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# Dietary Sodium Butyrate Supplementation Alleviates High-Fat Diet-Induced Liver Injury by Activating Nrf2 in Common Carp (*Cyprinus carpio*)

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## ABSTRACT

To evaluate the effects of sodium butyrate (NaBT) supplementation in high-fat diets on the growth performance and liver health of common carp (*Cyprinus carpio*), we formulated three isonitrogenous diets: the control diet (5.8% crude lipid, Control diet), the high-fat diet (10.8% crude lipid, HF diet), and the NaBT diet (10.8 % crude lipid and 0.1% NaBT, NaBT diet). Each diet was assigned to triplicate tanks (100 L) with 24 fish (14.52±0.08 g) in each tank. Experimental fish were fed twice daily for 8 weeks. The results showed that fish growth performance was not affected by experimental diets. Fish at HF group demonstrated higher content of triacylglyceride (TG) and total cholesterol (TCHO) in the liver. In addition, diet HF significantly increased hepatic oxidative stress by increasing malondialdehyde (MDA) content, decreasing activity levels of antioxidant enzymes and contents of reduced glutathione (GSH). Furthermoure, diet HF significantly decreased the mRNA expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), and simultaneously increased the mRNA expression of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and IL-6 in liver of common carp compared to control diet ($P$<0.05). However, diet NaBT significantly improved fish liver health by decreasing contents of TCHO and MDA, down-regulating mRNA expression of pro-inflammatory cytokines (e.g., TNF-α, IL-1β and IL-6), increasing the activity levels of antioxidant enzymes, and upregulating the mRNA expression of Nrf2 and HO-1 in the liver ($P$<0.05). In conclusion, dietary NaBT supplementation could ameliorate the detrimental effects of high-fat diets on liver health by activating Nrf2 in common carp.

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**YW and JC carried out the experiment, wrote the manuscript, and contributed to the biochemical analysis. PS and XS assisted in the biochemical analysis. SG and WC contributed to the study conception and design, revised the manuscript, and acquired the funding.**

**Key words**
***Cyprinus carpio*, Sodium butyrate, High-fat diets, Oxidative stress, Inflammation**

## INTRODUCTION

Aquafeed costs are a major consideration in aquaculture, generally accounting for 40%-50% of the total cost of production (Craig and Helfrich, 2002). Protein is considered as the most expensive part in aquafeed (Craig and Helfrich, 2002). Given this, many strategies have been implemented in aquaculture to decrease the protein content with other nutrients (Sargent *et al*., 2003), such as lipids. The protein-sparing effects of dietary lipids have been proved in many fish species, such as Atlantic cod (*Gadus morhua*) (Morais *et al*., 2001), blunt snout bream (*Megalobrama amblycephala*) (Li *et al*., 2012), and hybrid fish tambatinga (female *Colossoma macropomum* × male *Piaractus brachypomus*) (Welengane *et al*., 2019).

Common carp (*Cyprinus carpio*) is a worldwide-distributed species (cultured in over 100 countries) and accounts for up to 10% (over 4 million metric tons in 2018) of annual freshwater aquaculture production in the world (FAO, 2020). In China, the aquaculture production of common carp has reached 2.89 million metric tons (China Fishery Stastical Yearbook, 2020). For the sake of maximum culture profit, inclusion of large amounts of non-protein energy (especially high fat) into diets of common carp has been a common phenomenon in China (Abasubong *et al*., 2018). Intake of high-fat diets reduced the growth performance and disrupted the lipid metabolism in common carp (Abasubong *et al*., 2018; Ze *et al*., 2015). Fish liver is a multifunctional organ acting in detoxification, metabolism of carbohydrates and fat,

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and scavenging foreign substances (Dalmo *et al*., 1997; Moeller *et al*., 2014). Therefore, it is becoming increasing urgent to explore dietary strategies for counteracting the adverse effects of high-fat diets on the liver health in common carp.

