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Dietary butylated hydroxytoluene improves lipid metabolism, antioxidant and anti-apoptotic response of largemouth bass (*Micropterus salmoides*)

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ABSTRACT

A 10-week growth trail was conducted to investigate the efficacy and tolerance of dietary butylated hydroxytoluene (BHT) by evaluating inflammation, apoptosis and hepatic disease related to oxidative stress in largemouth bass (*Micropterus salmoides*). Four experimental diets were prepared with BHT supplement levels of 0 (B0), 150 (B150), 300 (B300) and 1500 (B1500) mg/kg, in which B150 was at the maximum recommended level established by European Union Regulation, and the B300 and B1500 levels were 2 and 10-fold of B150, respectively. Each diet was fed to 6 replicates with 30 largemouth bass (initial body weight, IBW = 6.20 ± 0.01 g) in each tank. The BHT inclusion level did not affect the specific growth rate, but fish in the B150 group showed the lowest feed conversion rate ($P < 0.05$). BHT inclusion significantly decreased the levels of plasma TC, TG, LDL, ALT and AKP, and increased the (HDL-C)/TC ratio ($P < 0.05$). Plasma MDA was significantly decreased in the B150 group and GSH-Px was extremely enhanced in each BHT inclusion group ($P < 0.05$). Hepatic T-AOC was significantly enhanced and $O_2^{-}\cdot$ was significantly decreased in each BHT inclusion group compared to the B0 group ($P < 0.05$), as well as hepatic MDA was significantly decreased in B1500 group ($P < 0.05$). Dietary BHT inclusion down-regulated the hepatic mRNA levels of inflammation, apoptosis and fibrosis related genes, including TNFα, TGF-β1, α-SMA, IL8, IL11β and caspase-9. Moreover, BHT could improve hepatic lipid metabolism via up-regulating the mRNA levels of APOA1, CYP7A1, CYP8B1, and down-regulating the mRNA levels of PPAR-γ and APOB. Histological examination of the liver morphology with H & E and Sirius Red staining showed that BHT inclusion decreased necrotic degenerative changes and collagen deposition in largemouth bass. An immunofluorescence examination revealed significantly decreased cleaved caspase-3 signals in the BHT groups. In conclusion, the results demonstrated that ROS induces hepatic cell apoptosis and fibrosis via the intrinsic pathway of apoptosis by activating caspase-9 in the mitochondria and then initiates apoptosis by activating caspase-3. Consuming 2.32–23.80 mg/kg·bw/d (150–1500 mg/kg in diet) of BHT effectively improved the plasma and hepatic lipid metabolism, antioxidant response as well as reduced ROS production, protecting hepatic cells from injury. It is implied that even a 10-fold increase of the maximum level of BHT (150 mg/kg) is safe for the largemouth bass.

1. Introduction

The largemouth bass (*Micropterus salmoides*) is a freshwater carnivorous species that has been widely cultured in the world and has become one of the species with rapid production growth in China [1]. Nowadays, more than 80% of cultured largemouth bass is relying on chilled fish because of the varying degrees of hepatosis, anorexia, and further decreased growth performance induced by non-integrated nutrition in artificial diet in China (MOA, 2016) [2]. The factors that induce hepatosis for fish could be oxidative stress [1,3,4], high levels of dietary digestible carbohydrate (> 19%) [5,6], mycotoxins [7,8], etc. Hepatosis in largemouth bass had become the first limiting factor for the sustainable development of the species in the world.

Largemouth bass have a limited ability to synthesize highly unsaturated fatty acids (HUFAs) from 18-carbon precursors, and the optimal dietary lipid level for largemouth bass was 100–154.5 g/kg [9].

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n-3 HUFAs supplied by fish oil are very susceptible to oxidative degeneration to produce toxic reactive oxygen species (ROS), such as peroxides, superoxide, hydroxyl radicals, and singlet oxygen, causing damage to cellular substances such as protein and DNA, thereby affecting cellular integrity [10]. Furthermore, in commercial situations, lipids are readily susceptible to be peroxided in feedstuffs, and the heating process or improper storage could also aggravate the dietary lipid oxidation [1,11]. To maintain animal feed quality and prevent fish from oxidative lesions, there must be effective antioxidant systems operating in fish, such as endogenous free radical scavenging enzymes and exogenous antioxidants [12,13].

