

Postprint: Evaluation of Tolerance of Largemouth Bass (*Micropterus salmoides*) to Dietary Butylated Hydroxyanisole

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Abstract

This experiment aimed to evaluate the tolerance of largemouth bass (*Micropterus salmoides*) to dietary butylated hydroxyanisole (BHA) through growth performance, plasma biochemical indices, tissue antioxidant indices, and histological changes in liver and intestine. Largemouth bass with an initial body weight of (6.20 ± 0.01) g were used as the target animal, and BHA was added to the basal diet at levels of 0 (D0 group, as control), 150 (D150 group), 300 (D300 group), and 1,500 mg/kg (D1500 group), where 300 and 1,500 mg/kg represent 2 and 10 times the maximum recommended dose (150 mg/kg), respectively, for a 10-week feeding trial. Each group had 6 replicates with 30 fish per replicate. The results showed that among the 4 groups, the D150 group exhibited the best growth performance, while no significant differences were observed in final mean weight, specific growth rate, weight gain rate, and feed intake among the remaining groups ($P > 0.05$). No significant differences were found in condition factor, viscerosomatic index, and hepatosomatic index among all groups ($P > 0.05$). The control group showed significantly higher contents of total cholesterol and triglycerides and alkaline phosphatase activity in plasma than the other groups ($P < 0.05$). The plasma high-density lipoprotein cholesterol/total cholesterol ratio in D150 and D300 groups was significantly higher than that in the control and D1500 groups ($P < 0.05$). The D1500 group exhibited significantly lower aspartate aminotransferase and alanine aminotransferase activities in plasma than the control group ($P < 0.05$), with no significant differences from the D150 and D300 groups ($P > 0.05$). The D150 and D300 groups showed significantly lower superoxide dismutase activity in liver than the control group ($P < 0.05$), with no significant difference from the D1500 group ($P > 0.05$). Dietary supplementation of 1,500 mg/kg BHA significantly increased the total antioxidant capacity in liver ($P < 0.05$); dietary supplementation of 150, 300, and 1,500 mg/kg BHA all significantly reduced

malondialdehyde contents in plasma, heart, and liver ($P < 0.05$). The control, D150, and D1500 groups all exhibited varying degrees of damage in liver and intestine of largemouth bass, but the damage was relatively less severe in the D150 and D1500 groups. These results indicate that dietary supplementation of 150 mg/kg BHA exerts certain promoting effects on lipid metabolism and antioxidant protection functions in largemouth bass, and is safe for largemouth bass, with a safety factor of 10.

Full Text

Tolerance Evaluation of Butyl-Hydroxyanisole in Diets of Largemouth Bass (*Micropterus salmoides*)

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Abstract: A 10-week feeding trial was conducted to evaluate the tolerance of largemouth bass (*Micropterus salmoides*) to dietary butyl-hydroxyanisole (BHA) based on growth performance, plasma biochemical indices, tissue antioxidant parameters, and histological changes in liver and intestine. Largemouth bass with initial body weight of (6.20 ± 0.01) g were fed experimental diets supplemented with 0 (D0 group, control), 150 (D150 group), 300 (D300 group), and 1,500 mg/kg BHA (D1500 group), where 300 and 1,500 mg/kg represent 2- and 10-fold multiples of the maximum recommended dose (150 mg/kg), respectively. Each dietary treatment had six replicates with 30 fish per replicate. The results showed that fish in the D150 group exhibited the best growth performance, while no significant differences were observed among other groups in final mean weight, specific growth rate, weight gain rate, or feed intake ($P > 0.05$). No significant differences were found in condition factor, viscerosomatic index, or hepatosomatic index among all groups ($P > 0.05$). Plasma total cholesterol, triglyceride contents, and alkaline phosphatase activity in the control group were significantly higher than those in other groups ($P < 0.05$). The plasma high-density lipoprotein cholesterol/total cholesterol ratio in D150 and D300 groups was significantly higher than that in control and D1500 groups ($P < 0.05$). Plasma aspartate aminotransferase and alanine aminotransferase activities in D1500 group were significantly lower than those in the control group ($P < 0.05$), but showed no significant differences with D150 and D300 groups ($P > 0.05$). Liver superoxide dismutase activity in D150 and D300 groups was significantly lower than that in the control group ($P < 0.05$), but did not differ significantly from D1500 group ($P > 0.05$). Dietary supplementation of 1,500 mg/kg BHA significantly increased liver total antioxidant capacity ($P < 0.05$), while BHA supplementation at 150, 300, and 1,500 mg/kg all significantly reduced malondialdehyde content