Sodium butyrate (NaBT) is a salt of butyric acid and a commonly used additive to improve fish gut health and growth performance (Abdel-Latif *et al*., 2020; Tran *et al*., 2018). Recently, numerous studies on mice have suggested that dietary supplementation with NaBT could reduce the negative effects of high-fat diets on liver health (Fang *et al*., 2019; Matheus *et al*., 2017; Zhai *et al*., 2019). In addition, ingestion of 300 mg/kg NaBT via gavage in rats fed high-fat diets reduced the oxidative stress (Sun *et al*., 2019), fat accumulation and inflammation in the liver (Sun *et al*., 2018). However, much less research has investitgated the effects of NaBT on the liver health of aquatic animals fed high-fat diets. Given this, we hypothesized that dietary supplementation with NaBT could improve the liver health of common carp fed high-fat diets.

To that end, the present study investigated the effect of dietary NaBT supplementation on the growth performance, lipid deposition, oxidative stress, and inflammation in liver of common carp fed high-fat diets.

## MATERIALS AND METHODS

*Experimental feed*

Three isonitrogenous (31% crude protein) diets with different fat content were formulated. The control diet (Control) contained medium fat (5.8%); the high fat diet (HF) contained high crude lipid (10.8%); the NaBT diet (NaBT) supplemented 0.1% NaBT in HF diet (Table I).

All ingredients were mixed thoroughly. Then the mixture were pelleted to pellets (2 mm) using a feed machine (Laifu Tk-12B, Guangdong, China). Pellets were dried to a moisture content of 8-10% and kept at -20 °C until used.

*Experimental fish and feeding management*

Common carp juveniles were purchased from a local fish pond (Luoyang, China) and were acclimated to laboratory conditions for 2 weeks. After starvation for 24 h, 216 experimental fish were randomly divided into 9 tanks (100 L). Each diet was assigned to triplicate tanks. Experimental fish were fed twice daily (8:00 and 17:00). Feed consumption of each tank was adjusted based on fish body weight which were weighted every two weeks. The experiment lasted for 8 weeks, during which water temperature was 25.7±1.4 °C, dissolved oxygen was above 6 mg/L, ammonia-nitrogen and nitrite were both below 0.1 mg/L.

**Table I. Feed formula and feed proximate composition (g/kg dry matter).**

| | Control | HF | NaBT |
|---|---|---|---|
| **Ingredients** | | | |
| Casein | 28.0 | 28.0 | 28.0 |
| Gelatin | 7.0 | 7.0 | 7.0 |
| Dextrin | 25.0 | 25.0 | 25.0 |
| Soybean oil | 5.0 | 10.0 | 10.0 |
| Mineral and vitamin premix | 1.0 | 1.0 | 1.0 |
| Vitamin C | 1.0 | 1.0 | 1.0 |
| $Ca(H_2PO_4)_2$ | 2.5 | 2.5 | 2.5 |
| Choline chloride | 0.5 | 0.5 | 0.5 |
| Sodium butyrate[1] | 0.0 | 0.0 | 0.1 |
| Cellulose | 30.0 | 25.0 | 24.9 |
| **Proximate composition (% dry matter)** | | | |
| Moisture | 9.97 | 9.83 | 9.45 |
| Crude protein | 31.43 | 31.63 | 31.54 |
| Crude lipid | 5.80 | 10.84 | 10.80 |
| Crude ash | 2.48 | 2.60 | 2.85 |

[1]Sodium butyrate was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China.

*Sampling*

At the end of the feeding trial, all fish were starved for 24 h before sampling. All fish in each tank were anesthetized with benzocaine (50 mg/L), counted and weighed. Then the body weight and body length of 3 fish in each tank were recorded for the determination of condition factor (CF) and their blood were drawn from caudal vein to detect the activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Then liver samples of another 9 fish in each tank were sampled and divided to three portions. The first portion was collected and preserved at 4% paraformaldehyde for Oil Red O staining, and the second and third portions were stored at -80 °C for enzyme activity assay and quantitative real-time polymerase chain reaction (PCR).