BHT is an exogenous synthetic phenolic antioxidant that is widely used to preserve and stabilize the freshness, nutritive value, flavor and color of foods and animal feed [14]. BHT inhibits colon cancer, gland cancer and also liver cancer in mammals by reducing the production of a wide variety of chemical carcinogens [15–18]. The largemouth bass is a species that is sensitive to dietary peroxidation [3]; 150–1500 mg/kg ethoxquin (EQ) in the diet of largemouth bass could effectively relieve oxidative stress induced by fish oil oxidation and alleviate hepatosis symptoms [4]. In Europe, EQ, BHT and BHA (butyl-hydroxyanisole) are currently authorized in feed for all animal species with a maximum concentration of 150 mg/kg feed, alone or in combination with other authorized synthetic antioxidants, except for EQ in dog feed, where only 100 mg kg^{-1} feed may be added (Council Directive 70/524/EEC, Commission, 2004) [19]. An overdose of BHT may have adverse effects on cancer development in a variety of tissues and organs, such as hepatotoxicity and nephrotoxicity in rats [20,21], pulmonary toxicity in mice [22], hemorrhagic death in rats [23], chronic carcinogenicity in rodents [24,25], etc. Therefore, the objectives of the present study were to evaluate the efficacy and tolerance of dietary BHT by its effects on growth performance, lipid metabolism, inflammation, anti-oxidant response, apoptosis and hepatosis induced by oxidative stress in largemouth bass.

2. Materials and methods

During the feeding period, the experimental fish were maintained in compliance with the Laboratory Animal Welfare Guidelines of China (Decree No. 2 of Ministry of Science and Technology, issued in 1988).

2.1. Experimental diets

The tested BHT (99%) was supplied by Zhenglong Chemical Co., Ltd, Shandong, China. Four experimental diets were prepared with BHT supplement levels at 0 (B0), 150 (B150), 300 (B300) and 1500 (B1500) mg/kg. The highest level (1500 mg/kg) was designed to be 10-fold of the maximum approved level (150 mg/kg) for the tolerance test to obtain the safety margin of BHT utilized in fish feed. Each diet was extruded into 2 mm diameter pellets under the following extrusion condition as: feeding section (90 °C/5 s), compression section (150 °C/5 s) and metering section (120 °C/4 s) using a Twin-screwed extruder (EXT50A, YANGGONG MACHINE, Beijing, China). All diets were stored at room temperature (25–30 °C) in the summer and kept out of the sun for 10 weeks, the same storage conditions used at fish farms. The diet formulation and analyzed chemical compositions are shown in Table 1.

Table 1
Formulation and compositions of experimental diets(g/kg).

Ingredients	B0	B150	B300	B1500
Fish meal[a]	180	180	180	180
Soy protein concentrate	200	200	200	200
Soybean meal	100	100	100	100
Krill meal	30	30	30	30
Wheat gluten meal	130	130	130	130
$Ca(H_2PO_4)_2$	23.4	23.4	23.4	23.4
Soy Lecithin	20	20	20	20
Fish oil[a]	80	80	80	80
Vitamin and mineral premix[b]	14	14	14	14
Wheat flour	210.9	210.8	210.6	209.4
Yeast extract	10	10	10	10
DL-Met	1.7	1.7	1.7	1.7
BHT[c]	0	0.15	0.30	1.50
Total	1000	1000	1000	1000
Analyzed nutrients compositions (g/kg, in dry matter basis)				
Moisture	107.1	114.1	110.4	105.5
Crude protein	502.7	510.8	513.3	508.3
Crude lipid	134.7	146.3	147.5	150.9
Crude ash	77.0	70.2	70.2	69.7
Starch[d]	163.5	163.4	163.3	162.5
Gross energy(MJ/kg)	181.4	182.7	182.6	182.6

