Inhibition of Farnesoid X Receptor Rescues Fat Depression Induced by Dietary Berberine in Grass Carp, *Ctenopharyngodon Idella*

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Abstract

Berberine (BBR) depresses lipid accumulation in fish, but the mechanism remains unknown. In this study, we hypothesize FXR signaling participates in this physiological process of grass carp. Three diets, namely the control, BBR (1.0 g/kg), and BBR + Gly- β -MCA (an FXR inhibitor) were formulated to feed juvenile grass carp (9.90 ± 0.07) for 8 weeks. Fish fed BBR presented significantly lower IPF index, hepatic TG and TC contents, as well as whole body lipid levels, whereas these were rescued by Gly- β -MCA. Serum TG and HDL-c contents were significantly decreased in fish fed BBR compared to those in the control. The serum ALT activity, combined with the TG, TC, HDL-c, and LDL-c concentrations were all significantly increased in fish fed BBR + Gly- β -MCA than those fed BBR. Dietary BBR significantly increased the mRNA and protein expression of FXR, decreased the mRNA level of FGF19 in the intestine, whereas these were reversed by Gly- β -MCA. In the hepatopancreas, the inhibitor recovered the suppression of the CYP7A1, CYP8B1, and CYP27A1 expression induced by dietary BBR. Fish fed BBR showed significantly lower mRNA expression of SREBP-1c and FAS, whereas these two genes were all up-regulated in response to inhibitor. Dietary BBR increased the gene expression of PPAR α , ATGL, CPT-1, which were all abolished by dietary Gly- β -MCA treatment. Fish fed BBR and BBR + Gly- β -MCA showed significantly lower total OTUs, ACE index, chao 1 index, and simpson index of the gut microbiota. Overall, our results demonstrate that inhibition of FXR leads to the rescue of lipid suppression induced by dietary BBR in grass carp.

Introduction

Aquaculture is the most promising strategy in solving increasingly demand for high quality protein in society (Reverter et al., 2020). In China, grass carp (*Ctenopharyngodon idella*) is the most cultured freshwater fish with nearly 20% in all the freshwater fish production (Fisheries Bureau of Ministry of Agriculture, 2019). However, there are some issues in front of the farmers and scientists that should be addressed, one of them is the excessively accumulated fat in the visceral, such as in the mesentery and liver, which is a big threaten to the health and market value of these cultured fish and sustainable development of aquaculture (Sun et al., 2021a; Tian et al., 2020).

Berberine (BBR, C₂₀H₁₈NO₄) is a natural plant alkaloid extracted from *Berberis aristate* and *Coptis chinensis* (Huanglian), an ancient anti-diarrhoeal medication. In mammals, BBR has antimicrobial activity against a variety of microorganisms (Zhou et al.), inhibits inflammatory process (Lin et al., 2019), and remits anti-oxidative stress (Moghaddam et al., 2014). Remarkably, BBR has been proved to reduce lipid accumulation by inhibiting lipogenesis and triggering fatty acid oxidative in mice (Kim et al., 2009; Yang et al., 2019b; Zhang et al., 2020). In aquaculture, there are increasingly evidences that demonstrate the fat-suppression role of BBR, such as *Megalobrama amblycephala* (Zhou et al., 2019), *Ctenopharyngodon idella* (Pan et al., 2019; Yang et al., 2019a), and *Acanthopagrus schlegelii* (Wang et al., 2021). However, though marked molecular in related to the lipogenesis and lipid catabolism has been tested, the primary mechanism that induced these changes is still not understood. BBR is poorly absorbed into the systemic circulation but is significantly accumulated in the intestine (Pan et al., 2019), implying an interestingly potential mechanism that BBR may influence the body's physiological changes through the intestine.

Actually, BBR has been found to impact the abundance and composition of gut microbiota both in mammals and fish (Pan et al., 2019; Zhang et al., 2020). Hence, it is supposed intestine is a target of BBR in depressing lipid accumulation in fish.

Farnesoid X receptor (FXR, NR1H4), a member of the nuclear receptor superfamily, is an intestinal molecular that has a vital role in regulating lipid metabolism of the whole body (Sun et al., 2021b). The natural ligands of FXR are bile acids (BAs) (Forman et al., 1995), which are reabsorbed by enterocytes and activate FXR that induce the expression of fibroblast growth factor (FGF) 19/15 hormone. After exiting the portal circulation, FGF19/15 binding to its liver FGFR4/ β -Klotho co-receptor complex ultimately leads to the inhibition of the rate-limiting enzyme of BAs synthesis: the cholesterol 7 α -hydroxylase (CYP7A1) (De Magalhaes Filho et al., 2017). In addition to influence the homeostasis of BAs, FXR also acts as an important target in modulating lipid accumulation by regulating lipid anabolism and lipid catabolism molecular (Jiao et al., 2015). Recently, we demonstrate that activating FXR decreased triglyceride (TG) and total cholesterol (TC) content in the liver, whereas inhibiting FXR increased the lipid accumulation in the liver and adipose tissue. Mechanically, inhibition of FXR would increase the expression of SREBP-1c and induce the TGs/cholesterol synthesis and lipid droplets formation (Tian et al., 2021). Interestingly, gut microbiota promote diet-induced obesity and associated phenotypes through FXR in mice (Parsus et al., 2017). Similarly, by modulating gut microbiota via antibiotic mixture, the FXR signaling of grass carp was attenuated and the lipid content in liver was increased (Tian et al., 2021). These studies indicate FXR is an effective molecular in controlling fat deposition in the intestine and may be influenced by the gut microbiota composition.