in plasma, heart, and liver ($P < 0.05$). Varying degrees of liver and intestinal damage were observed in control, D150, and D1500 groups, though damage was relatively less severe in D150 and D1500 groups. These results indicate that dietary supplementation of 150 mg/kg BHA can promote lipid metabolism and provide antioxidant protection in largemouth bass, and is safe for this species with a safety factor of 10-fold.

Keywords: largemouth bass (*Micropterus salmoides*); butyl hydroxyanisole; tolerance; growth; blood indices; antioxidant; histology

Introduction

Largemouth bass (*Micropterus salmoides*), also known as California bass, is native to California, USA, and was introduced to China in the 1980s. Due to its rapid growth, strong disease resistance, delicious meat, and rich nutritional value, it has become an important economic species in China's freshwater aquaculture [1]. As a typical carnivorous fish, largemouth bass has high dietary lipid requirements and is highly sensitive to feed oxidation [2]. Fish feeds contain large amounts of polyunsaturated fatty acids that are prone to oxidative deterioration, forming various toxic products such as hydroperoxides, fatty acid alkoxy radicals, and aldehydes, which damage cellular components including proteins, DNA, and small molecules, thereby affecting cell integrity [3]. To maintain fish health and prevent feed oxidation, effective antioxidant systems must be employed, including endogenous free radical scavengers and exogenous antioxidants.

Butyl hydroxyanisole (BHA) is an exogenous phenolic antioxidant that terminates chain reactions by generating relatively stable phenoxy radicals through hydrogen abstraction [4]. BHA is relatively heat-stable, lipid-soluble, resistant to destruction under weak alkaline conditions, does not color when interacting with metal ions, possesses strong antibacterial properties, and can inhibit the growth of *Aspergillus flavus* [5]. Previous studies have reported that 100 mg/kg BHA provides significant protective effects against adriamycin-induced liver and myocardial tissue damage in mice [6], while 0.2% and 0.5% BHA significantly protect against acetaminophen-induced liver injury in mice [7]. Additionally, appropriate amounts of BHA can delay aflatoxin-induced hepatocarcinogenesis in rats, significantly reduce liver cancer incidence, and postpone the occurrence of other tumors [8].

However, reports on BHA toxicity are inconsistent. Sgaragli et al. [9] demonstrated that high doses of BHA can damage biological membrane systems and induce methemoglobin formation in human erythrocytes. Kahl et al. [10] found that long-term BHA intake induces forestomach tumors in rats. Verhagen et al. [11] reported that BHA not only induces forestomach carcinoma in rats but also causes carcinogenesis in glandular stomach, small intestine, and rectum. Moreover, BHA toxicity is dose-dependent: 1,250 mg/kg causes forestomach ep-

ithelial cell hyperplasia, 5,000 mg/kg induces forestomach tumors, and 10,000 mg/kg triggers forestomach carcinoma in rats [12]. Conversely, other studies showed that 20,000 mg/kg BHA does not cause forestomach carcinoma in rats [13]. Currently, most BHA research focuses on detection methods in food, with limited studies on its application in feed and aquatic animals. Therefore, this study referenced the EU' s maximum recommended dose of 150 mg/kg for BHA used alone or in combination with other antioxidants in animal feed, using largemouth bass as the target species to evaluate BHA tolerance and determine safe limits for aquafeed applications.

