al*. (1969). Oligosaccharides content was analyzed using HPLC (HP 1100, USA) according to Delente and Ladenburg (1972). Saponin content was measured using the method of Gurfinkel and Rao (2002). Briefly, saponin preparations, after pretreatment to remove nonsaponin components, were spotted in rows on a thin-layer chromatography plate, along with soyasaponin standards. The plate, without solvent development, was directly treated with sulfuric acid and heated. Violet spots were developed, with a density proportional to the amount of saponin present. The contents of soy glycinin and β-conglycinin were determined using the immunological method of Iwabuchi and Yamauchi (1987). The SBM or FSBM was extracted once with 400 mL of 0.03 mol L^{-1} Tris-HCl buffer (pH 8.0) containing 10 mmol L^{-1} 2-mercaptoe- thanol (ME) at room temperature for 1 h and centrifuged at 15 000 × g for 20 min at 4℃. The resulting supernatant, soy glycinin- and β-conglycinin-rich fraction with high yield, was sterilized by passing through 0.22 μm filters.

2.3.4 Digestive enzyme assays

The samples were homogenized in ice-cold 50 mmol L^{-1} Glycine-HCl buffer (pH 2.5) for stomach and 50 mmol L^{-1} Tris-HCl buffer (pH 8.0) for pancreas and intestine, then centrifuged at 8 000 × g for 5 min at 4℃ and the supernatant was stored at −20℃ prior to enzymatic assays. The supernatants of stomach were used for assay of pepsin activity, while those of pancreas and segments were used for assay of trypsin and amylase, respectively. Pepsin activity was analyzed using the Folin-phenol reagent method (Lowry *et al*., 1951) as modified by Long *et al*. (1997). One unit pepsin activity was defined as a change in absorbance of at 660 nm min^{-1} at pH 2.0 – 2.5 and 37℃. Assays for trypsin activity were carried out as described by Stellmach (1992). Enterokinase was added to the homogenate and allowed to convert the trypsinogen into trypsin. Trypsin activity was then measured using benzoyl DL-arginine-p-nitroanilide as a substrate by the in-

crease absorbance of 0.003 min^{-1} at 253 nm (pH 7.5–8.0, 37℃). Alpha-amylase (E.C. 3.2.1.1) activity of tissue supernatants was measured according to Worthington (1993) using specific analytical procedures and commercially available kits (Jiancheng Bioengineering Institute., Nanjing, China). Enzyme activities were expressed as specific activity (U (mg protein)$^{-1}$).

Protein content was determined according to Bradford (1976) using bovine serum albumin (Sigma A-2153) as a standard.

2.3.5 Analysis of serum glutamic-oxalacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) activities, and uric acid concentration

The enzymatic activities of SGOT and SGPT in serum were assayed according to the method of Reitman and Frankel (1957) as modified by Bergmeyer *et al.* (1978). The enzyme activities in 100 mL serum could be deduced according to the standard curves at 340 nm and 37℃. An enzymatic unit of SGOT or SGPT was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol nicotinamide adenine dinucleotide (NADH) min^{-1}. Uric acid (UA) concentration in serum was determined using a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China) following a carbonate phosphotungstate method (Henry *et al*., 1957).

2.3.6 Analysis of lysozyme (LSZ), alkaline phosphatase (AKP) and total superoxide dismutase (T-SOD) activities

The LSZ activity of serum sampled from experimental turtle was measured using dried *Micrococcus lysodeikticus* as an indicator of serum lysis according to Hultmark *et al*. (1980) and Wang *et al*. (1994). *Micrococcus lysodeikticus* obtained from the Chinese Academy of Preventive Medicine was suspended in ice-cold 0.067 mol L^{-1} potassium phosphate buffer (pH 6.4). A 50 μL sample of serum was added to 3 mL bacterial suspension in an ice-bath, and the absorbance of this mixture was measured at 570 nm (A_0). The mixture was incubated at 37℃ for 30 min, and then transferred back to the ice-bath for 10 min to stop the reaction. The absorbance of the mixture was measured again at 570 nm (A). The LSZ activity (U) was estimated using the following formula: $U=(A_0-A)/A_0$. The activity of AKP was measured using the method of King (1965) as modified by Liu *et al*. (2010a). The optical density was measured at 520 nm. The unit of AKP enzymatic activity was defined as the degradation of 1 mg phenol per g protein at 37℃ within 15 min. The results were expressed as specific activity (U (g protein)$^{-1}$). The activity of total superoxide dismutases (T-SOD) activity was measured by its ability to inhibit superoxide radical-dependent reactions using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Jiangshu, China). One unit of T-SOD was defined as the amount of enzyme required to inhibit the rate of xanthine reduction by 50%. The specific activity of T-SOD was expressed as U mL^{-1} (Biagini *et al*., 1995).