Collectively, considering the role BBR in depressing lipid content in fish and its characteristic of deposition in the intestinal and impact on the gut microbiota composition in grass carp. We hypothesize that FXR is a key molecular that mediates the function of BBR in controlling fat in the viscera. Therefore, in this study, we designed three semi-purified diets utilizing a FXR inhibitor glycine- β -muricholic acid (Gly- β -MCA), which was used to feed grass carp for 8 weeks, aiming to investigate the role of FXR in the inhibition of lipid accumulation in grass carp responding to dietary BBR.

Material And Methods

Experimental diets

Three isonitrogenous and isoenergetic semi-purified diets with 36.0% crude protein and 6.0% crude lipid were designed and formulated based on the method previous described (Lovell and Tom, 1998). Table 1 shows the formation and composition of the experimental diets. Casein and gelatine were used as the protein sources, the fish oil and soybean oil were used as the oil sources. The first diet that was not added extra chemicals was designed as the control. The second diet was added BBR (10g/kg; MedchemExpress LLC, Monmouth Junction, NJ, USA) at the expense of cellulose. The third diet was extra added the FXR inhibitor Gly- β -MCA (2.5mg/kg; MedchemExpress LLC) on the base of the second diet. The concentration of BBR and Gly- β -MCA were referred the study previously (Pan et al., 2019; Tian et al., 2021). We added

0.1% butylated hydroxytoluene (BHT; Sigma-Aldrich, USA) to the diets as the antioxidant (Table 1). The ingredients were manually mixed at the sequence from less to more by the magnitude of the quantity, which were blended with 70% sterile pure water to make a dough, which was pressed into noodle-like pellets (2-mm diameter). The pellets were dried under forced air at 25°C for 24h, packaged and stored at -20°C until utilization. The proximate composition of diets was determined according to the Association of Official Analytical Chemists (AOAC) procedures.

Experimental procedure

Experimental grass carp were purchased from Tongwei Aquaculture Co., Ltd. (Foshan, Guangdong, China), which were originated from the same parental stock. Fish were reared in aquaria (0.73×0.46×1.0m) and fed the commercial diet for 1 week, then the control diet for 2 week to acclimatize them to the experimental environment. The fish were not starved for 24h until the feeding experiment. A total of 180 healthy fish with uniform size (9.90 ± 0.07) were randomly distributed into nine aquaria (20 fish/aquarium). Each diet was randomly assigned into three aquaria. Fish were hand-fed to apparent satiation twice daily (at 8:30 and 16:30) for 8 weeks and the feed intake was recorded daily. During the feeding experiment, the water-dissolved O₂ was 5.6–6.4 mg/L, pH was 6.8–7.1, the temperature was 28.0–33.0°C. The photoperiod was 12h light-12h dark (from 8:00 to 20:00).

Sampling procedure

After 8 weeks of feeding experiment, all of the fish were fasted for 24h. Afterwards, the fish were weighed and anaesthetized with MS-222 at the concentration of 0.06g/L to maximally reduce the pain of fish during sampling. Six fish per aquarium were randomly selected for blood collection from the caudal vein by using 1ml injection. The obtained blood was placed at 4°C (at least 6h) for clotting; the serum was collected after centrifugation (825 *g*, 4°C, 10 min) of the blood. The sampled serum was frozen in liquid N₂ and stored at -80°C for biochemistry analysis. The remaining nine fish were dissected, the viscera, hepatopancreas, intraperitoneal fat (IPF) were stripped and weighed. Samples of the hepatopancreas from three fish per aquarium were fixed in 4% paraformaldehyde solution for histology analysis. Samples of hepatopancreas and mid-intestine from another six fish per aquarium were frozen in liquid N₂ then stored at -80°C for TG, TC, gene expression, and protein analysis. The intestine from three fish in each aquarium were aseptically removed, and the faecal matter were obtained by squeezing and scrapping the intestinal mucosa using a sterile spatula. The last five fish per aquarium were stored at -80°C for whole body fat analysis. Specific growth rate (SGR), feed conversion ratio (FCR), visceral index (VI), hepatopancreas index (HI), intraperitoneal fat index (IPFI), and survival rate (SR) were calculated using the following formulae:

$$\text{SGR} = (\text{Ln final weight} - \text{Ln initial weight}) \times 100/\text{days},$$

$$\text{FCR} = \text{amount of feed given}/\text{weight gain (g)},$$

$$\text{VI (\%)} = \text{visceral weight (g)} \times 100/\text{body weight (g)},$$

HI (%) = hepatopancreas weight (g) × 100/ body weight (g),

IPFI (%) = IPF weight × 100/body weight (g),

SR (%) = final number of fish×100/initial number of fish.