Materials and Methods

Experimental Animals

The target aquatic animal was largemouth bass purchased in June 2014 from Foshan Sanshui Platinum Aquatic Seedling Co., Ltd. Prior to the formal experiment, fish were acclimated in the culture system for three weeks and fed the basal diet during this period.

Experimental Diets

This experiment was conducted according to the Ministry of Agriculture' s “Guidelines for Tolerance Evaluation Tests of Feed Raw Materials and Additives in Aquatic Target Animals (Trial).” BHA was added to the basal diet at levels of 0, 150, 300, and 1,500 mg/kg, where 150 mg/kg represented the maximum recommended dose, and 300 and 1,500 mg/kg were 2- and 10-fold multiples, respectively. Four experimental diets with 2.0 mm diameter were produced as hard pellets, air-dried, and stored. The diets were designated D0 (control), D150, D300, and D1500. Moisture, ash, crude protein, crude lipid, and gross energy were determined using 105°C atmospheric drying, 550°C incineration, Kjeldahl nitrogen analysis, total fat extraction, and bomb calorimetry, respectively. Diet composition and nutrient levels are presented in Table 1 .

Table 1 Composition and nutrient levels of experimental diets (DM basis)

Items	Diets
Ingredients	
Fish meal	
Soy protein concentrate	
Soybean meal	
Krill meal	
Wheat gluten	
Ca(H ₂ PO ₄) ₂	
Choline chloride	
Soy lecithin	
Fish oil	

Items	Diets
Premix ¹⁾	
Wheat flour	
Yeast extract	
Met	
Total	
BHA ²⁾	
Nutrient levels	
Crude protein	
Ash	
Crude lipid	
Gross energy (MJ/kg)	

¹⁾ The premix provided the following per kg of diet: VA 20 mg, VB₁ 10 mg, VB₂ 15 mg, VB₆ 15 mg, VB₁₂ 8 mg, VE 400 mg, VK₃ 20 mg, VD₃ 10 mg, niacinamide 100 mg, vitamin C acetate 1,000 mg, inositol 200 mg, calcium pantothenate 40 mg, biotin 2 mg, folic acid 10 mg, corn gluten meal 150 mg, CuSO₄ · 5H₂O 10 mg, FeSO₄ · H₂O 300 mg, ZnSO₄ · H₂O 220 mg, MnSO₄ · H₂O 25 mg, KIO₃ 3 mg, Na₂SeO₃ 5 mg, CoCl₂ · 6H₂O 5 mg, MgSO₄ 4,000 mg, powdered zeolite 4,632 mg.

²⁾ BHA was added in fish oil.

Experimental Design and Management

The experiment was conducted at the National Aquafeed Safety Assessment Station (Nankou, Beijing) using an indoor recirculating aquaculture system. Healthy largemouth bass with uniform size [average body weight (6.20±0.01)g] were randomly stocked into conical tanks (0.26m³ volume). Four experimental groups corresponding to the four diets were established, with six replicates per group and 30 fish per tank.

Fish were hand-fed to apparent satiation twice daily at 08:00 and 16:00. Water quality was monitored regularly, maintaining dissolved oxygen >7.0 mg/L, total ammonia nitrogen <0.3 mg/L, pH 7.5-8.5, and water temperature at (23±1)°C. The feeding trial lasted 70 days from July 15 to September 23, 2014.

Sampling and Analysis

Growth Parameters After 70 days of culture, fish in each tank were weighed and feed intake (FI) and survival numbers were recorded to calculate growth parameters using the following formulas:

- Survival rate (SR, %) = 100 × final fish number / initial fish number
- Weight gain rate (WGR, %) = 100 × weight gain / initial body weight

- Specific growth rate (SGR, %/d) = $100 \times (\ln \text{ final mean weight} - \ln \text{ initial mean weight}) / \text{trial days}$
- Feed conversion ratio (FCR) = feed intake / weight gain

