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Safety level evaluation of dietary 2-hydroxy-4-(methylthio) butanoic acid (HMTBa) for turbot *Scophthalmus maximus* based on growth performances, anti-oxidative responses, and liver and intestine conditions

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ABSTRACT

A 56-day feeding trial was conducted to evaluate the safety level of 2-hydroxy-4-(methylthio) butanoic acid (HMTBa) as a dietary methionine source for turbot *Scophthalmus maximus*. Four isonitrogenous and isolipidic diets were formulated with graded levels of HMTBa (0%, 1%, 5% and 10%). These four experimental diets were named as H-0, H-1, H-5 and H-10, respectively. The effects of dietary HMTBa on growth performances, anti-oxidative responses, and liver and intestine conditions of turbot were analyzed. Results showed that the survival rate in H-10 treatment (95.83%) was significantly lower than that in H-1 (99.58%) ($P < 0.05$). The specific growth rate, feed efficiency, protein efficiency ratio and productive protein value were significantly higher in H-1 and H-5 treatments than those in H-0 and H-10 treatments ($P < 0.05$). The feed intake in H-5 and H-10 treatments was significantly higher than that in H-0 and H-1 treatments ($P < 0.05$). The activities of serum superoxide dismutase, serum glutathione peroxidase, liver glutathione peroxidase, liver catalase as well as the content of serum ascorbic acid and serum thiobarbituric acid reactive substance were significantly influenced by dietary HMTBa. Compared with those in H-0 and H-10 treatments, the significantly higher anti-oxidative abilities were observed in H-1 and H-5 treatments ($P < 0.05$). Deficient (H-0) or excessive (H-10) HMTBa in the diet had significantly negative effects on liver morphology. The same thing occurred on the intermediate and distal intestine structures. The significantly lower intestinal fold height and impaired integrity of intestinal structures were found in H-0 and H-10 treatments. The results in the present study indicated that the supplementation of HMTBa in the diet less than 5% is safe for turbot.

1. Introduction

Limited supplies and potential high costs of fish meal have forced feed manufacturers to use less expensive plant ingredients alternative for fish meal in fish feed (Naylor et al., 2009; Olsen and Hasan, 2012; United States Department of Agriculture, 2009). In the fish feed containing high level of plant protein feedstuffs, methionine is the first limiting essential amino acid, and its deficiency in fish diets decreased fish growth performance and ultimately limited the use of plant ingredients in fish feed (Gatlin and Harrell, 1997; Ma et al., 2013; Mai et al., 2006). Studies indicated that sufficient addition of commercial crystal methionine or methionine analogue in fish feed containing high level plant feedstuffs could increase fish growth performance and feed utilization (Li et al., 2009; Ma et al., 2013; Mai et al., 2006; Mukhopadhyay and Ray, 2001; Opstvedt et al., 2003; Takagi et al., 2001).

The 2-hydroxy-4-(methylthio) butanoic acid (HMTBa) is a commercial synthetic methionine analogue (Dibner, 2003). It is a hydroxy mono-carboxylic acid which bears a hydroxyl group on the α-carbon instead of the amino group found in methionine (Dibner, 2003). As it can be rapidly assimilated in the intestine and converted to methionine within the animal body through broadly distributed enzymatic systems (Dibner, 2003; Yi et al., 2006), HMTBa was utilized as dietary methionine source for red drum (*Sciaenops ocellatus*) (Goff and Gatlin, 2004), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Li et al., 2009), and Jian carp (*Cyprinus carpio* var. Jian) (Feng et al., 2011; Xiao et al., 2011). Our previous study on turbot (*Scophthalmus maximus* L.),

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which is a highly valued marine flatfish in China since the 1990s, also found that turbot could use HMTBa as effectively as or better than crystal methionine to achieve a higher maximum specific growth rate (Hou et al., 2012; Ma et al., 2013). On the basis of SGR, the dietary total methionine requirements based on L-Methionine and HMTBa for juvenile turbot were 1.58% and 1.56% respectively, and the optimum addition levels of L-Methionine and HMTBa are 0.99% and 0.97%, separately, with 0.59% methionine in the basis diet (Ma et al., 2013).

However, study on Jian carp indicated that fish growth performance, feed utilization, digestive enzyme activities, liver and intestine health conditions (Xiao et al., 2011) as well as host anti-oxidative capacities (Feng et al., 2011; Xiao et al., 2012) decreased or impaired when the supplementation level of HMTBa in fish diet excessed its safety level (12.7 g/kg diet). However, the information on the safety level of dietary HMTBa for turbot is still not available (Hou et al., 2012). Therefore, before HMTBa is used as a commercial feed additive in turbot feed industry, it is necessary to confirm its safety level for turbot. The purpose of this study was to evaluate the safety level of dietary HMTBa for turbot based on the growth performance, anti-oxidative responses, and liver and intestine histology of turbot.

2. Materials and methods

2.1. Experimental diets

The basal diet (H-0, Table 1), containing 48% crude protein, 12% crude lipid, 0.59% methionine and 0.41% cysteine, was similar with that of our previous studies (Hou et al., 2012; Ma et al., 2013). The crystalline L-amino acid premix was supplemented to diet according to the whole-body amino acid pattern of turbot except for methionine and cystine (Table 2) (Kaushik, 1998). Ma et al. (2013) suggested that the dietary total methionine requirement for juvenile turbot was estimated to be 1.56% (1% of HMTBa + 0.56% of L-methionine). According to the "Technical Guidance: Tolerance and efficacy studies in target animals" (European Food Safety Authority, EFSA, 2011) and the "Guidelines for Tolerance Test of Feeds and Feed Additives in Target Aquatic Animals" (Ministry of Agriculture of China, 2012), the 0% of HMTBa was used as the control level, 1% of HMTBa was the use-level, and 5% and 10% of HMTBa were two tolerance levels (5× and 10× folds of the use-level). Graded levels of HMTBa were added respectively to formulate four isonitrogenous and isolipidic experimental diets. They were named as H-0, H-1, H-5 and H-10, respectively. The HMTBa was supplemented in the form of Mera™ Met (an 84% Ca salt of HMTBa, Novus International Inc., St. Charles, MO, USA). The final L-methionine levels of four diets were 0.60%, 0.59%, 0.58% and 0.59%, separately, as determined by amino acid analyzer (S7130, Sykam, Munich, Germany). Following the method of Ontiveros et al. (1987), the final HMTBa contents of the four experimental diets (H-0, H-1, H-5, H-10) were 0%, 1.01%, 4.89% and 10.07%, respectively, as analyzed by the reverse-phase high-performance liquid chromatography (HPLC, HP 1100, Agilent, Santa Clara, USA). The HPLC with a Zobar C18 column (4.6 mm × 250 mm) was used. Mobile phase was 0.05% trifluoroacetic acid in water, and the effluent was monitored by a UV detector (wave length 210 nm).