Serum biochemical parameters

Serum samples from of two fish were pooled and three two pooled samples for each aquarium were tested with the following methods. The serum biochemical parameters were assayed by enzymic procedures using an automatic analyzer (Mindray, Shenzhen, China). All of the serum biochemical parameters, including the alanine aminotransferase (ALT), aspartate aminotransferase (AST), TG, TC, high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) were tested using assay kits from Mindray.

Histological analysis of hepatopancreas

For haematoxylin and eosin (H&E) staining, the fixed hepatopancreas were washed in tap water for 12h, followed by dehydration in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 95%, and 100%) twice. samples were equilibrated in xylene and embedded in paraffin based on standard histological techniques previously described (Tian et al., 2017). Sections (5 µm) were cut with a rotary microtome (RM2235, Leica Camera AG, Wetzlar, Germany), mounted on glass slides, and stained with H&E. Histological samples were observed and photographed using an upright microscope (Olympus BX41, Olympus Corporation, Tokyo, Japan).

For oil red O staining, fixed hepatopancreases were incubated with 30% sucrose at 4°C for three days. They were then embedded in an optimal cutting temperature compound (Leica), cut into 6–10 µm sections, and rinsed with distilled water. Slides were permeated with 60% isopropanol for 20–30 s and stained with oil red O (Sigma-Aldrich) for 10 min. Slides were immediately destained in 60% isopropanol for 3 min and washed with distilled water to clean the background. The sections were counterstained with Mayer's haematoxylin for 1 min, and washed with distilled water for 10 min. The slides were seal-capped with glycerogelatin and were photographed using a light microscope (Olympus BX41, Olympus Corporation).

Hepatic TG and TC content

The hepatopancreases from three fish per aquarium were used for the TG and TC content assays, which were determined using TG and TC assay kits, respectively (Jian Cheng Bioengineering Institute, Nanjing, China). The manufacturer's instructions were followed, and each sample was analysed in triplicate.

Whole body lipid content

The whole body fat content (3 individuals/aquarium) was determined according the Association of Official Analytical Chemists (AOAC) procedures (1995) methods. Briefly, the whole fish was homogenized

and its crude lipid was measured by ether extraction using the Soxhlet method after freeze drying to a constant weight at -40°C.

Quantitative real-time PCR

The total RNA of the sampled hepatopancreases or intestines was extracted based on the method described above. After removing the DNA in the total RNA, cDNA was synthesised using a PrimeScript® RT reagent kit (TaKaRa) according to the manufacturer's protocol. The primers are listed in Table S1. Based on a series of 6-step 10-fold dilutions of the target template, the amplification efficiency of these primers ranged from 92.46–103.68% after qRT-PCR assay. The determination coefficient (R^2) values were > 0.99. The β -actin was used as the reference gene based on preliminary tests using geNorm (version 3.5) and NormFinder algorithms. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed three times (LightCycler 96, Roche Diagnostics, Basel, Switzerland) in a final volume of 20 μ L containing 0.6 μ L of each primer (0.5 μ M), 1 μ L diluted first-strand cDNA product, 10 μ L 2 $\times$ SYBR Premix Ex Taq II (TaKaRa), and 7.8 μ L sterilised double-distilled water. The cycling parameters were: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. After the PCR reaction, the melting curve was analysed over a range of 60–95°C (in 5 s steps) to confirm a single product. Negative controls, including a no-cDNA control and a DNase-treated non-reverse transcribed tissue RNA sample, were used to ensure that only the cDNA was quantified in each sample. A relative quantification method was used to calculate the gene expression values using the comparative CT method ($2^{-\Delta\Delta C_t}$) previously described in the literature (Livak and Schmittgen, 2001; Pfaffl, 2001).

Western-blotting

The intestine and hepatopancreas were homogenized with glass tenbroeck tissue grinders on ice (3 individuals per aquarium). The cell lysis buffer supplemented with protease and phosphatase inhibitor cocktails (Beyotime, Nanjing, China) was added before homogenization. Afterwards, the crude lysates were centrifuged for 10 min (4°C; 13,000g), the upper mixture were collected for further analysis. The total protein concentration was determined by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc., United States). The protein samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Beyotime, Nanjing, China) by electroblotting. The membranes were incubated with the primary antibodies overnight at 4°C. After washing, the secondary antibody was added and incubated for 2h at room temperature. The protein bands were visualized by ECL Plus (Beyotime, Nanjing, China). The membranes were then stripped and reprobed with anti- β -actin antibody. Densitometric quantitation was performed using a Sagecreation imaging system with Sagecreation Quantity One software (Sagecreation Co., Ltd.). The following antibodies were used: anti-bodies against anti-FXR (56kDa; Rabbit; Abcam, Cambridge, MA); anti-bodies against anti-CYP7A1 (57kDa; Rabbit; Invitrogen)

Gut microbiota diversity analysis

The total gut microbial DNA was extracted using the NucleoSpin Soil kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. DNA integrity and quality were monitored on 1% agarose gels. The quantity of the DNA was measured using an Implen NanoPhotometer (Implen, Inc.).