Morphometric Parameters Three fish were randomly selected from each tank to measure body length, body weight, viscera weight, and liver weight for calculating morphometric indices:

- Condition factor (CF, g/cm³) = average body weight / average body length³
- Hepatosomatic index (HSI, %) = $100 \times \text{liver weight} / \text{body weight}$
- Viscerosomatic index (VSI, %) = $100 \times \text{viscera weight} / \text{body weight}$

Plasma Biochemical Indices Six fish were randomly selected from each tank, anesthetized with chlorbutanol, and blood was collected from the caudal vein using sodium fluoride-potassium oxalate as anticoagulant. Plasma was separated by centrifugation at 4,000 r/min for 10 min at 4°C and stored at -80°C for analysis. Plasma biochemical indices were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer' s instructions.

Antioxidant Indices Five fish were randomly selected from each tank for blood collection and tissue sampling (liver, heart, and muscle), which were stored at -80°C for analysis. Antioxidant parameters in plasma and tissues were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer' s protocols.

Histological Examination Liver and posterior intestine tissues were sampled from two fish per tank in D0, D150, and D1500 groups. Tissues were fixed in neutral formalin, dehydrated through graded alcohol series, cleared, paraffin-embedded, sectioned, and stained with hematoxylin-eosin (HE) for microscopic observation.

Statistical Analysis

Data are presented as mean \pm standard error (SE). All data were analyzed using one-way ANOVA with SPSS 17.0 software. Duncan' s multiple range test was used to examine differences among groups, with significance level set at $P < 0.05$.

Results

Effects of BHA on Growth Performance

The effects of dietary BHA on growth performance are shown in Table 2 . No significant differences were observed in SR or FCR among groups ($P > 0.05$). The D150 group showed significantly higher final body weight, SGR, WGR,

and FI compared with D300 and D1500 groups ($P < 0.05$), while no significant differences were detected among other groups ($P > 0.05$).

Table 2 Effects of BHA on growth performance of largemouth bass (*Micropterus salmoides*) (n=6)

Items	Groups
	D0
Final body weight (g)	34.60 \pm 1.06 ^{ab} 37.46 \pm 0.91 ^b 33.38 \pm 2.14 ^a 33.36 \pm 1.18 ^a SGR(\pm 0.05 ^{ab}) 2.8

In the same row, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while different small letter superscripts mean significant difference ($P < 0.05$). The same as below.

Effects of BHA on Morphometric Parameters

Table 3 shows that no significant differences were observed in CF, VSI, or HSI among all groups ($P > 0.05$).

Table 3 Effects of BHA on morphometric parameters of largemouth bass (*Micropterus salmoides*) (n=3)

Items	Groups
	D0
CF (g/cm ³)	2.03 \pm 0.03 1.97 \pm 0.03 1.99 \pm 0.03 2.07 \pm 0.05 VSI(\pm 0.26) 7.54 \pm 0.27 7.53

Effects of BHA on Plasma Biochemical Indices

As shown in Table 4, no significant differences were observed in plasma total protein (TP), glucose (GLU), or urea nitrogen (UN) contents among groups ($P > 0.05$). Plasma total cholesterol (TC), triglyceride (TG) contents, and alkaline phosphatase (AKP) activity in the control group were significantly higher than those in other groups ($P < 0.05$), with no significant differences among the BHA-supplemented groups ($P > 0.05$). Plasma high-density lipoprotein cholesterol (HDL-C) in D150 and D300 groups was significantly higher than in D1500 group ($P < 0.05$), but did not differ significantly from the control group ($P > 0.05$). The HDL-C/TC ratio in D150 and D300 groups was significantly higher than in control and D1500 groups ($P < 0.05$). Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in D1500 group were significantly lower than in the control group ($P < 0.05$), but showed no significant differences with D150 and D300 groups ($P > 0.05$).