Diet ingredients were ground through 80-mesh size. After mixed with the progressive enlargement method, all ingredients were thoroughly blended with oil and water. Then diets were pelleted with a pelletizer and dried for 12 h in a ventilated oven at 50 °C. After drying, feeds were packed in double plastic bags and stored at −20 °C until used.

2.2. Feeding trial

Turbot juveniles were obtained from a commercial farm in Haiyang, Shandong, China. Prior to the start of the feeding trial, fish were acclimated to a commercial diet (Qingdao Great Seven Bio-Tech Co. Ltd., Qingdao, China) for two weeks. Then the fish were fasted for 24 h and weighed. A total of 960 fish with similar size (initial weight: 3.76 ± 0.01 g, means ± S.E.M.) were randomly distributed to 4 treatments with 6 replicates. There were 24 cylindrical fiberglass tanks, and each tank (500 L) was stocked with 40 fish.

Table 1
Formulation and compositions of the experimental diets (%).

Ingredient	H-0	H-1	H-5	H-10
	0%	1% HMTBa	5% HMTBa	10% HMTBa
Fish meal[a]	23.60	23.60	23.60	23.60
Soybean meal[a]	22.60	22.60	22.60	22.60
Beer yeast[a]	5.00	5.00	5.00	5.00
Crystalline amino acid premix[b]	16.57	16.57	16.57	16.57
Microcrystalline cellulose	19.03	17.84	13.08	7.13
Fish oil	8.50	8.50	8.50	8.50
Lecithin	1.00	1.00	1.00	1.00
Mineral premix[c]	0.50	0.50	0.50	0.50
Vitamin premix[d]	0.50	0.50	0.50	0.50
Choline chloride	0.25	0.25	0.25	0.25
$Ca(H_2PO_4)_2 \cdot H_2O$	0.30	0.30	0.30	0.30
Alginate	1.50	1.50	1.50	1.50
Attractant	0.50	0.50	0.50	0.50
Mold inhibitor	0.10	0.10	0.10	0.10
Antioxidant	0.05	0.05	0.05	0.05
Mera Met [e]	0	1.19	5.95	11.90
Total	100	100	100	100
Proximate composition (n = 6)				
L-Methionine	0.60	0.59	0.58	0.59
HMTBa	0.00	1.01	4.89	10.07
Lysine	3.11	3.13	3.08	3.05
Cystine	0.41	0.40	0.42	0.41
Moisture	6.62	6.14	5.84	6.21
Crude protein	47.50	47.81	48.03	47.32
Crude lipid	11.67	11.31	12.04	11.87
Ash	6.80	7.10	7.00	7.00

[a] Fish meal, obtained from Great Seven Bio-tech (Qingdao, China), crude protein 71.65% and crude lipid 6.89%; soybean meal, obtained from Great Seven Bio-tech, crude protein 45.60% and crude lipid 1.70%; beer yeast, obtained from Great Seven Bio-tech, crude protein 53.40% and crude lipid 0.98%.

[b] Crystalline amino acid premix (g/100 g diet): arginine 1.44, histidine 0.52, isoleucine 0.49, leucine 0.82, lysine 1.18, phenylalanine 0.75, threonine 0.77, valine 0.58, alanine 1.49, aspartic acid 1.41, glutamic acid 1.98, glycine 2.57, serine 0.86, tyrosine 0.50, and proline 1.21. According to the whole body amino acid composition of turbot (Kaushik, 1998).

[c] Mineral premix (mg/kg diet): $MgSO_4 \cdot H_2O$, 1200; $CuSO_4 \cdot 5H_2O$, 10; $FeSO_4 \cdot H_2O$, 80; $ZnSO_4 \cdot H_2O$, 50; $MnSO_4 \cdot H_2O$, 45; $CoCl_2 \cdot 6H_2O$ (1%), 50; $Ca(IO_3)_2$ (1%), 60; Na_2SeO_3 (1%), 20; and zeolite, 3485.

[d] Vitamin premix (mg/kg diet): thiamin, 25; riboflavin, 45; pyridoxine HCl, 20; vitamin B12, 10; vitamin K3, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, 60; retinal acetate, 32; cholecalciferol, 5; α-tocopherol, 240; ascorbic acid, 2000; ethoxyquin 3; and microcrystalline cellulose, 1470.

[e] An 84% Ca salt of HMTBa, Novus International Inc., St. Louis, MO, Shanghai, China.

The feeding trial was carried out in an indoor flow-through water system for 8 weeks, with water flow rate at about 4.7 tank volumes/h. Fish were carefully hand-fed little by little till apparent satiation twice daily at 07:30 and 19:30, respectively. The uneaten feeds were removed by siphoning twice daily at 8:30 and 20:30. During the feeding trial, the feed consumptions were recorded weekly. The number and the weight of dead fish were recorded daily. The water temperature ranged from 19 to 22 °C, salinity ranged from 24‰ to 26‰, and dissolved oxygen was higher than 7 mg L^{-1}.

2.3. Sample collection and analysis

2.3.1. Growth performance and compositions of diets and the whole-body

Before the feeding trial, 25 fish were randomly collected and stored at −20 °C for the determination of the initial whole-body proximate composition. At the termination of the feeding trial, fish were fasted for 24 h, then were counted and weighed. Six fish per tank were randomly collected and stored at −20 °C for the determination of the whole-body composition.

The compositions of the experimental diets and the fish whole-body were analyzed for the contents of moisture, crude protein, crude lipid and ash using the standard methods of the Association of Official

Table 2
Amino acid composition of experimental diets (g/100 g).

Amino acids	Amount in				Total	The whole body amino acid pattern of turbot[a]
	23.6 g FM	22.6 g SBM	5 g yeast	AAP		
EAA						
Arginine	0.99	0.72	0.11	1.44	3.26	3.26
Histidine	0.45	0.33	0.04	0.52	1.34	1.34
Isoleucine	0.65	0.45	0.09	0.49	1.68	1.68
Leucine	1.19	0.79	0.16	0.82	2.96	2.96
Lysine	1.12	0.66	0.15	1.18	3.11	3.11
Methionine	0.37	0.18	0.04	Variable	Variable	1.58
Phenylalanine	0.62	0.49	0.08	0.75	1.94	1.94
Threonine	0.66	0.40	0.10	0.77	1.93	1.93
Valine	0.75	0.48	0.11	0.58	1.92	1.92
NEAA						
Alanine	0.91	0.48	0.13	1.49	3.01	3.01
Aspartic acid	1.45	1.08	0.20	1.41	4.14	4.14
Cystine	0.20	0.18	0.03	–	0.42	0.49
Glutamic acid	2.18	1.80	0.32	1.98	6.28	6.28
Glycine	0.97	0.48	0.10	2.57	4.12	4.12
Serine	0.72	0.50	0.10	0.86	2.18	2.18
Tyrosine	0.53	0.35	0.22	0.50	1.60	1.60
Proline	0.57	0.44	0.11	1.21	2.33	2.33

FM, fish meal; SBM, soybean meal; WM, wheat meal; AAP, amino acid premix; EAA, essential amino acid; NEAA, non-essential amino acid.
[a] Amino acid composition of experimental diet was in accordance with the whole body amino acid composition of turbot (Kaushik, 1998).