The V3 + V4 regions of the 16S rDNA were then amplified by the broad fusion primers of 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCRs were performed using 10 µL of reaction volume: 5 µL of KOD FX Neo Buffer, 2µL of dNTP, 50 ng of DNA, 0.3 µL of each primer (10 µM), 0.2 µL of KOD FX Neo, and ddH₂O up to 10 µL. The amplification program had an initial denaturation phase at 95°C for 3 min followed by 25 cycles of denaturation at 95°C for 30 s, an annealing stage at 50°C for 30 s, and an extension stage at 72°C for 40 s, followed by an extension at 72°C for 7 min. The amplifications were extracted from 1.8% agarose gels and purified using Agencourt® AMPure XP Beads (A63881, Beckman Coulter Life Sciences, Indianapolis, IN, USA). Then, the gene quality was assessed using a Qubit® 2.0 Fluorometer (Q32866, Invitrogen, Carlsbad, CA, USA) and an Agilent 2100 Bioanalyzer (G2939AA, Agilent Technologies, Santa Clara, CA, USA). Finally, the qualified amplifications were run on an Illumina HiSeq 2500 platform from Biomarker Technologies Co., Ltd. (Beijing, China).

The obtained raw data were then jointed using FLASH v1.2.7 software to produce raw tags with an overlap length > 10 bp and a maximum mismatch rate < 0.2. High-quality tags were obtained by filtering raw tags using the Trimmomatic v 0.33 software with average Phred scores < 20. Chimeric sequences were discarded with the use of UCHIME v 4.2 software. The tags were clustered into one operational taxonomic unit (OTU) at a 97% identify threshold. The OTUs were classified and annotated using the QIIME software based on the Silva database (<http://www.arb-silva.de>). The alpha diversity indexes, including the number of OTUs, abundance-based coverage estimator (ACE), and Chao1, and the Shannon and Simpson diversity indexes were calculated.

Statistical analyses

The data are expressed as the mean ± standard deviation (S.D.). One way analysis of variance was used to compare differences, followed by Tukey's post hoc test. Percentage data were arcsine-transformed prior to analysis. All analyses were conducted using PASW Statistics 18 (SPSS, Chicago, IL, USA). A significance level of $P < 0.05$ was used for all tests.

Results

Growth performance, feed utilization, and biological parameters

During the feeding experiment, all fish accepted the diets well and no dead fish was found (Table 2). As shown in table 2, grass carp fed with BBR+Gly-β-MCA showed significantly higher final weight and SGR than fish fed the control and BBR diets ($P < 0.05$). The FCR of the BBR+ Gly-β-MCA group was significantly lower than that of the BBR group ($P < 0.05$). Fish fed with BBR showed lower VI, HI, and IPFI than the other two groups, of which VI was significant difference than fish fed Gly-β-MCA, and IPFI was significant difference than the other two groups ($P < 0.05$).

Serum biochemical parameters

Table 3 shows the serum biochemical parameters in fish fed different diets. The serum from fish fed BBR showed significantly lower TG, and HDL-c contents than those from fish fed the control diet ($P<0.05$). However, when the grass carp was fed the diet supplemented with Gly- β -MCA, the ALT activity, as well as the TG, TC, HDL-c, and LDL-c concentrations were all significantly increased in comparison to the fish fed the BBR diet ($P<0.05$).

Hepatic lipid content

The hepatopancreas was stained with H&E and oil red O to directly observe the lipid accumulation of the fish (Fig. 1A). Histological section showed that the hepatopancreas of fish fed BBR contained distinctly less lipid droplets than those of the control fish, whereas there were obviously larger lipid droplets in the hepatopancreas of fish fed the BBR+Gly- β -MCA than in those of fish fed the control and BBR feed. Quantitatively, hepatic TG and TC contents were significantly lower in the BBR group than those in the control and BBR+Gly- β -MCA groups ($P<0.05$, Fig. 1B).

Whole body lipid content

The whole body of fish fed different diets were analyzed the crude fat contents (Fig. 2). It is found that fish fed BBR had significantly lower lipid content than the fish fed the control diet, whereas the whole body lipid content was significantly recovered after administration Gly- β -MCA in the BBR diet ($P<0.05$).

FXR signaling gene and protein expression

The mRNA and protein expression of the molecular related to FXR signaling are shown in Fig. 3. Fish fed BBR showed significantly higher expression level of FXR than the control in the intestine ($P<0.05$, Fig. 3A), whereas the relative expression of the mRNA for FXR was significantly decreased in the fish fed BBR+Gly- β -MCA than that in the BBR group ($P<0.05$, Fig. 3A). The target gene of FXR, FGF19, was significantly down-regulated in the intestine of the BBR fed fish, whereas this was recovered to a significant level by Gly- β -MCA administration ($P<0.05$, Fig. 3A). Similarly, the protein expression of FXR was significantly increased in the intestine of fish fed BBR, whereas was suppressed by Gly- β -MCA ($P<0.05$, Fig. 3B).