The study protocol and all experimental procedures were approved by Experimental Animal Ethics Committee of Henan University of Science and Technology.

*Proximate composition of experimental feed*

Proximate composition of experimental diets was tested according to the procedures described by AOAC (1995). Moisture content was determined through drying samples to a constant weight at 105°C. Crude protein content was measured with the Kjeldahl method. Crude lipid content was determined in a Soxtec system. Ash content

was determined with a muffle furnace for 2 h at 600°C.

*Oil red O staining*

Fixed liver samples were first dehydrated in a graded series of ethanol with concentration increasing from 75% to 100% and were embedded in parafflin. Then liver samples were sliced into sections (4 μm). Liver sections were stained with Oil Red O. The area stained with Oil Red O solution was analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

*Assay of biochemical parameters in blood and liver samples*

Activity levels of ALT and AST in plasma were measured according to 2, 4-dinitrophenyl hydrazine (DNPH) method (Reitman and Frankel, 1957). Triglyceride (TG) content was measured with a peroxidase-coupled method (McGowan *et al*., 1983) and TCHO content were tested following the method described by Allain *et al*. (1974).

*Determination of antioxidant parameters in liver*

MDA content was determined with thiobarbituric acid (TBA) following the method of Esterbauer and Cheeseman (1990). Commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, China) were used to detect the activity levels of catalase (CAT) and glutathione peroxidase (GPx), and contents of glutathione (GSH). Activity level of total superoxide dismutase (T-SOD) was assayed with xanthine/xanthine oxidase method (McCord and Fridovich, 1969). Protein content in liver samples was tested with coomassie brilliant blue method following Bradford (1976).

*Quantitative real-time PCR*

Total RNA in liver was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. After determination of RNA concentration and quality, total RNA (1 μg) was used to synthesize first-strand cDNA for RT-PCR with a commercial reagent kits (TransGen Biotech Co., Ltd.)

Primers were synthesized commercially from Tsingke Biotechnology (Wuhan, China) (Table II). Real time PCRs were performed on a Light Cycler 96 (Roche Diagnostics, Meylan, France). The total reaction volume was 15 μL, containing 7.5 μL Light Cycler 480 SYBR Green I Master mix (Roche Diagnostics), 2.0 μL cDNA template, 1.5 μL of primer (2.5 μM), and 4.0 μL PCR-grade water. Each sample was analyzed in duplicate with the following thermal cycling conditions: 95 °C for 10 min; followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. Relative quantification of target gene transcripts were calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

*Calculations and statistical analysis*

The growth performance parameters were calculated according to following formulae:

SR (%) = 100 × (1 - dead fish number/initial fish number)

WGR (%) = 100 × (final body weight - initial body weight)/initial body weight

SGR (% $d^{-1}$) = 100 × [ln (final body weight) - ln (initial body weight)]/ days

Feed efficiency (FE, %) = 100 × (fresh body weight gain)/dry feed intake

CF ($g/cm^3$) = 100 × body weight/body $length^3$

**Table II. Primer sequences for RT-PCR in the experiment.**

| Gene | Sequence (5'-3') | Product size (bp) | GenBank ID |
|---|---|---|---|
| *Nrf2* | F: TTCCCGCTGGTTTACCTTAC | 158 | JX462955 |
| | R: CGTTTCTTCTGCTTGTCTTT | | |
| *HO-1* | F: TCAGCCCATCTACTTCCCTCA | 106 | JX257180.1 |
| | R: GGCAGGCACTGTTACTCTCT | | |
| *TNF-α* | F: AGCCAGGTGTCTTTCCACAT | 110 | XM_019088899.1 |
| | R: ATGTAGCCGCCATAGGAATCG | | |
| *IL-1β* | F: AAGGAGGCCAGTGGCTCTGT | 69 | AB010701 |
| | R: CCTGAAGAAGAGGAGGCTGTCA | | |
| *IL-6* | F:CATCTGGGGACGAGGTTCAG | 195 | XM_019073058.1 |
| | R:AGGGTTTGAGGAGAGGGGTT | | |
| *β-actin* | F: TTGCTCCCTCCACCATGAAG | 126 | JQ619774.1 |
| | R: ACTCCTGCTTGCTGATCCAC | | |

Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; IL-6, interleukin-6.

