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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