In the hepatopancreas, the mRNA expression of CYP7A1, sterol 12 α -hydroxylase (CYP8B1), and sterol 27-hydroxylase (CYP27A1) were all significantly decreased after feeding with BBR ($P<0.05$, Fig. 3C). However, when the diet was added the FXR inhibitor, these three genes were all significantly increased their expression in comparison to BBR treatment ($P<0.05$, Fig. 3C). Accordingly, dietary BBR significantly decreased the protein level of CYP7A1, which was recovered by Gly- β -MCA administration ($P<0.05$, Fig. 3D).

Lipid metabolism related gene expression

The mRNA expression of genes related to lipid anabolism in the hepatopancreas are shown in Fig. 4. There was no significant difference of the mRNA expression of peroxisome proliferator activated receptor γ (PPAR γ) between the control and BBR groups ($P>0.05$). However, fish fed with BBR diet showed

significantly decreased mRNA expression of sterol-regulatory element binding proteins-1c (SREBP-1c) and fatty acid synthase (FAS) ($P<0.05$). When the diet was supplemented with BBR+Gly- β -MCA, the mRNA expression of PPAR γ , SREBP-1c and FAS were all significantly increased compared to the BBR group ($P<0.05$).

Several genes related to lipid catabolism in the hepatopancreas are shown in Fig. 5. Significant difference of the relative mRNA expression of peroxisome proliferator activated receptor α (PPAR α), adipose triglyceride lipase (ATGL), and carnitine palmitoyltransferase 1 (CPT-1) was found between the control and BBR groups ($P<0.05$), they were all up-regulated in the fish consuming BBR. Moreover, these three genes were down-regulated to a significant level in the fish fed inhibitor Gly- β -MCA diet than fish fed the BBR diet ($P<0.05$).

Gut microbiota composition

The alpha-diversity of the gut microbiota in fish feeding different diets are shown in table 4. Fish fed with BBR and BBR+Gly- β -MCA showed significantly lower total OTUs, ACE index, chao1 index, and simpson index than fish fed the control diet ($P<0.05$, Table 4). However, no significant difference of the shannon index was found amongst the treatments.

At the phylum level, the most abundant phyla were *Proteobacteria*, *Fusobacteria*, *Bacteroidetes*, and *Firmicutes* (Fig. 6A, Fig. S1). No significant difference of the relative abundance of these phyla was found among groups. However, numerically, the abundance of *Proteobacteria* was higher in BBR group (49.67%) than in control (35.75%), whereas this was suppressed by dietary Gly- β -MCA to 22.57%. The abundance of *Firmicutes* was decreased from 5.58% to 1.24% after feeding BBR, but was increased to 10.87% after feeding inhibitor. At the genus level, *Cetobacterium*, *Rhodobacter*, *Phreatobacter* were the most abundance genus (Fig. 6B, Fig. S1). *Phreatobacter* was the significant abundance genus, which was increased from 7.25% to 14.90% after feeding with BBR, whereas significantly decreased to 2.38% in response to BBR+Gly- β -MCA diet ($P<0.05$). No significant difference of the other genus of the gut microbiota was found among the groups ($P>0.05$).

Discussion

BBR has been used in traditional Chinese medicine and Ayurvedic medicine and current research evidences support its use for various therapeutic areas (Singh and Mahajan, 2013). One of its remarkable function is reduction of lipid accumulation, this is conservative from fish to mammals (Hao et al., 2017; Liang and Wang, 2018; Wang et al., 2021; Yang et al., 2019a; Yang et al., 2019b; Zhou et al., 2011). Interestingly, although it has direct role in reducing lipid deposition in hepatocytes or adipocytes (Yang et al., 2019a; Yang et al., 2019b; Zhou et al., 2011), BBR seldom enter into the circulatory system and mainly accumulates in the intestine *in vivo* (Pan et al., 2019; Sun et al., 2017). Thus, the mechanism of dietary BBR on the lipid metabolism still remain elusive. In the present study, we explored the role of FXR, a modulator of lipid metabolism molecular in the intestine, in this process of grass carp through pharmacological methods. We show that dietary BBR activated the FXR signaling pathway, and inhibition

of FXR abolished the fat suppression function, as well as some lipogenic genes induced by dietary BBR in grass carp.

During the feeding experiment, all fish accepted the diets well, and no dead fish was recorded, suggesting that dietary BBR and FXR inhibitor had no negative impact on the experimental fish. Dietary BBR had no impact on the growth of grass carp, which is consistent with previous studies in grass carp (Pan et al., 2019) and black sea bream (Wang et al., 2021). The decreased VI, HI, and IPFI in fish fed BBR are in line with the lipid content of the whole fish, reflecting BBR decreased lipid accumulation in a macroscopic view. On the contrary, dietary BBR + Gly- β -MCA increased the growth, VI, HI, and IPFI in fish compared to the BBR group, which can be explained by the fat accumulation in the viscera. However, the compensatory growth of the fish (such as intestine, hepatopancreas, IPF *etc.*) in responses to Gly- β -MCA to meet the FXR signalling requirements in tissues cannot be excluded.