2.4 Calculations and Statistical Analysis

The following calculations were performed:

$$\text{Feeding rate (FR, \% g}^{-1}\text{ day}^{-1}) = \frac{W_d}{\frac{1}{2}(W_f + W_i)D} \times 100,$$

$$\text{Special growth rate (SGR, \% day}^{-1}) = \frac{\text{Ln}W_f - \text{Ln}W_i}{D} \times 100,$$

$$\text{Feed efficiency ratio (FER)} = \frac{W_f - W_i}{W_d - W_e},$$

$$\text{Survival rate (\%)} = \frac{N_f}{N_i} \times 100,$$

where W_f and W_i are the final and initial wet body weights of turtle (g), respectively; W_d and W_e are total amounts of the feed intake and the waste and uneaten feed (g), respectively; D is the duration of experimental days; N_f and N_i are the final and initial numbers of turtles, respectively.

In order to detect statistically significant differences, experimental values were compared using a one-way analysis of variance (ANOVA), and the significance of mean differences was tested using a Duncan's multiple range test. The significance level was set at $P<0.05$. Statistical analyses were performed using SPSS 13.0 for Windows. Values are presented as means±S.D. (Standard Deviation of 4 replicates).

3 Results

3.1 Feeding Rate, Growth Performance and Survival Rate

The turtles accepted all diets equally throughout the experiment. The FR of turtles fed with the diet containing 3% FSBM (0.79% g^{-1} day^{-1}–0.83% g^{-1} day^{-1}) was higher than that of turtles fed with the diet containing 0% FSBM (the control group) on day 60 (0.78% g^{-1} day^{-1}–0.82% g^{-1} day^{-1}), but associated differences were not statistically significant between these two dietary treatments ($P>0.05$) (Table 3). The FR of turtles fed with the diet containing 6% FSBM was significantly lower than that of turtles in the control group on days 60 and 120 ($P<0.05$).

The SGR and FER of selected turtles were affected by the level of dietary FSBM (Table 3). There were no significant differences in SGR and FER between turtles respectively fed with the diets containing 3% FSBM and 0% FEBM (the control group) on days 60 and 120 ($P>0.05$). However, both SGR and FER of turtles fed with the diet containing 6% FSBM were significantly lower than those of the control group ($P<0.05$).

The survival rate of turtles averaged 97.3%–98.1% on day 60 and 91.4%–91.8% on day 120 (Table 3). The survival rate of turtles showed no significant difference among dietary treatments ($P>0.05$).

Table 3 Feeding rate (FR), special growth rate (SGR), feed efficiency rate (FER) and survival rate of turtles fed with experimental diets on days 60 and 120[1, 2]

Diet No. (Substitution levels of BFM protein)	Initial body weight (g)	Final body weight (g)	FR (% $g^{-1} d^{-1}$)	SGR (% d^{-1})	FER	Survival rate (%)
Day 60						
Diet 1 (0%, control)	116.34±0.89	198.59±2.98[b]	0.80±0.02[b]	0.89±0.03[b]	1.05±0.05[b]	97.56±0.63
Diet 2 (4.72%)	115.15±0.84	200.64±3.71[b]	0.81±0.02[b]	0.93±0.02[b]	1.09±0.04[b]	98.13±0.66
Diet 3 (9.44%)	115.06±1.09	186.40±5.15[a]	0.77±0.01[a]	0.80±0.03[a]	0.98±0.04[a]	97.31±0.43
Day 120						
Diet 1 (0%, control)	116.34±0.89	321.69±4.31[b]	0.77±0.01[b]	0.85±0.01[b]	0.96±0.01[b]	91.44±0.55
Diet 2 (4.72%)	115.15±0.84	325.99±6.08[b]	0.77±0.01[b]	0.87±0.01[b]	0.98±0.01[b]	91.75±0.65
Diet 3 (9.44%)	115.06±1.09	295.73±4.89[a]	0.76±0.00[a]	0.79±0.02[a]	0.91±0.02[a]	91.63±0.43

Notes: [1] Values are presented as means±S.D. (n=4); [2] Means in the same column with different superscript letters are significantly different determined by the Duncan's multiple test (P<0.05).