Analytical Chemists (AOAC) (1995). The samples were dried to a constant weight at 105 °C to determine the moisture content. The crude protein contents were determined by measuring nitrogen (N × 6.25) using the Kjeldahl method (2300-Auto-analyzer, FOSS, Denmark), crude lipid content by ether extraction using Soxhlet method (36680-analyzer, BUCHI, Switzerland), and ash content by combustion at 550 °C. The amino acid compositions, except for the methionine and cysteine, in dietary ingredients and the experimental diets were determined by automatic amino acid analyzer (Biochrom 30 Ltd®, Cambridge, UK) after acid hydrolysis in 6 N HCl for 24 h at 110 °C (Ma et al., 2013). While for the methionine and cysteine contents, the samples were oxidized with performic acid, then hydrolyzed and analyzed by the amino acid analyzer (S7130, Sykam, Germany).

2.3.2. Enzyme activities

Another ten fish per tank were anesthetized with eugenol (1:10,000) (Shanghai Reagent Corp, China). Blood samples were collected from the caudal vein with 1 ml syringes, and stored at 4 °C for 5 h. After that, the samples were centrifuged at 4000 *g* for 10 min. And then, the serum samples were stored at −20 °C. Liver samples were initially frozen in liquid nitrogen, and then stored at −80 °C before analysis. The serum samples and liver samples were used for the analysis of enzyme activity.

The contents of ascorbic acid (AA) and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), in the liver and serum were analyzed by using the commercial assay kits (Nanjing Jiancheng Bioengineer Institute, Nanjing, China), respectively. Thiobarbituric acid reactive substance (TBARS) contents in the liver and serum were analyzed by QuantiChrom™ TBARS assay kit (Bas-biotech, Inc., Chengdu, China).

The activities of glutamic–oxaloacetic transaminase (GOT), glutamic–pyruvic transaminase (GPT) and alkaline phosphatase (AKP) in the liver and serum were determined by using the commercial assay kits (Nanjing Jiancheng Bioengineer Institute, Nanjing, China).

2.3.3. Somatic indexes and liver and intestine morphologies

The liver and total digestive tract of three fish per tank were dissected and weighed to calculate the hepatosomatic index (HSI) and viscerosomatic index (VSI). The middle part (about 0.5 cm long) of the intermediate intestine and distal intestine was collected, respectively (Bonaldo et al., 2011). Then these samples were rinsed in saline (9 g L^{-1}) to remove eventual remaining gut contents. Liver samples (size: 0.5 cm × 0.5 cm × 0.5 cm) were also collected.

All the samples, including liver samples and intestine samples, were fixed by immersion in Bonn's stationary liquid (saturated water solution of picric acid/40% formol/acetic acid, 15/5/1). After 24 h fixation in Bonn's stationary liquid, the samples were transferred and kept in 70% ethanol. After that, the intestine and liver samples were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin wax according to the standard histological procedures (Amin et al., 1992; Hu et al., 2015). The liver samples were oriented so that the liver was cut in the median zone. The intermediate and distal intestine samples were cut following the axis of gut lumen by Lecia Jung RM 2016 rotary microtome. All sections were stained with Hematoxylin–Eosin (H&E) for the histological analysis. The histological observations were performed using the Nikon eclipse Ti–S microscope (Nikon, Japan). The livers and intestines were observed for the lesions generally described (Amin et al., 1992; Baeverfjord and Krogdahl, 1996; Refstie et al., 1999; Hu et al., 2015). The intestine structures, such as the height of the simple fold (hSF), the height of the complex fold (hCF), the total length of the intestinal epithelium over 500 μm distance (lIE), the height of the microvillus (hMV), the number of the goblet cell from one section (nGC) and the thickness of the muscularis (tM), were examined to assess the modifications of the intestinal morphometry according to the methods described by Escaffre et al. (2007), Bonaldo et al. (2011) and Hu et al. (2015).

2.4. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using SPSS 16.0 for windows. When overall differences were significant ($P < 0.05$), Tukey's test was used to compare the means among individual treatments.

3. Results

3.1. Survival rate, growth performance and somatic indexes

The survival rate (SR) of fish in H-0, H-1 and H-5 treatments showed no significant difference and ranged from 96.67% to 99.58% ($P > 0.05$) (Table 3). However, SR in H-10 treatment (95.83%) was significantly lower than that in H-1 treatment (99.58%) ($P < 0.05$). Fish fed the basal diet (H-0) significantly showed the lowest specific growth rate (SGR) (2.04%/day) ($P < 0.05$). The SGRs in H-1 and H-5 treatments were significantly higher than those in H-0 and H-10 ($P < 0.05$). Moreover, the highest SGR was found as 3.38%/day in H-1 treatment.

Generally, the feed efficiency (FE), protein efficiency ratio (PER) and productive protein value (PPV) of turbot significantly increased and thereafter significantly decreased with the increasing dietary HMTBa supplemented levels ($P < 0.05$) (Table 3). The FE, PER and PPV of fish fed with H-1 diet significantly had the highest values ($P < 0.05$). The feed intake (FI) of fish fed with H-5 and H-10 diets was significantly higher than those in H-0 and H-1 treatments ($P < 0.05$). However, no significant difference in FI was observed between the H-5 and H-10, or between the H-0 and H-1 ($P > 0.05$).

Condition factor (CF), viscera somatic index (VSI) and hepatic somatic index (HSI) were not significantly influenced by dietary HMTBa levels ($P > 0.05$) (Table 3).

3.2. Whole-body composition

Fish fed with H-0 diet significantly had the lowest whole-body crude protein and crude lipid contents and highest moisture and ash contents ($P < 0.05$) (Table 4). However, the contents of crude protein, crude lipid,

Table 3
Effects of dietary HMTBa on the survival, growth responses, feed utilization and somatic indexes of turbot.