Table 4 Effects of BHA on plasma biochemical indexes of largemouth bass (*Micropterus salmoides*) (n=6)

Items	Groups
	D0
TP (g/L)	17.11 \pm 1.42 15.62 \pm 2.14 12.87 \pm 2.12 13.66 \pm 1.95 <i>GLU</i> (mmol/L) 2.96 \pm 0.45 1.45 \pm 0.12ab 1.61 \pm 0.11b 1.57 \pm 0.21b 1.07 \pm 0.12a <i>HDL-C</i> (mmol/L) 0.26 \pm 0.02a 0.49 \pm 0.06b 0.44 \pm 0.06b 0.28 \pm 0.05a <i>UN</i> (mmol/L) 7.3

Effects of BHA on Antioxidant Indices

Liver Antioxidant Indices Table 5 shows that no significant differences were observed in catalase (CAT) activity among groups ($P>0.05$). Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity in the control group were significantly higher than those in D150 and D300 groups ($P<0.05$), but did not differ significantly from D1500 group ($P>0.05$). Total antioxidant capacity (T-AOC) was highest in D1500 group, significantly higher than in other groups ($P<0.05$), with no significant differences among the remaining groups ($P>0.05$). Glutathione S-transferase (GST) activity was lowest in D150 group, significantly lower than in other groups ($P<0.05$), while no significant differences were observed among the other three groups ($P>0.05$).

Table 5 Effects of BHA on antioxidant indexes in liver of largemouth bass (*Micropterus salmoides*) (n=5)

Items	Groups
	D0
SOD (U/mg prot)	6.65 \pm 0.60b 4.76 \pm 0.42a 4.28 \pm 0.44a 5.64 \pm 0.49ab <i>T-AOC</i> (U/mgprot) 1.12 \pm 0.07a 1.18 \pm 0.10a 1.12 \pm 0.08a 1.70 \pm 0.10b <i>GST</i> (U/mgprot) 0.13 \pm 0.02 0.17 \pm 0.01 0.15 \pm 0.01 0.14 \pm 0.01

Muscle Antioxidant Indices Table 6 shows that no significant differences were observed in SOD, GST, CAT activities, or T-AOC among groups ($P>0.05$). However, MDA content in D150 and D300 groups was significantly lower than that in control and D1500 groups ($P<0.05$).

Table 6 Effects of BHA on antioxidant indexes in muscle of largemouth bass (*Micropterus salmoides*) (n=5)

Items	Groups
	D0
SOD (U/mg prot)	3.81 \pm 0.34 3.72 \pm 0.29 3.68 \pm 0.23 3.62 \pm 0.28 <i>T-AOC</i> (U/mgprot) 0.13 \pm 0.02 0.17 \pm 0.01 0.15 \pm 0.01 0.14 \pm 0.01 <i>GST</i> (U/mgprot) 0.13 \pm 0.02 0.17 \pm 0.01 0.15 \pm 0.01 0.14 \pm 0.01

Heart Antioxidant Indices Table 7 shows that no significant differences were observed in T-AOC or CAT activity among groups ($P>0.05$). SOD activity in the control group was significantly higher than that in D150 group ($P<0.05$),

but did not differ significantly from D300 and D1500 groups ($P>0.05$). MDA content was highest in the control group, significantly higher than in all other groups ($P<0.05$).

Table 7 Effects of BHA on antioxidant indexes in heart of largemouth bass (*Micropterus salmoides*) (n=5)

Items	Groups
	D0
SOD (U/mg prot)	3.23 \pm 0.21b 2.30 \pm 0.19a 2.88 \pm 0.28ab 2.82 \pm 0.27ab T– AOC(U/mgprot) 0.75 \pm 0.03 0.74 \pm 0.08 0.75 \pm 0.06 0.75 \pm 0.03 GST(U/m
MDA (nmol/mg prot)	–

“–” indicated not detected.