3.2 Whole Body Composition of Turtle

The turtle whole body composition analysis showed that as dietary FSBM increased, whole body protein, lipid and ash exhibited a decreasing trend, and the whole body moisture had an increasing trend. However, no significant differences were observed in the moisture (65.9%–66.4%), crude protein (20.6%–20.8%), lipid (3.7%–4.7%) or ash (7.1%–8.2%) among dietary treatments (P>0.05)(Table 4).

Table 4 Whole body composition of turtles (wet weight) fed with experimental diets on days 60 and 120

Diet No. (Substitution levels of BFM protein)	Moisture (%)	Crude protein (%)	Crude lipid (%)	Crude ash (%)
Day 60				
Diet 1 (0%, control)	66.17±0.24	20.85±0.42	4.70±0.24	7.30±0.27
Diet 2 (4.72%)	66.29±0.44	20.75±0.28	4.55±0.50	7.22±0.21
Diet 3 (9.44%)	66.40±0.23	20.63±0.43	4.47±0.05	7.12±0.27
Day 120				
Diet 1 (0%, control)	65.87±0.34	20.61±0.15	3.76±0.12	8.21±0.22
Diet 2 (4.72%)	66.06±0.20	20.68±0.06	3.72±0.17	8.23±0.32
Diet 3 (9.44%)	66.29±0.50	20.57±0.24	3.66±0.09	8.18±0.16

Notes: Data represent mean±S.D. (n=4). Values in the same column without superscript letter are not significantly different determined by the Duncan's test (P<0.05).

3.3 Activities of Digestive Enzymes

The activities of digestive enzymes in turtles respectively fed with diets containing 3% and 6% FSBM generally increased on days 60 and 120 compared with those of turtles in the control group (Table 5). An exception was that the activity of amylase in pancreas slightly decreased, whereas that of trypsin in intestine slightly increased. The dietary FSBM level did not significantly affect the activities of digestive enzymes in tested turtles, and associated changes in the digestive enzyme activities were not significantly different among dietary treatments (P>0.05) (Table 5).

Table 5 Activities of digestive enzymes of turtles fed with experimental diets on days 60 and 120

Diet No. (Substitution levels of BFM protein)	Pepsin in stomach (U $(mg\ prot)^{-1}$)	Trypsin in pancreas (U $(mg\ prot)^{-1}$)	Amylase in pancreas (U $(mg\ prot)^{-1}$)	Trypsin in intestine (U $(mg\ prot)^{-1}$)	Amylase in intestine (U $(mg\ prot)^{-1}$)
Day 60					
Diet 1 (0%, control)	56.79±9.53	7673.25±578.80	27.43±2.52	724.50±60.61	1.26±0.03
Diet 2 (4.72%)	62.01±15.35	8174.75±401.70	26.62±3.14	753.54±104.61	1.26±0.03
Diet 3 (9.44%)	59.82±11.99	8089.67±227.92	28.03±1.88	774.92±98.60	1.27±0.04
Day 120					
Diet 1 (0%, control)	52.58±6.05	7302.72±622.96	31.04±1.79	738.97±33.14	1.27±0.09
Diet 2 (4.72%)	57.08±6.72	7533.81±262.34	29.19±1.86	741.12±26.42	1.30±0.06
Diet 3 (9.44%)	52.53±8.61	7684.42±571.89	30.86±2.28	721.60±43.47	1.33±0.05

Notes: Data represent mean±S.D. (n=4). Values in the same column without superscript letter are not significantly different determined by the Duncan's test (P<0.05).

3.4 SGOT and SGPT Activities and UA Concentration

The SGOT and SGPT activities of turtles averaged 35.9–36.4 UL^{-1} on day 60 and increased to 53.2–57.0 UL^{-1} on day 120; SGPT activities averaged 19.0–20.3 UL^{-1} on day 60 and increased to 29.9–31.4 UL^{-1} on day 120 (Table 6). There were no significant differences in the activities of SGOT and SGPT among dietary treatments (P>0.05). The serum UA concentration of 0% FEBM-fed turtles (the control group) was significantly higher than that of

3% or 6% FSBM-fed turtles ($P<0.05$). However, no significant differences were observed between serum UA concentrations of 3% FSBM-fed and 6% FSBM-fed turtles ($P>0.05$) (Table 6).