The activities of serum ALT and AST are generally important indicators of the liver function, they also reflect the health of other tissues, such as spleen, kidney, *etc.* (Tian et al., 2014). Our study showed that the activity of serum ALT was decreased in the BBR feeding fish, whereas increased in the BBR + Gly- β -MCA feeding fish. The serum AST also showed similar trends. The changes of these two enzymes are consistent with the VI, HI, and IPFI, suggesting that dietary BBR may be beneficial for the function of tissues in the viscera of grass carp. However, administration with FXR inhibitor may do harm to the health of the cells in the fish. This is possibly due to the lipid droplets accumulation in cells that disordered the cellular homeostasis (Hyun et al., 2005). Serum TG and TC are indicators for the body lipid content both in mammals and fish (De Giorgio et al., 1982; Tian et al., 2019). Our study proved the serum TG and TC were all decreased in fish consuming BBR, which is in line with early study (Pan et al., 2019; Wang et al., 2021). We further proved that administration with Gly- β -MCA rescued the suppression of these parameters, suggesting that inhibition of FXR could recover the serum fat homeostasis induced by dietary BBR.

By staining with H&E and oil red O, the lipid droplets in the hepatocytes were decreased the accumulation by the dietary BBR (marked in vacuole and red particles, respectively). Because lipid droplets are composed by TG and sterol esters (Lundquist and Susanto, 2020), our quantitative data also showed significantly decreased TG and TC content in BBR feeding fish, suggesting the component of the lipid droplets were also reduced. Our results are in line with previous studies in grass carp (Yang et al., 2019a), black sea bream (Wang et al., 2020), and blunt snout bream (Zhou et al., 2019). As a contrast, BBR + Gly- β -MCA increased the lipid content in the hepatopancreas, suggesting that FXR signalling play a negative role in fat deposition. Our previous study showed that solely treatment with Gly- β -MCA also increased the lipid content in grass carp (Tian et al., 2021), combined with this study, we could conclude that inhibition of FXR could eliminate the fat depression function of dietary BBR. However, it is still not confirmed BBR decreased the lipid accumulation via FXR signalling. We then explored the molecular events in the FXR signalling. In the intestine, activated FXR stimulates the expression of FGF19 hormone, which binds to the liver FGFR4/ β -Klotho co-receptor complex to inhibit the rate-limiting enzyme of the bile acid synthesis CYP7A1 after transportation in the portal circulation (Zheng et al., 2017). Intriguingly, dietary BBR increased the mRNA (and protein) expression of FXR and decreased its downstream gene FGF19 in the

intestine, as well as decreased the gene (or protein) expression of CYP7A1, CYP8B1, and CYP27A1 in the hepatopancreas, suggesting that BBR did activate the signalling of FXR. Expectedly, the decreased molecular expression of FXR combined with the up-regulation of the downstream of the FXR genes (or protein) by Gly- β -MCA treatment implied that pharmacological treatment succeeded in abolishment in the FXR signaling induced by BBR. These data also indirectly indicate that FXR signalling participated in the fat suppression of dietary BBR in grass carp.

Previous studies have shown that BBR suppresses lipid accumulation via inhibiting lipogenesis and promoting lipid oxidative in fish (He et al., 2021; Wang et al., 2021). In the present study, we show that dietary BBR decreased the relative mRNA expression of lipogenic genes, SREBP-1c and FAS. Moreover, we also did find the difference of lipid catabolism genes, PPAR α , ATGL and CPT-1. These results are consistent with early studies. Importantly, our results provide evidences that inhibition of FXR abolished the down-regulation of lipid anabolism genes, as well as the up-regulation of lipid catabolism genes induced by dietary BBR, suggesting FXR signalling plays an important role in the modulation of lipid metabolism related transcripts induced by dietary BBR, similar to our early study (Tian et al., 2021).

The results that dietary BBR decreased the total OTUs, ACE index, and Chao 1 index suggest that BBR decreased the richness of gut microbiota in grass carp. This might be due to the antibacterial property of BBR (Zhang et al., 2020). Interestingly, dietary BBR also decreased the simpson index, which implied increased diversity of the gut microbiota in BBR treated fish, in line with early studies (Pan et al., 2019). The total decreased richness but increased diversity may be due to increased community evenness for the composition of gut microbiota. Furthermore, we also showed that dietary Gly- β -MCA had no obvious difference of the alpha-diversity of the gut microbiota, indicating that the drug used in this study did not influence the gut microbiota composition. In mammals, the richness and diversity of gut microbiota are regarded to be a key factor in impacting the body fat accumulation and metabolic disease (Clemente et al., 2012; Turnbaugh et al., 2006). Obesity people have more diversity of the gut microbiota than the thin ones (Turnbaugh et al., 2009). Similarly, the changes of the gut microbiota in fish are also linked to the changes in fat content (Sheng et al., 2018; Tian et al., 2021). It is generally accepted that gut microbiota decougate and further metabolise primary BAs into secondary BAs through bile salt hydrolase (BSH) (Wahlström et al., 2017). BAs differ widely in their ability to activate FXR (de Boer et al., 2020), the changes in the BAs composition altered by the may altered the signalling of FXR. From this view, BBR may influence the FXR via modulating gut microbiota, which altered the composition of BAs and activated FXR signalling, further decreased the lipid accumulation in grass carp.