Items	H-0	H-1	H-5	H-10
Survival rate (SR, %)[a]	98.33 ± 0.83[ab]	99.58 ± 0.42[b]	96.67 ± 0.83[ab]	95.83 ± 1.05[a]
Initial weight (g)	3.77 ± 0.01	3.76 ± 0.01	3.75 ± 0.01	3.76 ± 0.01
Specific growth rate (SGR, %/day)[b]	2.04 ± 0.05[a]	3.38 ± 0.06[c]	3.23 ± 0.02[c]	2.64 ± 0.02[b]
Feed efficiency (FE)[c]	0.87 ± 0.03[a]	1.17 ± 0.01[c]	1.00 ± 0.03[b]	0.86 ± 0.03[a]
Feed intake (FI, %/day)[d]	2.12 ± 0.05[a]	2.25 ± 0.05[a]	2.53 ± 0.07[b]	2.56 ± 0.09[b]
Protein efficiency ratio (PER)[e]	1.82 ± 0.07[a]	2.46 ± 0.03[c]	2.11 ± 0.07[b]	1.82 ± 0.06[a]
Productive protein value (PPV, %)[f]	25.01 ± 1.06[a]	37.28 ± 0.52[c]	31.16 ± 1.33[b]	26.37 ± 1.00[a]
Condition factor (CF, %)[g]	3.39 ± 0.09	3.68 ± 0.13	3.66 ± 0.05	3.52 ± 0.07
Viscera somatic index (VSI, %)[h]	6.03 ± 0.12	5.66 ± 0.12	5.79 ± 0.19	5.78 ± 0.19
Hepatic somatic index (HSI, %)[i]	1.21 ± 0.12	1.30 ± 0.07	1.32 ± 0.04	1.06 ± 0.09

Values are means ± S.E.M. of six replicates and values within the same row with different letters are significantly different ($P < 0.05$).
[a] Survival rate (SR, %) = 100 × (final amount of fish) / (initial amount of fish).
[b] Specific growth rate (SGR, %/day) = 100 × ln [(final mean body weight, g) / (initial mean body weight, g)] / (days of feeding trial, day).
[c] Feed efficiency (FE) = (body weight gain, g) / (total feed consumed, g).
[d] Feed intake (FI, %/day) = 100 × (total feed consumed, g) / {(days of feeding trial, day) × [(initial body weight, g) + (final body weight, g)] / 2}.
[e] Protein efficiency ratio (PER) = (body weight gain, g) / (total protein intake, g).
[f] Productive protein value (PPV, %) = 100 × (body protein gain, g) / (protein intake, g).
[g] Condition factor (CF, %) = 100 × (body weight, g) / (body length, cm)^3.
[h] Hepatosomatic index (HSI, %) = 100 × (liver weight, g) / (body weight, g).
[i] Viscerosomatic index (VSI, %) = 100 × (viscera weight, g) / (body weight, g).

Table 4
Effects of dietary HMTBa on the whole-body composition of turbot.

Items	H-0	H-1	H-5	H-10
Moisture (%)	78.55 ± 0.25[b]	76.16 ± 0.11[a]	76.26 ± 0.51[a]	76.67 ± 0.36[a]
Crude protein (%)	13.74 ± 0.16[a]	15.16 ± 0.07[b]	14.78 ± 0.31[b]	14.53 ± 0.15[b]
Crude lipid (%)	2.79 ± 0.17[a]	4.83 ± 0.10[b]	5.05 ± 0.26[b]	4.84 ± 0.19[b]
Ash (%)	4.49 ± 0.05[b]	3.67 ± 0.07[a]	3.53 ± 0.14[a]	3.43 ± 0.06[a]

Values are means ± S.E.M. of six replicates and values within the same row with different letters are significantly different ($P < 0.05$).

moisture and ash in the whole body did not show significant differences among the HMTBa supplemented diets (H-1, H-5 and H-10) ($P > 0.05$).

3.3. Anti-oxidative responses in the serum and liver

The SOD activity and the AA content in the serum of fish fed with HMTBa diets (H-1, H-5 and H-10) were significantly higher than those in control (H-0) ($P < 0.05$) (Table 5). The H-1 treatment had significantly higher serum AA content than H-10 ($P < 0.05$). The fish in H-1 treatment had significantly higher serum GSH-Px activity and lower content of serum TBARS than those in H-0 and H-10 treatments ($P < 0.05$). Dietary HMTBa did not significantly affect the activities of serum CAT ($P > 0.05$).

The activities of CAT and GSH-Px in the liver in H-1 treatment were significantly higher than those in H-0 and/or H-10 treatments ($P < 0.05$) (Table 5). No significant differences were observed in the activities of liver SOD and the contents of liver TBARS among all treatments ($P > 0.05$).

3.4. Liver health parameters

The activities of serum AKP in H-1 treatment and the activities of serum GPT in H-5 treatment were significantly lower than those in H-0 and H-10 treatments ($P < 0.05$) (Table 6). The fish fed with H-1 and H-5 diets showed significantly lower activities of serum GOT than that in H-0 treatment, while had significantly higher activities of liver GPT than that in H-10 treatment ($P < 0.05$). Liver GOT activities showed no significant difference among treatments ($P > 0.05$).

3.5. Liver histology

The impaired integrity of liver histological structures, especially for the increased hepatic sinusoids (hc) or cell vacuolus structures (cvs), was observed in fish fed with H-0 and H-10 diets (Fig. 1 1-1 and 1-4). No significant lesion or damage was observed in the liver histological structures of fish fed with H-1 and H-5 diets (Fig. 1 1-2 and 1-3).

3.6. Intestine histology

No significant lesion or damage was observed in the intermediate and distal intestines of fish fed with H-1 and H-5 diets (Figs. 2 2-2, 2-3, 3 3-2 and 3-3). All sections in these treatments showed integrated histological structures with an intact mucosa, unbroken tidy villus folds and enterocyte with regular basal nucleus. However, the sections of the intermediate and distal intestines in H-0 and H-10 treatments showed the impaired integrity of intestinal histological structures, such as the hyperplasia of loose connective tissue (lct), adipose cells

Table 5
Effects of dietary HMTBa on the serum and liver anti-oxidant responses of turbot.

Items	H-0	H-1	H-5	H-10
Serum superoxide dismutase (SOD, U/ml)	108.56 ± 4.58[a]	130.89 ± 4.27[b]	141.89 ± 4.17[b]	130.73 ± 3.42[b]
Serum catalase (CAT, U/ml)	1.68 ± 0.25	2.10 ± 0.48	1.71 ± 0.15	2.06 ± 0.11
Serum glutathione peroxidase (GSH-Px, U/ml)	108.06 ± 13.19[a]	154.74 ± 12.95[b]	127.00 ± 5.96[ab]	111.65 ± 9.39[a]
Serum ascorbic acid (AA, μg/ml)	27.68 ± 0.41[a]	34.91 ± 0.82[c]	32.41 ± 0.70[bc]	30.98 ± 0.70[b]
Serum thiobarbituric acid reactive substance (TBARS, μM MDA equivalents)	2.86 ± 0.11[b]	2.08 ± 0.21[a]	2.65 ± 0.18[ab]	2.95 ± 0.19[b]
Liver SOD (U/mg prot)	51.82 ± 3.88	55.99 ± 4.16	54.58 ± 6.53	51.05 ± 5.27
Liver CAT (U/mg prot)	7.69 ± 0.67[a]	12.44 ± 0.75[b]	10.18 ± 0.73[ab]	7.49 ± 1.43[a]
Liver GSH-Px (U/mg prot)	17.81 ± 1.36[a]	25.54 ± 1.40[b]	23.07 ± 1.12[ab]	23.42 ± 2.21[ab]
Liver TBARS (μM MDA equivalents)	43.69 ± 4.10	62.37 ± 15.73	51.40 ± 4.00	67.66 ± 16.61

Values are means ± S.E.M. of six replicates and values within the same row with different letters are significantly different ($P < 0.05$).