Plasma Antioxidant Indices Table 8 shows that no significant differences were observed in SOD, CAT activities, or T-AOC among groups ($P>0.05$). GST activity in D150 group was significantly lower than that in control and D300 groups ($P<0.05$), but did not differ significantly from D1500 group ($P>0.05$). Glutathione peroxidase (GSH-Px) activity was highest in D1500 group, significantly higher than in other groups ($P<0.05$), with no significant differences among the remaining groups ($P>0.05$). MDA content decreased significantly with increasing BHA supplementation ($P<0.05$), reached the lowest level in D300 group, then increased slightly in D1500 group, but remained significantly lower than in control and D150 groups ($P<0.05$).

Table 8 Effects of BHA on antioxidant indexes in plasma of largemouth bass (*Micropterus salmoides*) (n=5)

Items	Groups
	D0
SOD (U/mL)	10.36 \pm 1.69 14.49 \pm 1.32 14.65 \pm 2.05 13.18 \pm 1.24 T– AOC(U/mL) 3.84 \pm 0.60 4.06 \pm 0.42 4.12 \pm 0.60 3.99 \pm 0.49 GST(U/mL) 1 Px(U/mL) 81.79 \pm 13.58a 72.44 \pm 10.47a 86.48 \pm 17.56a 246.05 \pm 35.98b M

Histological Observations

Liver Histology As shown in Figure 1 [Figure 1: see original paper], varying degrees of liver damage were observed across groups. In the control group (D0), 12 fish were examined: 1 showed normal liver, 5 exhibited hepatic steatosis, and 6 showed hepatic fibrosis with cell disintegration. In D150 group, 12 fish were examined: 4 showed normal liver, and 8 exhibited varying degrees of hepatic fibrosis, cell disintegration, and steatosis. In D1500 group, 12 fish were examined: 3 showed normal liver, 4 exhibited hepatic steatosis, and 5 showed hepatic fibrosis.

White arrows indicate normal liver, red arrows indicate hepatic fibrosis, and yellow arrows indicate adipose infiltration.

Figure 1 Observation on hepatic histology of largemouth bass (*Micropterus salmoides*) in groups D0, D150 and D1500

Posterior Intestine Histology As shown in Figure 2 [Figure 2: see original paper], in the control group (D0), 12 fish were examined: 9 showed normal intestine, while 3 exhibited damage including intestinal epithelial cell injury, detachment of epithelial cells from the lamina propria, and mild microvilli damage. In D150 group, 12 fish were examined: 11 showed normal intestine and 1 exhibited mild epithelial cell damage. In D1500 group, 12 fish were examined: 11 showed normal intestine and 1 exhibited intestinal epithelial cell detachment.

A and B represent normal and damaged intestinal tissue, respectively; 1 and 2 represent 10× and 20× magnification, respectively.

Figure 2 Observation on distal intestinal histology of largemouth bass (*Micropterus salmoides*) in groups D0, D150 and D1500

Discussion

Effects of BHA on Growth Performance

Feed oxidation during processing and storage produces various toxic substances that affect fish growth. To maintain fish health and prevent feed oxidation, effective antioxidant systems are essential. This study demonstrated that dietary supplementation of 150 mg/kg BHA significantly improved largemouth bass growth performance, likely because this level effectively delayed feed oxidation and protected fish from oxidative stress. Hansen et al. [14] and Würtzen et al. [15] reported that 400 mg/kg BHA significantly reduced weight gain in pregnant sows and caused liver and thyroid enlargement, suggesting that high BHA doses may affect animal growth and exacerbate tissue damage. However, in this study, D300 and D1500 groups showed no significant differences in growth performance compared with the control group, indicating that high BHA doses did not suppress feed intake in largemouth bass. This discrepancy may be related to species-specific tolerance differences or variations in dietary lipid sources and contents.

Effects of BHA on Plasma Biochemical Indices

Blood biochemical indices are closely related to fish metabolism, nutritional status, and disease, reflecting both normal physiological states and pathological changes, and serving as important indicators of relative health status [16]. This study showed that BHA supplementation had no significant effects on plasma TP, GLU, or UN contents, indicating that BHA did not significantly affect protein regulation or glucose metabolism. TC and TG are blood lipid components whose levels primarily reflect lipid absorption and metabolism status [17].