Prior to analysis, the Shapiro-Wilk and Levene tests were used to examine data normality and homogeneity of variance, respectively. Then Data that met the requirements were analyzed with one-way ANOVAs in SPSS v.20.0. $P$ <0.05 was considered as statistically significant. All data were expressed as mean ± standard error of means (SEM).

## RESULTS

*Growth performance*

There was no difference among groups in final body weight (FBW), WGR, SGR, FE, SR, and CF (Table III).

**Table III. Growth performance of common carp fed experimental diets for eight weeks.**

| | Control | HF | NaBT |
|---|---|---|---|
| IBW (g) | 14.55±0.08 | 14.51±0.04 | 14.51±0.02 |
| FBW (g) | 33.38±0.70 | 31.34±1.51 | 36.37±1.70 |
| WGR (%) | 129.43±5.16 | 115.90±10.31 | 150.58±11.41 |
| SGR (%/d) | 1.47±0.03 | 1.37±0.07 | 1.63±0.09 |
| FE (%) | 64.71±1.95 | 66.85±7.24 | 76.48±1.38 |
| SR (%) | 100.00±0.00 | 98.13±1.87 | 98.13±1.87 |
| CF (g/cm$^3$) | 2.56±0.11 | 2.56±0.08 | 2.69±0.05 |

IBW, initial body weight; FBW, final body weight; WGR, weight gain rate; SGR, specific growth rate; FE, feed efficiency; SR, survival rate; CF, condition factor.

*Liver function*

Experimental diets did not affect plasma ALT activity level. Diet HF increased AST activity level compared with the control. However, diet NaBT significantly decreased ALT activity level compared to diet HF ($P$<0.05) (Fig. 1).

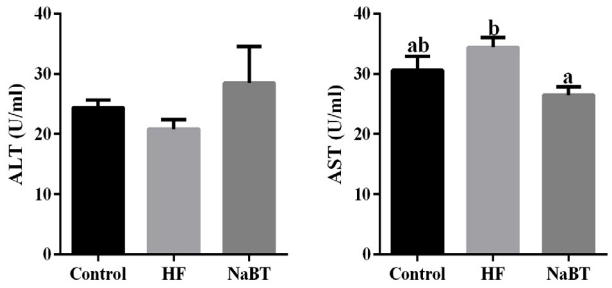


Fig. 1. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity levels of common carp fed experimental diets. Control, the control diet; HF, the high-fat diet; NaBT, the high-fat diet added with 0.1% sodium butyrate. Bars marked with different letters are significantly different ($P$<0.05).

*Hepatic lipid accumulation*

Common carp fed diet HF significantly increased liver TG and TCHO contents compared with that fed control diet. However, fish fed diet NaBT had a lower TG ($P$>0.05) and TCHO ($P$<0.05) contents in liver compared to that fed diet HF (Fig. 2). More lipid droplets in fish fed diet HF were observed than that in fish fed diets Control and NaBT ($P$<0.05) (Fig. 3).

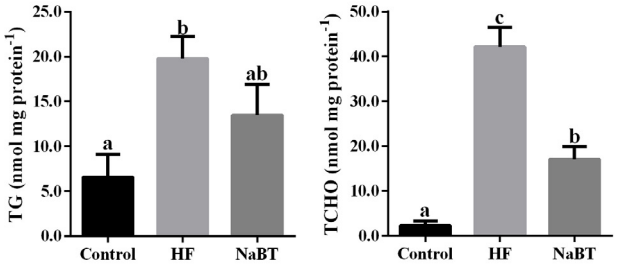


Fig. 2. Liver triacylglyceride (TG) and total cholesterol (TCHO) contents of common carp fed experimental diets. Control, the control diet; HF, the high-fat diet; NaBT, the high-fat diet added with 0.1% sodium butyrate. Bars marked with different letters are significantly different ($P$<0.05).