Table 6
Effects of dietary HMTBa on the liver health parameters of turbot.

Items	H-0	H-1	H-5	H-10
Serum alkaline phosphatase (AKP, U L^{-1})	16.28 ± 1.43^{b}	10.07 ± 1.86^{a}	11.57 ± 1.50^{ab}	16.07 ± 1.57^{b}
Serum glutamic–oxaloacetic transaminase (GOT, U L^{-1})	7.39 ± 0.50^{b}	4.71 ± 0.57^{a}	3.23 ± 0.38^{a}	5.20 ± 0.37^{ab}
Serum glutamic–pyruvic transaminase (GPT, U L^{-1})	1.04 ± 0.11^{b}	0.83 ± 0.05^{ab}	0.37 ± 0.08^{a}	0.95 ± 0.17^{b}
Liver GOT (U/g prot)	1.11 ± 0.44	1.96 ± 0.41	2.03 ± 0.65	2.58 ± 0.69
Liver GPT (U/g prot)	78.72 ± 10.41^{ab}	90.81 ± 8.77^{b}	90.32 ± 9.77^{b}	54.73 ± 6.76^{a}

Values are means ± S.E.M. of six replicates and values within the same row with different letters are significantly different ($P < 0.05$).

(ac) in the submucosa or the increased invagination (i) in the mucosa (Figs. 2 2-1, 2-4, 3 3-1 and 3-4).

The lIE in the intermediate intestine and the hCF and lIE in the distal intestine of turbot in H-1 treatment were significantly higher than those of fish in H-0 treatment ($P < 0.05$) (Table 7). More dietary HMTBa supplementation (>1%) did not result in further significant increases for these parameters ($P > 0.05$). The hCF in the intermediate intestine of turbot in H-5 treatment was significantly higher than that in H-0 ($P < 0.05$). The hSF in the intermediate and distal intestines of turbot from H-1 treatment was significantly higher than those of fish from H-0 and/or H-10 treatments ($P < 0.05$). No significant differences were observed in the hMV, the nGC and the tM among all treatments both in the intermediate intestine and the distal intestine ($P > 0.05$).

4. Discussion

It was confirmed in the present study that HMTBa, as a commercial synthetic methionine analogue, could be used to meet turbot's requirement for methionine and increase turbot growth performance and feed utilization when methionine was deficient in the diets. Similar results were also reported in previous studies on turbot (Ma et al., 2013), rainbow trout (*Oncorhynchus mykiss*) (Cheng et al., 2003), hybrid striped bass (Li et al., 2009) and Jian carp (Xiao et al., 2011).

However, when the supplementation level of dietary HMTBa increased to 10%, the survival and growth of turbot were significantly decreased. Depressed growth performance was also reported in the studies of overdosing dietary HMTBa on Jian carp (Xiao et al., 2011) and overdosing dietary methionine on Indian major carp (*Cirrhinus mrigala*) (Ahmed et al., 2003), large yellow croaker (*Pseudosciaena crocea*) (Mai et al., 2006) and Cobia (*Rachycentron canadum*) (Zhou et al., 2006). Actually, in the present study, when dietary HMTBa supplementation was higher than 1% (H-5 and H-10), the FE, PER and PPV significantly decreased, while the FI significantly increased. From this point of view, dietary HMTBa supplementation for turbot should be less than 5%. Until now, the reason why the high level of dietary HMTBa depressed growth performance and feed utilization of fish has not been clearly understood. As the HMTBa could convert to methionine within animal body through broadly distributed enzymatic system, high level of methionine in the host body would generate a relatively toxic characteristic for animals (Baker, 2006; Yi et al., 2006). This could be the reason why the high level of dietary HMTBa depressed growth performance and feed utilization in turbot. Meanwhile, methionine could work as feed attractants to stimulate fish feed intake by increasing feed palatability (Hubbard et al., 2011; Pérez-Jiménez et al., 2013). This could explain that the high level of dietary HMTBa increased the feed intake.

Previous studies reported that dietary HMTBa could improve the anti-oxidative capacities of fish, such as turbot and Jian carp (Feng et al., 2011; Xiao et al., 2011; Hou et al., 2012; Ma et al., 2013). In the present study, fish fed the basal diet (H-0) showed the lowest serum and liver anti-oxidative capacities. The activities of serum SOD, liver CAT, and serum and liver GSH-Px and the content of serum AA of turbot increased with the addition of HMTBa in the diets, while the content of

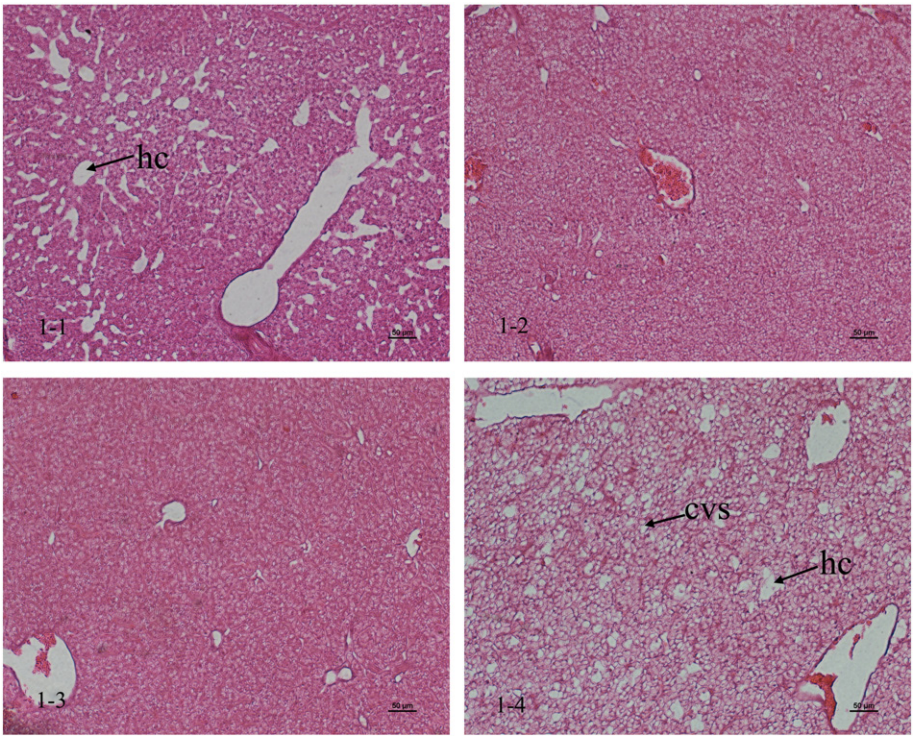

Fig. 1. Effects of dietary HMTBa on liver histology of turbot.