In this study, all BHA-supplemented groups showed significantly lower plasma TC and TG contents than the control group, and 150 and 300 mg/kg BHA significantly increased plasma HDL-C content and HDL-C/TC ratio, suggesting that BHA supplementation significantly improved blood lipid transport and metabolism, reduced blood lipid levels, and helped maintain fish health. AKP, AST, and ALT activities primarily reflect liver damage degree; under normal physiological conditions, these enzyme activities are low in fish plasma, but increase significantly when hepatocytes are damaged, with the elevation degree correlating with damage severity [18]. This study found that BHA supplementation significantly reduced plasma AKP activity, and AST and ALT activities in D1500 group were significantly lower than in the control group, indicating that BHA supplementation effectively alleviated dietary lipid oxidation and oxidative damage in largemouth bass, thereby reducing liver oxidative stress and damage.

Effects of BHA on Antioxidant Function in Liver, Muscle, Heart, and Plasma

Oxidative stress disrupts the balance between oxidation and antioxidation, causing a surge in reactive oxygen species and producing large amounts of oxidative intermediates. If excess reactive oxygen radicals are not promptly eliminated, lipid peroxidation damage occurs [19]. To defend against free radical damage and maintain metabolic balance, organisms must establish effective defense mechanisms [20]. These include specific antioxidant enzyme systems (SOD, GSH-Px, GST, CAT) and non-enzymatic systems comprising synthetic antioxidants, vitamins, and trace elements [21].

This study showed that BHA supplementation had no significant effect on liver CAT activity, while liver SOD activity and MDA content in the control group were significantly higher than in D150 and D300 groups, but did not differ from D1500 group. SOD is a metalloenzyme encoded by nuclear genes and the only enzyme using superoxide anion (O_2^-) as substrate [22], catalyzing O_2^- conversion to hydrogen peroxide (H_2O_2) and oxygen to eliminate O_2^- and maintain free radical homeostasis. MDA is a major lipid peroxidation product whose level indirectly reflects the severity of free radical attack on cells. SOD and MDA often work coordinately to reflect free radical metabolism status. The significantly higher liver SOD activity in the control group may be due to long-term consumption of oxidized feed without exogenous antioxidants, leading to increased reactive oxygen species concentrations and elevated antioxidant enzyme substrate levels, thereby enhancing liver SOD activity. Liver GST activity in D150 group was significantly lower than in other groups. GST is a liver detoxification-related enzyme abundant in hepatocytes that eliminates peroxides and provides detoxification; elevated GST activity often serves as a sensitive indicator of liver damage [23]. Some studies showed that BHA significantly increased GST activity in rat and mouse livers [24], contrary to our results. Liver damage occurs in at least three stages: steatosis, fibrosis, and carcinogenesis. Currently, HE

staining only reveals morphological changes without clarifying their relationship with physiological status. Therefore, the significantly lower GST activity in D150 group is primarily related to liver function and antioxidant system responses. The highest T-AOC in D1500 group indicated that 1,500 mg/kg BHA significantly enhanced liver antioxidant capacity.

This study found that muscle MDA content in D150 and D300 groups was significantly lower than in the control group, demonstrating that BHA supplementation effectively alleviated muscle lipid peroxidation. Free radicals have high affinity for polyunsaturated fatty acids in muscle, initiating lipid oxidation to form unstable hydroperoxides that rapidly degrade into MDA and other substances, increasing MDA content [25]. Previous studies reported that 120 mg/kg BHA provided good antioxidant effects on pork fat with minimal dosage [26]; Sebranek et al. [27] found that 2,500 mg/kg BHA showed significant antioxidant effects in fresh pork sausage; Ahn et al. [28] reported that BHA was more effective than natural rosemary extract in inhibiting oxidation in cooked beef. However, muscle MDA content in D1500 group was significantly higher than in D150 and D300 groups, possibly because excessive antioxidants accompanied by free radical damage may reduce antioxidant efficacy at high BHA doses [29].