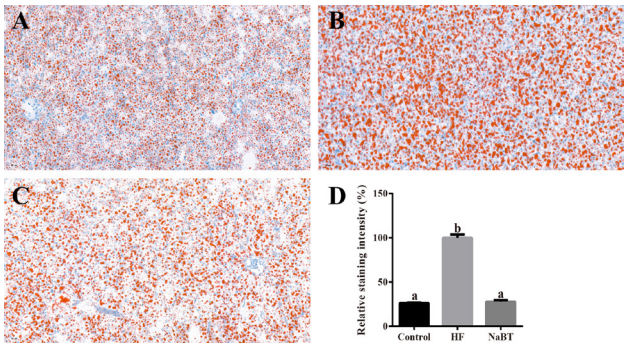


Fig. 3. Effect of dietary NaBT supplementation on hepatic lipid accumulation of common carp. Lipid droplets and nuclei are dyed in red and blue by oil red staining, respectively (200× magnification). A, the control diet; B, the high-fat diet; C, the high-fat diet added with 0.1% sodium butyrate. D. lipid accumulation was quantified by measuring the intensity of the stained oil droplets. Bars marked with different letters are significantly different ($P$<0.05).

*Hepatic oxidative stress*

Compared with diet control, diet HF significantly increased MDA content, and simultaneously decreased activity levels of T-SOD, CAT and GPx, as well as contents of GSH. However, diet NaBT significantly decreased MDA contents, and significantly increased T-SOD and

CAT activity levels ($P$<0.05), compared with diet HF (Fig. 4).

Compared to control, common carp at the HF group had a lower expression level of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) ($P$<0.05). However, common carp fed diet NaBT showed a significantly higher expression levels of Nrf2 and HO-1 compared with that fed the HF diet and exhibited a significantly lower expression levels of Nrf2 and HO-1 relative to that fed diet control ($P$<0.05) (Fig. 5).

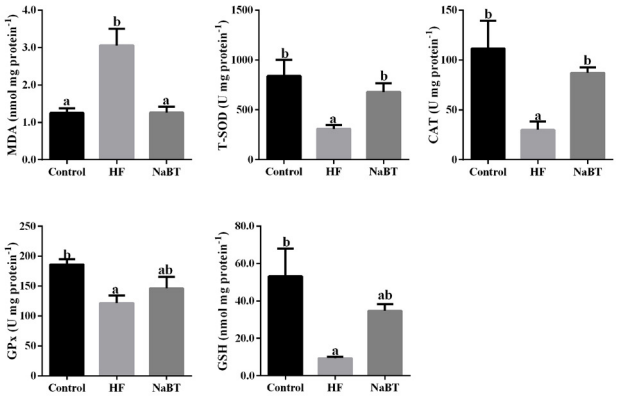


Fig. 4. Liver antioxidant status of common carp fed experimental diets. Notes, MDA, malondialdehyde; T-SOD, total superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione. Control, the control diet; HF, the high-fat diet; NaBT, the high-fat diet added with 0.1% sodium butyrate. Bars marked with different letters are significantly different ($P$<0.05).

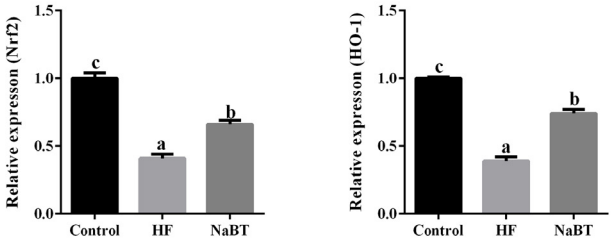


Fig. 5. Relative expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in liver of common carp fed experimental diets. Control, the control diet; HF, the high-fat diet; NaBT, the high-fat diet added with 0.1% sodium butyrate. Bars marked with different letters are significantly different ($P$<0.05).