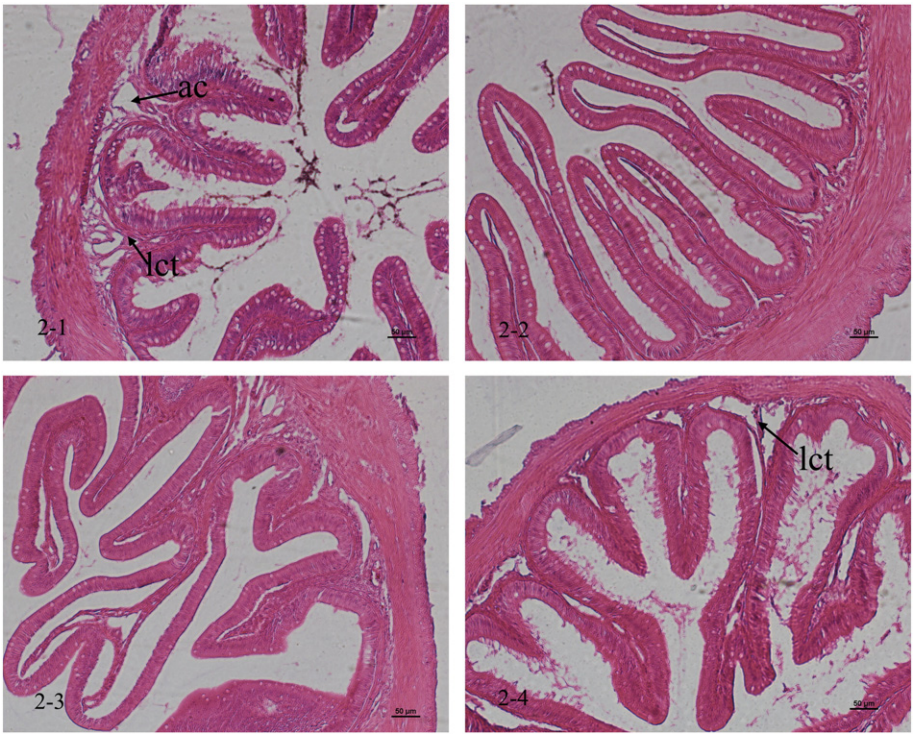

Fig. 2. Effects of dietary HMTBa on intermediate intestine histology of turbot.

serum TBARS of turbot decreased. However, dietary HMTBa supplementation higher than 1% did not result in further significant increase of the anti-oxidative capacities or decrease of the oxidative stress (TBARS) in turbot. Furthermore, when dietary HMTBa supplementation was higher than 5% (H-10), the anti-oxidative capacities were significantly decreased, while the oxidative stress was significantly increased. Similarly negative effects by high level of dietary HMTBa were also reported in Jian carp (Feng et al., 2011; Xiao et al., 2012). From this point of view, dietary HMTBa supplementation for turbot should be less than 5%. Previous studies on HMTBa suggested that high level of dietary HMTBa had a similar role like methionine to make DNA hypermethylated and down-regulated some gene expression, which could decrease the synthesis of antioxidant enzymes (Waterland, 2006; Feng et al., 2011).

Serum GOT and GPT as well as AKP often act as markers of tissue injury in terrestrial animals, as it would leak out from the cell into the blood when tissue injury occurred (Yoshikawa et al., 2002). In the

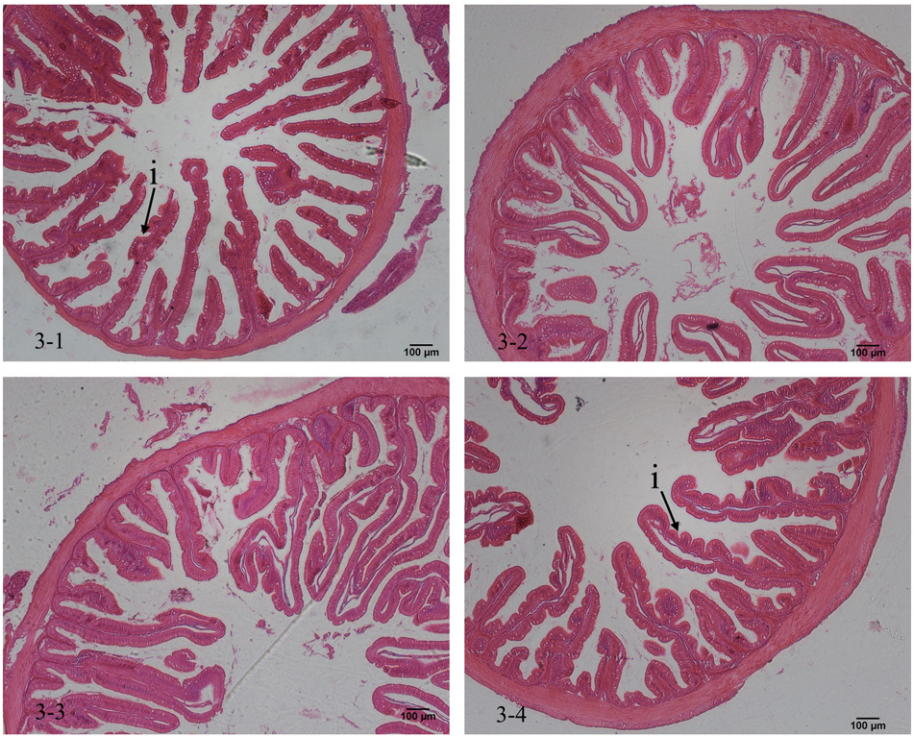

Fig. 3. Effects of dietary HMTBa on distal intestine histology of turbot.

Table 7
Effects of dietary HMTBa on intermediate and distal intestine histology of turbot.

Diet	H-0	H-1	H-5	H-10
Intermediate intestine				
hSF (μm)[a]	272.80 ± 18.51[a]	387.23 ± 13.37[c]	372.04 ± 5.08[bc]	315.98 ± 9.21[ab]
hCF (μm)[b]	676.96 ± 87.26[a]	1003.30 ± 40.92[ab]	1171.73 ± 82.47[b]	841.71 ± 66.36[ab]
lIE (μm)[c]	2060.56 ± 75.90[a]	2628.99 ± 122.67[b]	2412.72 ± 104.70[ab]	2361.30 ± 129.56[ab]
hMV (μm)[d]	3.25 ± 0.16	3.36 ± 0.08	3.36 ± 0.13	3.48 ± 0.12
tM (μm)[e]	88.49 ± 10.65	113.62 ± 16.21	118.24 ± 16.55	115.21 ± 12.46
nGC (unit)[f]	536.60 ± 77.83	775.50 ± 101.53	712.33 ± 84.34	572.00 ± 157.00
Distal intestine				
hSF (μm)[a]	450.51 ± 3.25[ab]	534.54 ± 24.13[b]	433.59 ± 26.06[ab]	417.12 ± 36.84[a]
hCF (μm)[b]	1063.31 ± 58.95[a]	1422.25 ± 9.87[b]	1390.39 ± 53.26[ab]	1120.08 ± 108.65[ab]
lIE (μm)[c]	2905.42 ± 246.17[a]	4522.00 ± 402.51[b]	4224.01 ± 382.15[ab]	3141.87 ± 305.07[ab]
hMV (μm)[d]	3.78 ± 0.03	3.74 ± 0.25	3.75 ± 0.02	3.80 ± 0.23
tM (μm)[e]	120.10 ± 10.98	106.65 ± 4.57	91.62 ± 6.42	100.81 ± 5.74
nGC (unit)[f]	747.00 ± 62.00	715.00 ± 63.00	817.00 ± 65.86	712.67 ± 102.35