SOD and CAT activities in mouse heart are significantly lower than in liver [30], consistent with our results. However, heart SOD activity in the control group was significantly lower than in D150 group, possibly because 150 mg/kg BHA reduced heart MDA content and oxidative stress, decreasing the need for SOD to scavenge free radicals. Studies showed that adriamycin significantly increased MDA content in mouse myocardium, while pre-feeding 100 mg/kg BHA significantly inhibited this increase [6], consistent with our finding that BHA supplementation significantly reduced heart MDA content. GST is widely distributed in mammalian tissues and plays an important role in protecting cardiomyocytes from oxidative damage [31], cooperating to protect membrane thiol groups from free radical destruction. However, GST activity is very low in myocardial tissue, and GST isozymes are undetectable, making cardiomyocytes vulnerable to free radical attack. GST was not detected in largemouth bass heart in this study, possibly because GST is not expressed or is insensitive to cardiac stress responses in this species [32].

This study showed that BHA supplementation had no significant effects on plasma SOD, CAT activities, or T-AOC, but significantly reduced plasma MDA content. This may be because BHA supplementation enhanced endogenous antioxidant enzyme activity, effectively alleviating systemic lipid peroxidation and reducing oxidative product levels in plasma [6]. Plasma GST activity in D150 group was significantly lower than in control and D300 groups. BHA is a monofunctional inducer of phase II enzymes that induces GST activity [33] to scavenge free radicals and detoxify harmful substances; GST is often released into blood when hepatocytes are damaged. The significantly lower plasma GST activity in D150 group may be due to less severe liver damage in fish fed 150 mg/kg BHA, resulting in less GST release into plasma, which aligns

with histological results showing that 150 mg/kg BHA alleviated liver damage. GSH-Px is an important enzyme catalyzing hydrogen peroxide decomposition, with selenocysteine at its active center. Five GSH-Px isoenzymes exist with tissue-specific expression patterns [34]; all use glutathione (GSH) as substrate to reduce H_2O_2 and alkyl hydroperoxides, protecting membrane structure and function. The highest GSH-Px activity in D1500 group plasma may be due to high-dose BHA significantly inducing GSH and promoting the reaction between GSH-Px, GSH substrate, and H_2O_2 to produce water and oxidized glutathione (GSSG).

Effects of BHA on Liver and Posterior Intestine Histology

Histopathological analysis was conducted on liver and posterior intestine tissues from control, D150, and D1500 groups. Liver sections revealed varying degrees of damage across all groups, particularly in the control group without exogenous antioxidants, which showed hepatic fibrosis, steatosis, numerous fat vacuoles, and cell disintegration. Posterior intestine sections also showed damage in all groups, with the control group exhibiting intestinal epithelial cell injury, detachment from lamina propria, and microvilli damage, while these lesions were alleviated in D150 and D1500 groups, demonstrating that BHA effectively relieved intestinal tissue damage. The occurrence of liver and intestinal lesions across groups may be attributed to simulating field feed storage conditions (room temperature, 3-month storage period) where feeds underwent oxidative deterioration during long-term summer storage under high temperature and humidity. Largemouth bass is highly sensitive to lipid oxidation [2], and long-term consumption of oxidized oils causes tissue damage. Alternatively, this may result from inadequate adaptation to artificial diets, causing slow growth and inducing liver and intestinal lesions in later culture stages. Guan et al. [35] reported that largemouth bass fed artificial diets for 20 days showed slow growth and liver lesions, consistent with our results. This study demonstrated that dietary supplementation of 150 mg/kg BHA effectively alleviated liver and posterior intestine damage, though BHA alone could not completely prevent tissue damage caused by oxidized oils.

Dietary supplementation of 150 mg/kg BHA can enhance lipid metabolism and provide antioxidant protection in largemouth bass, and is safe for this species with a safety factor of 10-fold.

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