Table 6 Serum glutamic-oxalacetic transaminase (SGOT) and glutamic-pyruvic transaminase (SGPT) activities and uric acid (UA) concentration of turtles fed with experimental diets on days 60 and 120

Diet No. (Substitution levels of BFM protein)	SGOT activity ($U\,L^{-1}$)	SGPT activity ($U\,L^{-1}$)	UA concentration ($umol\,L^{-1}$)
Day 60			
Diet 1 (0%, control)	35.92±7.00	19.02±2.84	118.67±6.53 [b]
Diet 2 (4.72%)	36.41±9.34	19.30±3.12	107.95±5.07 [a]
Diet 3 (9.44%)	35.87±3.82	20.31±2.10	100.56±6.81 [a]
Day 120			
Diet 1 (0%, control)	53.21±7.29	30.74±5.00	131.87±6.91 [b]
Diet 2 (4.72%)	57.01±5.81	29.91±3.89	116.20±10.84 [a]
Diet 3 (9.44%)	53.46±7.55	31.44±5.14	113.16±8.40 [a]

Notes: Data represent mean±S.D. ($n=4$). Values in the same column without superscript letters are not significantly different determined by the Duncan's test ($P<0.05$).

3.5 Activities of LSZ, AKP and T-SOD

The activities of LSZ averaged 0.19–0.20 U on day 60 and slightly increased to 0.20–0.23 U on day 120; the activities of AKP averaged 44.90–49.54 $U\,10^{-2}\,mL^{-1}$ on day 60 and increased to 56.59–63.36 $U\,10^{-2}\,mL^{-1}$ on day 120; the activities of T-SOD averaged 254.91–262.05 $U\,mL^{-1}$ on day 60, then slightly decreased to 243.25–252.40 $U\,mL^{-1}$ on day 120. Associated changes in the activities of LSZ, AKP and T-SOD of turtle serums were not statistically significant among dietary treatments ($P>0.05$) (Table 7).

Table 7 Activities of lysozyme, alkaline phosphatase (AKP) and total superoxide dismutase (T-SOD) in serum of turtles fed with experimental diets on days 60 and 120

Diet No. (Substitution levels of BFM protein)	Lysozyme activity (U)	AKP activity ($U\,10^{-2}mL^{-1}$)	T-SOD activity ($U\,mL^{-1}$)
Day 60			
Diet 1 (0 %, control)	0.19±0.02	44.90±4.04	254.91±24.08
Diet 2 (4.72 %)	0.20±0.02	49.54±3.05	261.16±12.47
Diet 3 (9.44 %)	0.20±0.02	46.77±5.71	262.05±14.67
Day 120			
Diet 1 (0 %, control)	0.20±0.01	56.59±5.66	252.40±5.97
Diet 2 (4.72 %)	0.22±0.01	63.36±6.16	243.25±13.00
Diet 3 (9.44 %)	0.23±0.03	58.77±5.77	250.79±18.62

Notes: Data represent mean±S.D. ($n=4$). Values in the same column without superscript letter are not significantly different determined by the Duncan's test ($P<0.05$).

4 Discussion

Results from this study showed that 4.72% BFM protein could be replaced by 3% FSBM without significantly affecting the growth, feed utilization, whole body composition and measured immune parameters of Chinese soft-shelled turtles under the proposed experimental conditions. This could be related to several previous studies on different aquatic animals. Luo *et al*. (2004) and Zhou *et al*. (2011) found that 10%–20% FM protein could be replaced by FSBM without negative effects on the growth of orange-spotted grouper and black sea bream, respectively. Leng *et al*. (2007) and Meunpol *et al*. (2009) reported that 20%–25% of FM could be replaced by FSBM without significant negative effects on weight gain of Pacific white shrimps and tiger prawns, respectively. The reason for lower substitution levels in diet for turtle may be that soft-shelled turtle is a typical carnivore, which prefer to feed on RFM rather than FSBM (Qian, 1995; Ren *et al*., 1997).