*Hepatic inflammation*

Compared with the control, diet HF significantly upregulated the mRNA expression of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and IL-6 in liver ($P$<0.05), whereas diet NaBT significantly reduced the mRNA expression of TNF-α, IL-1β, and IL-6 ($P$<0.05) compared to fish fed diet HF. There were no significant difference in these genes in liver of fish fed diets control and NaBT ($P$>0.05) (Fig. 6).

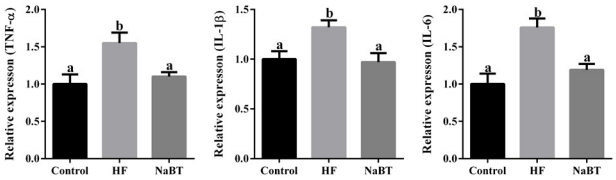


Fig. 6. Relative expression of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and IL-6 in liver of common carp fed experimental diets. Control, the control diet; HF, the high-fat diet; NaBT, the high-fat diet added with 0.1% sodium butyrate. Bars marked with different letters are significantly different ($P$<0.05).

## DISCUSSION

*Effect of dietary NaBT supplementation in high-fat diets on fish growth performance*

In the current study, high-fat diets did not affect fish growth and feed utilization, similar phenomenon were also found in previous studies on common carp (Abasubong *et al*., 2018) and blunt snout bream (Chen *et al*., 2016). Moreover, dietary supplementation with 0.1% NaBT has no influence on fish growth, in agreement with results of common carp fed diets containing 300 mg/kg microencapsulated sodium butyrate (MSB) (Liu *et al*., 2014). However, in other studies, enhancement of growth performance was observed in fish fed diets contacting NaBT, such as grass carp fed diets added with 0.1% powdery sodium butyrate or 0.05%-0.2% MSB (Tian *et al*., 2017), and rice field eel (*Monopterus albus*) fed with high soybean meal diets containing 0.025%-0.1% MSB (Zhang *et al*., 2020), and turbot (*Scophthalmus maximus*) fed 0.2% NaBT-containing diets (Liu *et al*., 2019). This discrepancy may be partly explained by the difference in fish species, dose and form of NaBT, feed composition, and rearing environment (Biagi *et al*., 2007; Liu *et al*., 2014, 2019).

*Dietary NaBT supplementation decreased lipid deposition and improved liver function*

In line with previous studies (Dai *et al*., 2019; Du *et al*., 2006), we found that high-fat diets significantly increased the deposition of TG and TCHO in the liver. However, dietary 0.1% NaBT supplementation significantly decreased hepatic TCHO content and lipid droplets. At present, few researches have examined the effect of dietary NaBT supplementation on the liver

TCHO and lipid droplets in aquaculture. However, reports on rats and pigs suggested that NaBT could reduce lipid deposition through peroxisome proliferator-activated receptor α-mediated activation of β oxidation (Sun *et al*., 2018). In addition, studies on pigs suggested that dietary 0.1% NaBT supplementation could reduce triglyceride content in liver by reducing lipogenesis and enhance lipolysis via regulating related hormones and genes, such as down-regulating the expression of fatty acid synthase (Jiao *et al*., 2020). Thus NaBT could decrease TCHO contents and lipid droplets by enhancing lipolysis and reducing lipogenesis, further study is needed to reveal the underlying mechanism.