Values are means ± S.E.M. of six replicates and values within the same row with different letters are significantly different ($P < 0.05$).
[a] The hSF, the height of the simple fold.
[b] The hCF, the height of the complex fold.
[c] The lIE, the total length of the intestinal epithelium over 500 μm distance.
[d] The hMV, height of the microvillus.
[e] The tM, the thickness of the muscularis.
[f] The nGC, the number of the goblet cell from one section.

present study, compared to those in H-1 and H-5 treatments, the fish fed with the basal diet (H-0) or the excessive HMTBa supplemented diet (H-10) had relatively higher activities of serum AKP, GOT and GPT, and relatively lower activity of liver GPT. These suggested that the fish fed diets of H-0 or H-10 had increased tissue injury in the liver. This is confirmed by the impaired integrity of liver histological structures in H-0 and H-10 treatments. The increased oxidative stress in fish in H-0 and H-10 treatments could be the reason for the impaired liver condition, as oxidative stress could induce serious peroxidation reaction on cell membrane lipid which would initiated the loss of membrane integrity in the liver and led to the leak of enzymes from the liver into the serum (Veena et al., 2006; Humtsoe et al., 2007). In addition, the utilization of high level of dietary HMTBa in turbot might accumulate more toxic metabolites in the liver, such as ketones and S-adenosylmethionine. These metabolites were reported to negatively affect the liver condition (Choo et al., 1991; Regina et al., 1993; Murthy and Varghese, 1998). When 1–5% HMTBa was added in the basal diet, the tissue injury in the liver was avoided and the integrity of liver structures was protected. Similar improvements by proper supplementation of dietary HMTBa were also reported in Jian carp (Feng et al., 2011; Xiao et al., 2011). In consideration of the above discussion on the anti-oxidative capacities, the improved liver condition of turbot in the present study may be due to the increases of the anti-oxidative capacities and the decreased oxidative stress in fish from H-1 to H-5 treatments.

The intestine is the main place for the digestion and absorption of HMTBa. Previous study reported that HMTBa could improve the growth and development of the intestine (Xiao et al., 2011). In the present study, the fish fed the basal diet (H-0) showed the lowest height of intestinal fold and the impaired integrity of intestine structures, including the hyperplasia of loose connective tissue and adipose cells in the submucosa of the intermediate intestine and the increased invagination in the mucosa of the distal intestine. With the addition of 1–5% dietary HMTBa in the diets, the intestinal morphological structures were improved. However, when 10% of HMTBa was added in the diet, the fish showed the decreased height of the simple fold and the impaired integrity of intestine structure. Though the definite reason for these negative effects is not clear, the depressed anti-oxidative abilities and the increased oxidative stress by the inclusion of 10% HMTBa in the diet might contribute to these negative effects on fish intestine. From this point of view, dietary HMTBa supplementation for turbot should be less than 5%.

5. Conclusion

In this study, the turbot fed with the basal diet and the excessive HMTBa supplemented diet (10%) showed the decreased growth performance, feed utilization and anti-oxidative abilities, and the impaired liver and intestine histology structures. These responses of turbot were generally improved as 1–5% dietary HMTBa added in the diet. The results suggest that the supplementation of HMTBa in the diet less than 5% was safe for turbot.

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References

Ahmed, I., Khan, M.A., Jafri, A.K., 2003. Dietary methionine requirement of fingerling Indian major carp, *Cirrhinus mrigala* (Hamilton). Aquac. Int. 11, 449–462.
Amin, A.B., Mortensen, L., Poppe, T.T., 1992. Histology atlas, normal structure of salmonids: a colour atlas, English, German, French and Spanish legends. Histology Atlas: Salmonids.
Association of Official Analytical Chemists (AOAC), 1995. Official Methods of Analysis of Official Analytical Chemists International. 16th ed. Association of Official Analytical Chemists, Arlington, VA.
Baeverfjord, G., Krogdahl, Å., 1996. Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: a comparison with the intestines of fasted fish. J. Fish Dis. 19, 375–387.
Baker, D.H., 2006. Comparative species utilization and toxicity of sulfur amino acids. J. Nutr. 136, 1670S–1675S.
Bonaldo, A., Parma, L., Mandrioli, L., Sirri, R., Fontanillas, R., Badiani, A., Gatta, P.P., 2011. Increasing dietary plant proteins affects growth performance and ammonia excretion but not digestibility and gut histology in turbot (*Psetta maxima*) juveniles. Aquaculture 318, 101–108.
Cheng, Z.J., Hardy, R.W., Blair, M., 2003. Effects of supplementing methionine hydroxy analogue in soybean meal and distiller's dried grain-based diets on the performance and nutrient retention of rainbow trout *Oncorhynchus mykiss* (Walbaum). Aquac. Res. 34, 1303–1310.
Choo, P., Smith, T.K., Cho, C.Y., Ferguson, H.W., 1991. Dietary excesses of leucine influence on growth and body composition of rainbow trout. J. Nutr. 121, 1932–1939.
Dibner, J.J., 2003. Review of the metabolism of 2-hydroxy-4-(methylthio) butanoic acid. Worlds Poult. Sci. J. 59, 99–110.
Escaffre, A.M., Kaushik, S., Mambrini, M., 2007. Morphometric evaluation of changes in the digestive tract of rainbow trout (*Oncorhynchus mykiss*) due to fish meal replacement with soy protein concentrate. Aquaculture 273, 127–138.
European Food Safety Authority (EFSA), 2011. Technical guidance: tolerance and efficacy studies in target animals. EFSA J. 9 (2175) (15 pp.).

Feng, L., Xiao, W.W., Liu, Y., Jiang, J., Hu, K., Jiang, W., Li, S., Zhou, X., 2011. Methionine hydroxy analogue prevents oxidative damage and improves antioxidant status of intestine and hepatopancreas for juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquac. Nutr. 17, 595–604.

Gatlin, D.M., Harrell, R.M., 1997. Nutrition and feeding of striped bass and hybrid striped bass. Striped Bass and other Morone Culture pp. 235–251.

Goff, J.B., Gatlin, D.M., 2004. Evaluation of different sulfur amino acid compounds in the diet of red drum, *Sciaenops ocellatus*, and sparing value of cystine for methionine. Aquaculture 241, 465–477.