It is suggested that obesity mice or human have higher content of *Firmicutes* and lower *Bacteroidetes* (Kallus and Brandt, 2012). This is possibly due to the differences of these two bacteria in the contents for the enzymes related to lipid/carbohydrate metabolism (Stephens et al., 2018). In our study, though have no obvious difference of the gut microbiota composition in the phyla level. The concentration of *Firmicutes* had a decreased trend in the BBR treatment, whereas had an increased trend in the BBR + Gly- β -MCA treatment, which is in line with the fat content of grass carp. In addition, several other bacteria have

similar trends with lipid content, such as *acidobacteria* and *gemmatimonadetes*, as well as the genus *Phreatobacter*, but the relationship between these bacteria and lipid metabolism are scarcely studied.

In conclusion, in this study, we explored the mechanism of dietary BBR on the lipid metabolism by using the FXR inhibitor Gly- β -MCA in grass carp. Dietary BBR modulated gut microbiota composition, activated FXR signalling, and suppressed lipid accumulation. Meanwhile, inhibition of FXR could recover the suppression of fat deposition induced by BBR. It is speculated that FXR signalling plays an important role in the function of BBR in modulating lipid accumulation in grass carp. More studies related to the gut microbiota and BAs composition that altered by BBR are needed to be further addressed.

Declarations

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Consent for publication: Not applicable.

Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability: Not applicable.

Authors' contributions: J.J. Tian, E.M. Yu, and J. Xie conceived and designed the experiments. Y. Q. Jin and J. J. Tian performed experiment and analyzed the data. J. J. Tian, Y. Q. Jin, and E.M. Yu co-wrote the paper. J. H. Sun, Y. Xia, K. Zhang, Z. F. Li, W. B. Gong, and G. J. Wang revised the manuscript.

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Tables

Table 1 Formulation and chemical composition of the experimental diets (g Kg⁻¹ dry matter)

Component	Control	BBR	BBR+Gly-β-MCA
Casein	320	320	320
Gelatin	80	80	80
Dextrin	280	280	280
Cellulose	189	188.8	187.975
Fish oil	30	30	30
Soybean oil	30	30	30
Carboxymethylcellulose	20	20	20
Butylated hydroxytoluene	1	1	1
BBR	0	1	1
Gly-β-MCA	0	0	0.025
Vitamin mixture ¹	10	10	10
Mineral mixture ²	40	40	40
Total	1000	1000	1000
Chemical composition			
Moisture%	8.94	8.94	8.74
Ash%	5.43	5.55	5.61
Crude protein%	36.02	35.96	36.03
Crude fat%	6.00	5.99	5.95

Note: ¹ The mineral mix contained (g/100 g of the total mineral): KAl(SO₄) 0.159, CaCO₃ 18.101, Ca(H₂PO₄)₂ 44.601, CoCl 0.070, MgSO₄ 5.216, MnSO₄·H₂O 0.070, KCl 16.553, KI 0.014, ZnCO₃ 0.192, NaH₂PO₄ 13.605, Na₂SeO₃ 0.006, CuSO₄·5H₂O 0.075, ferric citrate 1.338.

² The vitamin mix contained (mg/1000 g of diet): vitamin C, 200, thiamine, 10, riboflavin, 20, vitamin A, 3000 IU, vitamin E, 50 IU, vitamin D3, 1500 IU, menadione, 10, pyridoxine HCl, 10, cyanocobalamin, 0.02, biotin, 1.0, calcium pantothenate, 40, folic acid, 5, niacin, 20, inositol, 400, choline chloride, 2000, and cellulose was used as a carrier.

Table 2 Growth and biological parameters of grass carp fed berberine (BBR) supplemented with or without farnesoid X receptor (FXR) inhibitor Gly- β -MCA ^a

	Control	BBR	BBR+ Gly- β -MCA
Initial weight (g)	9.91±0.04	9.81±0.06	9.98±0.08
Final weight (g)	19.73±0.55 ^b	19.51±0.81 ^b	21.63±0.57 ^a
Specific growth rate (SGR)	1.23±0.04 ^b	1.20±0.08 ^b	1.38±0.06 ^a
Feed conversion ratio (FCR)	1.36±0.07 ^{ab}	1.50±0.11 ^a	1.25±0.07 ^b
Viscera index (VI, %)	10.01±0.22 ^{ab}	9.66±0.08 ^b	11.14±0.72 ^a
Hepatopancreas index (HI, %)	1.88±0.14	1.71±0.18	2.02±0.27
Intraperitoneal fat index (IPFI, %)	1.49±0.11 ^b	1.19±0.13 ^c	2.17±0.26 ^a
Survival rate (SR, %)	100	100	100

^a Different symbols denote significant differences ($P < 0.05$), values are means $\pm$ standard deviation, n = 3 per group.