The use of alternative protein sources is generally limited by three main factors: low FR, low feed digestibility and imbalance of essential amino acids (EAA) (Gallagher, 1994; Mambrini *et al*., 1999; Pereira *et al*., 2002; Ai *et al*., 2006). This can be illustrated by our experimental results. Firstly, we found that the replacement of 9.44% of BFM protein with FSBM significantly reduced the feeding rate of turtles (Table 3). This was consistent with previous studies, which showed the FR of aquatic animals decreased with increasing dietary FSBM due to the reduced palatability (Luo *et al*., 2004; Refstie *et al*., 2005). It has been suggested that FSBM lacks feeding stimulants that are abundant in FM, although it contains less ANFs and more small-size peptides than SBM (Lindsay, 1994; Francis *et al*., 2001). Hajen *et al*. (1993) and Refstie *et al*. (2005) also reported elevated FSBM or SBM content caused the diets to become relatively less attractive or palatable, leading to growth reduction. Therefore, lower FR was likely one of the main factors, which decreased the growth of turtles fed with elevated levels of dietary FSBM.

Secondly, the activities of pepsin, trypsin and amylase in pancreas and intestine did not significantly differ among dietary treatments (Table 5), suggesting the feed digestibility did not decrease with increasing dietary FSBM. Similarly, Meunpol *et al*. (2009) reported that the feed digestibility of black tiger prawn was not signifi-

cantly affected by 25% FSBM substitution in the diet, but decreased due to 25% BFM substitution. Zhou *et al*. (2011) also found that 10% FM substitution by FSBM in the diet for black sea bream had no significant effects on the apparent digestibility coefficient of dry matter, crude protein, crude lipid and gross energy compared with fish fed with the control diet (0% FSBM). It was likely that less ANFs and more small-size peptides enhanced the feed digestibility and absorbability of FSBM compared with SBM (Table 1), as higher inclusive levels of SBM could reduce the feed digestibility or activities of digestive enzymes via restriction of nutrient absorption by ANFs in animal (Riche *et al*., 2001; Choi *et al*., 2004; Førde-Skjærvik *et al*., 2006; Tibaldi *et al*., 2006). Hence, we suggest feed digestibility was not the limiting factor for growth depression induced by a high level of FSBM substitution in this study.

Thirdly, we found the EAA supply in turtle diet was compromised as the dietary FSBM increased. When FSBM level increased from 0% to 6%, the lysine and methionine contents in turtle diets declined from 3.68% to 3.61%, and from 1.06% to 0.98%, respectively (Table 2). Our dietary lysine levels (>3.60% dry diet) were all above the previously estimated values required by turtle (2.40% dry diet, and 5.32% dietary protein) (Zhou *et al*., 2001), whereas methionine levels in the diet with 6% FSBM (0.98% dry diet, and 1.98% dietary protein) were lower than the previously estimated values required by turtle (1.03% dry diet, 2.48% dietary protein) (Huang and Lin, 2002). Moreover, when 9.44% BMF was substituted by FSBM, the FR and FER of turtle decreased compared with that of the control group (Table 3). Thus, methionine could be the first limiting amino acid for the turtle growth, and the turtle growth reduction was partially related to EAA deficiencies at higher substitution level of BFM. Additionally, the replacement of BFM with FSBM had no significant effects on the activities of SGOT and SGPT (Table 6). However, the serum UA concentration was found decreasing on days 60 and 120, indicating slow amino acid catabolism due to the replacement of BFM with FSBM. The potentially slow amino acid catabolism could be attributed to EAA deficiency in the diet with 6% FSBM. Together these results indicated that the imbalance of EAA was another factor accounting for reduced turtle growth at higher substitution levels of FSBM in the diet.

To our knowledge, this work first determined the effects of FM substitution with FSBM on the immunity of aquatic animals such as Chinese soft-shelled turtle. Our results showed no significant differences in the survival rate, activities of LSZ, AKP and T-SOD of turtles among dietary treatments. These suggested that the immunity of turtles was not significantly affected by the dietary FSBM levels, even when 9.44% BFM protein was replaced by FSBM. However, the immunity of aquatic animals could decrease with increasing dietary SBM due to reduced antigenic soybean proteins such as glycinin and β-conglycinin (Burrells *et al*., 1999; Lin and Luo, 2011). Previous studies have demonstrated that replacement of SBM with FSBM reduced immunoreactivity of antigenic soybean proteins in newly weaned pigs (Frias *et al*., 2008; Song *et al*., 2008, 2010). The effects of replacement of RFM with FSBM on measured immune parameters could partially be related to the degradation of main antigenic soybean proteins in FSBM.

In conclusion, our results showed that 4.72% BFM protein could be replaced by 3% FSBM in diets without adverse effects on growth performance, feed efficiency and measured immune parameters of Chinese soft-shelled turtle.

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