[a] Low temperature steam-dried fishmeal from anchovy with a nature antioxidant NATUROX® at 250 mg/kg (Kemin, USA), TripleNine Fish Protein. Esbjerg, Denmark. Health product level sardine fish oil (cold storage, without anti-oxidant) was supplied by Coland Group, Fujian, China. The peroxide value, acid value, MDA and anisidine value of fresh fish oil (stored at −20 °C) were 7.35 mmoL/kg, 0.49 mg/g, 9.91 mg/kg and 17.1, and those of oxidized fish oil (stored at room temperature for 10 week) were 49.2 mmoL/kg, 1.10 mg/g, 33.5 mg/kg and 155, respectively.

[b] Vitamin premix (mg/kg diet): Vitamin A 20; Vitamin B_1 12; Vitamin B_2 10; Vitamin B_6 15; Vitamin B_{12} (1%) 8; Niacinaminde 100; Ascorbyl calcium phosphate (35%) 1000; Calcium pantothenate 40; Biotin (2%) 2; Folic acid 10; Vitamin E (50%) 400; Vitamin K_3 20; Vitamin D_3 10; Inositol 200; Choline chaoride (50%) 4000; Corn protein powder 150. Mineral premix (mg/kg diet): $CuSO_4{\cdot}5H_2O$ 10; $FeSO_4{\cdot}H_2O$ 300; $ZnSO_4{\cdot}H_2O$ 200; $MnSO_4{\cdot}H_2O$ 100; KIO_3 (10%) 80; Na_2SeO_3 (10% Se) 67; $CoCl_2{\cdot}6H_2O$ (10% Co) 5; NaCl 100; Zeolite 638.

[c] BHT was added into fish oil.

[d] Starch was calculated according to the wheat gluten meal (90 g/kg) and wheat flour (720 g/kg).

2.2. Experimental fish, feeding and sampling

Largemouth bass were obtained from the Sanshui platinum Aquafarm (Foshan, Guangdong, China). All fish were acclimated in laboratory conditions and fed the control experimental diet without BHT (B0) for 4 weeks before the commencement of the trial. Fish (initial body weight = 6.20 ± 0.01 g) were selected and distributed into 256 L tanks after 24 h of starvation with 30 fish per tank and six tanks per treatment. The water temperature was maintained at 23 ± 1 °C, pH = 7.5–8.5, dissolved oxygen (DO) > 7.0 mg/L and NH_4-N < 0.3 mg/L. Aeration was supplied to each tank 24 h per day, and the photoperiod was 12D: 12 L. Fish were fed to apparent satiation twice daily at 08:00 and 16:00 for 70 days.

The fish from each tank were batch weighed at the end of the growth trial. Twelve fish for each treatment (2 fish from each tank) were randomly selected and anaesthetized with chlorobutanol (300 mg/mL). The body weight, body length, liver and viscera weight were recorded individually to calculate condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI), respectively. Blood samples were drawn from the caudal part of the sedated fish using anticoagulant syringes with 2% NaF and 4% potassium oxalate. Blood samples were centrifuged at 4000 rpm for 10 min at 4 °C to obtain plasma. Two liver samples near to the bile duct were collected for histology (fixed in 10% phosphate-buffered formalin solution with a pH of 7.2) or fast frozen in liquid nitrogen for antioxidant analysis and RNA isolation. All samples were stored at −80 °C until analysis.

2.3. Chemical analysis

All chemical analyses of the diets were carried out in duplicate according to AOAC (2006). The dry matter was analyzed by drying the samples to a constant weight at 105 °C. Crude protein (CP) was determined using a Kjeltec™ 2300 Unit (Foss, Hillerød, Denmark) by the method of Kjeldahl, and the CP content was estimated by multiplying nitrogen by 6.25. Crude lipid was analyzed by acid hydrolysis with a Soxtex System HT 1047 Hydrolyzing Unit (Foss, Hillerød, Denmark), followed by Soxhlet extraction using a Soxtex System 1043 (Foss,