The increased activity level of AST in plasma usually indicates liver damage or dysfunction (Ashouri *et al*., 2015; Wang *et al*., 2006). In the current study, dietary NaBT supplementation in high-fat diets significantly decreased AST activity levels, indicating that NaBT decreased the impairment of liver function induced by high fat. At present, reports on the effect of NaBT supplementation on the blood AST activity levels are scarce. However, accumulating data in terrestrial animals suggested that dietary NaBT supplementation could significantly reduce AST activity levels in blood of mice or rats fed high-fat diets (Mattace-Raso *et al*., 2013; Zhou *et al*., 2017, 2018). The reduction of AST activity in plasma of common carp fed NaBT diets may be due to antioxidant effect of NaBT. NaBT could reduce the hepatic oxidative stress, thereby stabilizing the membrane permeability and reducing the leakage of AST into the blood (Nasr, 2014).

*Dietary NaBT supplementation in high-fat diets increased liver antioxidant capacity*

High-fat diets often leads to oxidative stress in fish (Jia *et al*., 2020; Lu *et al*., 2017; Zhou *et al*., 2020). These oxidative stress includes increasing MDA contents and decreasing activity levels of antioxidant enzymes (Jia *et al*., 2017; Zhong *et al*., 2020). In this study, high-fat diets significantly increased MDA content and decreased the activity levels of antioxidant enzymes (e.g, SOD and CAT), indicating that oxidative stress occurred in fish fed on high-fat diets. However, dietary 0.1% NaBT supplementation significantly decreased MDA content and increased the activity levels of SOD and CAT, suggesting that NaBT alleviated the high-fat diet-induced oxidative stress.

Decrease in hepatic oxidative stress by NaBT could be due to the activation of Nrf2/HO-1 pathway. Nrf2/HO-1 pathway plays an important role in defending oxidative stress (Loboda *et al*., 2016). Nrf2 controls the expression of many antioxidant response element dependent genes and has been reported to upregulate the mRNA expression of CAT and SOD (Ma, 2013). Nrf2 could activate the transcription of HO-1 which degrades heme and generates the antioxidant molecules (Loboda *et al*., 2016). In the present study, lower and higher expression levels of Nrf2 and HO-1 were found in fish fed high-fat diets and NaBT diet, respectively, indicating NaBT increased the activation of Nrf2/HO-1 pathway. NaBT is known as an activator of Nrf2 (Dong *et al*., 2017; Wu *et al*., 2018; Yaku *et al*., 2013). In mammals, it has been proved that NaBT increased the expression of Nrf2 by inhibiting histone deacetylase (Dong *et al*., 2017; Wang *et al*., 2012). Whether NaBT activated Nrf2 expression through inhibiting HDAC in fish is an interesting question which was worthy of further investigation.

*Dietary NaBT supplementation in high-fat diets decreased liver inflammation*

Liver inflammation is a common phenomenon in aquatic animals (Cao *et al*., 2020; Dai *et al*., 2019). TNF-α, IL-1β, and IL-6 are commonly proinflammatory cytokines (Rauta *et al*., 2012) and have been identified as markers of inflammation in fish (Dai *et al*., 2019; Urán *et al*., 2008). In this study, high-fat diets significantly upregulated the mRNA expression levels of these proinflammatory cytokines in the liver, implying the occurrence of the liver inflammation in fish fed high-fat diets. However, dietary addition with NaBT in the high-fat diets significantly decreased the mRNA expression of these proinflammatory cytokines. Oxidative stress may partly account for the phenomenon. Oxidative stress and inflammation are closed linked; continued oxidative stress is known for leading to chronic inflammation through activating a variety transcription factors (Reuter *et al*., 2010). In this study, NaBT reduced the oxidative stress, thus alleviating the liver inflammation.

## CONCLUSION

In the present study, dietary 0.1% NaBT supplementation significantly reduced hepatic fat deposition and improved liver function of common carp fed high-fat diets. In addition, dietary supplementation with 0.1% NaBT in high-fat diets reduced hepatic oxidative stress and inflammation in common carp by activating Nrf2/HO-1 pathway.

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*Statement of conflict of interest*

The authors have declared no conflict of interest.

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