Table 3 Serum biochemistry parameters in grass carp fed berberine (BBR) supplemented with or without farnesoid X receptor (FXR) inhibitor Gly- β -MCA.^a

Index	Control	BBR	BBR+ Gly- β -MCA
ALT ^b (U L ⁻¹)	20.00 $\pm$ 4.64 ^{ab}	12.50 $\pm$ 4.30 ^b	22.17 $\pm$ 0.24 ^a
AST ^c (U L ⁻¹)	106.67 $\pm$ 3.52	96.00 $\pm$ 11.78	107.33 $\pm$ 18.58
TG ^d (mmol L ⁻¹)	2.61 $\pm$ 0.01 ^b	2.30 $\pm$ 0.08 ^c	2.89 $\pm$ 0.07 ^a
TC ^e (mmol L ⁻¹)	5.86 $\pm$ 0.54 ^{ab}	5.30 $\pm$ 0.20 ^b	6.56 $\pm$ 0.17 ^a
HDL-c ^f (mmol L ⁻¹)	1.74 $\pm$ 0.02 ^b	1.60 $\pm$ 0.04 ^c	1.87 $\pm$ 0.02 ^a
LDL-c ^g (mmol L ⁻¹)	1.77 $\pm$ 0.05 ^b	1.53 $\pm$ 0.04 ^b	2.08 $\pm$ 0.16 ^a

^a Different symbols denote significant differences ($P < 0.05$), values are means $\pm$ standard deviation, n = 3 per group.

^b ALT: alanine aminotransferase

^c AST: aspartate aminotransferase

^d TG: triglyceride

^e TC: total cholesterol

^f HDL-c: high-density lipoprotein cholesterol

^g LDL-c: low-density lipoprotein cholesterol

Table 4 The α -diversity of gut microbiota in grass carp fed berberine (BBR) supplemented with or without farnesoid X receptor (FXR) inhibitor Gly- β -MCA^a

Index	Control	BBR	BBR+ Gly- β -MCA
Total OTUs	407.67 $\pm$ 11.58 ^a	378.67 $\pm$ 6.94 ^b	359.00 $\pm$ 13.64 ^b
Ace index	474.80 $\pm$ 5.52 ^a	439.84 $\pm$ 16.62 ^b	427.58 $\pm$ 6.41 ^b
Chao1 index	486.59 $\pm$ 14.76 ^a	449.03 $\pm$ 11.33 ^b	435.41 $\pm$ 11.82 ^b
Shannon index	2.47 $\pm$ 0.60	2.45 $\pm$ 0.52	3.20 $\pm$ 1.46
Simpson index	0.34 $\pm$ 0.01 ^a	0.23 $\pm$ 0.05 ^b	0.19 $\pm$ 0.05 ^b

^a Different symbols denote significant differences ($P < 0.05$), values are means $\pm$ standard deviation, n = 3 per group.

Figures

Figure 1

Histology section (A), triglyceride (TG) and total cholesterol (TC) levels (B) of the hepatopancreas of grass carp fed berberine (BBR) supplemented with or without farnesoid X receptor (FXR) inhibitor Gly- β -MCA. All results are presented as mean $\pm$ SD (error bars) (n = 3). Means in each panel without a common letter are significantly different ($P < 0.05$, ANOVA)

Figure 2

Whole body fat content of grass carp fed berberine (BBR) supplemented with or without farnesoid X receptor (FXR) inhibitor Gly-β-MCA. All results are presented as mean $\pm$ SD (error bars) (n = 3). Means in each panel without a common letter are significantly different (P < 0.05, ANOVA)

Figure 3

Farnesoid X receptor (FXR) signalling related genes and protein expression in the intestine (A, B) and hepatopancreas (C, D) of grass carp fed berberine (BBR) supplemented with or without FXR inhibitor Gly-β-MCA. All results are presented as mean $\pm$ SD (error bars) (n = 3). Means in each panel without a common letter are significantly different (P < 0.05, ANOVA).

Lipid anabolism

Figure 4

Lipid anabolism related genes in the hepatopancreas in grass carp fed berberine (BBR) supplemented with or without farnesoid X receptor (FXR) inhibitor Gly- β -MCA. All results are presented as mean $\pm$ SD (error bars) (n = 3). Means in each panel without a common letter are significantly different (P < 0.05, ANOVA).

Lipid catabolism

Figure 5

Lipid catabolism related genes in the hepatopancrease in grass carp fed berberine (BBR) supplemented with or without farnesoid X receptor (FXR) inhibitor Gly- β -MCA. All results are presented as mean $\pm$ SD (error bars) (n = 3). Means in each panel without a common letter are significantly different (P < 0.05, ANOVA).

Figure 6

Relative abundance of 16S gut microbiota at the phylum (A) and genus (B) levels in juvenile grass carp fed berberine (BBR) supplemented with or without farnesoid X receptor (FXR) inhibitor Gly- β -MCA.

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