Hou, H., Ma, R., Zhang, W., Mai, K., 2012. The influence of overdose dietary DL-Methionine of HMTBA on growth, feed utilization and anti-oxidant responses of Turbot (*Scophthalmus maximus* L.). Period. Ocean Univ. China 42, 036–043.

Hu, H., Mai, K., Zhang, Y., Ai, Q., Xu, W., Zhang, W., Li, Y., Liu, J., 2015. Effects of dietary xylan on growth performance, digestive enzyme activities and intestinal morphology of juvenile turbot (*Scophthalmus maximus* L.). Isr. J. Aquacult.-Bamid. 67, 1–10.

Hubbard, P.C., Barata, E.N., Ozório, R.O., Valente, L.M., Canário, A.V., 2011. Olfactory sensitivity to amino acids in the blackspot sea bream (*Pagellus bogaraveo*): a comparison between olfactory receptor recording techniques in seawater. J. Comp. Physiol. A. 197, 839–849.

Humtsoe, N., Davoodi, R., Kulkarni, B.G., Chavan, B., 2007. Effect of arsenic on the enzymes of the rohu carp *Labeo rohita* (Hamilton, 1822). Raffles Bull. Zool. 14, 17–19.

Kaushik, S., 1998. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*): gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. Aquat. Living Resour. 11, 355–358.

Li, P., Burr, G.S., Wen, Q., Goff, J.B., Murthy, H.S., Gatlin, D.M., 2009. Dietary sufficiency of sulfur amino acid compounds influences plasma ascorbic acid concentrations and liver peroxidation of juvenile hybrid striped bass (*Morone chrysops* × *M. saxatilis*). Aquaculture 287, 414–418.

Ma, R., Hou, H., Mai, K., Bharadwaj, A.S., Cao, H., Ji, F., Zhang, W., 2013. Comparative study on the effects of L-methionine or 2-hydroxy-4-(methylthio) butanoic acid as dietary methionine source on growth performance and anti-oxidative responses of turbot (*Psetta maxima*). Aquaculture 412, 136–143.

Mai, K., Wan, J., Ai, Q., Xu, W., Liufu, Z., Zhang, L., Zhang, C., Li, H., 2006. Dietary methionine requirement of large yellow croaker, *Pseudosciaena crocea* R. Aquaculture 253, 564–572.

Ministry of Agriculture of China, 2012. The Guidelines for Tolerance Test of Feeds and Feed Additives in Target Aquatic Animals (in Chinese). http://www.xmys.moa.gov.cn/biaozhun/201201/t20120119_2469472.htm.

Mukhopadhyay, N., Ray, A.K., 2001. Effects of amino acid supplementation on the nutritive quality of fermented linseed meal protein in the diets for rohu, *Labeo rohita*, fingerlings. J. Appl. Ichthyol. 17, 220–226.

Murthy, H.S., Varghese, T.J., 1998. Total sulphur amino acid requirement of the Indian major carp, *Labeo rohita* (Hamilton). Aquac. Nutr. 4, 61–66.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., Nichols, P.D., 2009. Feeding aquaculture in an era of finite resources. PNAS 106, 15103–15110.

Olsen, R.L., Hasan, M.R., 2012. A limited supply of fishmeal: impact on future increases in global aquaculture production. Trends Food Sci. Technol. 27, 120–128.

Ontiveros, R.R., Shermer, W.D., Berner, R.A., 1987. An HPLC method for the determination of 2-hydroxy-4-(methylthio)-butanoic acid (HMB) in supplemented animal feeds. J. Agric. Food Chem. 35, 692–694.

Opstvedt, J., Aksnes, A., Hope, B., Pike, I.H., 2003. Efficiency of feed utilization in Atlantic salmon (*Salmo salar* L.) fed diets with increasing substitution of fish meal with vegetable proteins. Aquaculture 221, 365–379.

Pérez-Jiménez, A., Peres, H., Rubio, V.C., Oliva-Teles, A., 2013. Effects of diet supplementation with white tea and methionine on lipid metabolism of gilthead sea bream juveniles (*Sparus aurata*). Fish Physiol. Biochem. 39, 661–670.

Refstie, S., Svihus, B., Shearer, K.D., Storebakken, T., 1999. Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch polysaccharide content in different soyabean products. Anim. Feed Sci. Technol. 79, 331–345.

Regina, M., Korhonen, V.P., Smith, T.K., Alakuijala, L., Eloranta, T., 1993. Methionine toxicity in the rat in relation to hepatic accumulation of *s*-adenosylmethionine: prevention by dietary stimulation of the hepatic transsulfuration pathway. Arch. Biochem. Biophys. 300, 598–607.

Takagi, S., Shimeno, S., Hosokawa, H., Ukawa, M., 2001. Effect of lysine and methionine supplementation to a soy protein concentrate diet for red sea bream *Pagrus major*. Fish. Sci. 67, 1088–1096.

United States Department of Agriculture, 2009. USDA National Nutrient Database for Standard Reference Release 18.

Veena, K., Shanthi, P., Sachdanandam, P., 2006. Protective effect of kalpaamruthaa on altered glycoprotein component levels and membrane stability in mammary carcinoma. Int. J. Cancer 2, 315–329.

Waterland, R.A., 2006. Assessing the effects of high methionine intake on DNA methylation. J. Nutr. 136, 1706S–1710S.

Xiao, W., Feng, L., Liu, Y., Jiang, J., Hu, K., Jiang, W., Li, S., Zhou, X., 2011. Effects of dietary methionine hydroxy analogue supplement on growth, protein deposition and intestinal enzymes activities of juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquac. Nutr. 17, 408–417.

Xiao, W., Feng, L., Kuang, S., Liu, Y., Jiang, J., Hu, K., Jiang, W., Li, S., Tang, L., Zhou, X., 2012. Lipid peroxidation, protein oxidant and antioxidant status of muscle and serum for juvenile Jian carp (*Cyprinus carpio* var. Jian) fed grade levels of methionine hydroxy analogue. Aquac. Nutr. 18, 90–97.

Yi, G.F., Gaines, A.M., Ratliff, B.W., Srichana, P., Allee, G.L., Perryman, K.R., Knight, C.D., 2006. Estimation of the true ileal digestible lysine and sulfur amino acid requirement and comparison of the bioefficacy of 2-hydroxy-4-(methylthio) butanoic acid and dl-methionine in eleven- to twenty-six-kilogram nursery pigs. J. Anim. Sci. 84, 1709–1721.

Yoshikawa, M., Ninomiya, K., Shimoda, H., Nishida, N., Matsuda, H., 2002. Hepatoprotective and antioxidative properties of *Salacia reticulata*: preventive effects of phenolic constituents on CCl4-induced liver injury in mice. Biol. Pharm. Bull. 25, 72–76.

Zhou, Q., Wu, Z., Tan, B., Chi, S., Yang, H., 2006. Optimal dietary methionine requirement for Juvenile Cobia (*Rachycentron canadum*). Aquaculture 258, 551–557.