Fish Physiol Biochem (2021) 47:499–513
https://doi.org/10.1007/s10695-021-00927-8

Dietary berberine can ameliorate glucose metabolism disorder of *Megalobrama amblycephala* exposed to a high-carbohydrate diet

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Received: 13 September 2020 / Accepted: 12 January 2021 / Published online: 26 January 2021

Abstract Blunt snout bream (*Megalobrama amblycephala*) were randomly assigned into three diets: normal-carbohydrate diet (NCD, 30% carbohydrate, *w/w*), high-carbohydrate diet (HCD, 43% carbohydrate), and HCB (HCD supplemented with 50 mg/kg berberine (BBR)). After 10 weeks' feeding trial, the results showed that higher levels of plasma glucose, triglyceride, and total cholesterol were observed in HCD-fed fish than in NCD-fed fish, while HCB feeding significantly ameliorated this effect. Moreover, HCB feeding remarkably reversed HCD-induced hepatic glycogen and lipid contents. In insulin signaling, BBR inclusion restored HCD-induced suppression of *insulin receptor substrate* mRNA expression and elevation of *forkhead transcription factor 1* mRNA expression. In glucose metabolism, upregulated *glucose transporter 2* and *glycogen synthase* mRNA expressions in the HCD group were observed compared to the NCD group. However, BBR adding reduced the mRNA expressions of *glycogen synthase*, *phosphoenolpyruvate carboxykinase*, and *glucose-6-phosphatase* and increased the transcriptional levels of *glucose transporter 2* and *pyruvate kinase*. In lipid metabolism, BBR supplementation could reverse downregulated hepatic *carnitine palmitoyl transferase I* mRNA expression and upregulated hepatic *acetyl-CoA carboxylase* and *fatty acid synthetase* mRNA expressions in the HCD group. Taken together, it demonstrates that BBR could improve glucose metabolism of this species via enhancing liver's glycolysis and insulin signaling, while inhibiting liver's glycogen synthesis and gluconeogenesis. It also indicates that BBR could reduce the metabolic burden of the liver by inhibiting fat synthesis and promoting lipid decomposition, and then enhance fat uptake in peripheral tissues.

Keywords Carbohydrate · Berberine · Glycolipid metabolism · Insulin signaling · *Megalobrama amblycephala*

Introduction

With the increasing demand for aquatic feed, the booming aquaculture industry has been facing a big problem, how to replace expensive and limited animal-origin feed with cheap plant-origin feed. Carbohydrate, rich in sugar and starch, is an inexpensive and widely sourced energy substance. Adding carbohydrates appropriately in the diets can promote fish growth, save protein, reduce ammonia emissions, cut down lipid catabolism, provide metabolic intermediates, and etc. (Azaza et al. 2015; Li

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et al. 2015; Watanabe 2002). However, due to lower carbohydrate utilization rate of fish than mammal, long-term intake of a carbohydrate-rich diet would cause a glucose metabolic disorder to fish (Moon 2001; NRC 2011). Functional foods may have the potential to improve glycemic control (Bai et al. 2015). For example, adding specific nutrients exogenously, such as thiamine and specific amino acids, is a very simple and effective way to attenuate hyperglycemia (Andoh 2007; Hansen et al. 2015; Kamalam et al. 2017). Additionally, Chinese herbal medicine, as a natural medicine, has been showing the characteristics of mild hypoglycemic effect and low cost to solve this issue (El Kaissi and Sherbeeni 2011; Li et al. 2004).

Berberine (BBR) is a natural compound in many Chinese herbaceous plants such as *Coptis chinensis* and has been recognized as having various medical potentials (Tillhon et al. 2012). Traditionally, BBR is often used to treat gastrointestinal infections due to its antibacterial and anti-inflammatory properties (Chow et al. 2016; Neag et al. 2018; Tillhon et al. 2012). Nowadays, BBR is attracting much attention because of its outstanding regulation of glucose and lipid metabolism (Ilyas et al. 2020; Zhang et al. 2008), and has also been confirmed in clinical trials (Ni 1988; Wei et al. 2004; Xie et al. 2005). Not only that, BBR also shows a good potential of regulating glucolipid metabolism in fish. For example, in *Ctenopharyngodon idella*, BBR-supplemented feed can significantly reduce the levels of glucose, total cholesterol (TC), and triglyceride (TG) in the blood, which might be through affecting the diversity and structure of intestinal microorganisms (Pan et al. 2019). And it was also reported that BBR could also reduce lipids by activating genes related to fatty acid oxidation and genes related to reducing fatty acid intake (Lu et al. 2016; Zhou et al. 2019). However, there are limited reports about the molecular mechanism of BBR regulating glycometabolism so far.

Blunt snout bream (*Megalobrama amblycephala*) is a herbivorous freshwater fish of key economic and cultural importance in China. Its herbivory and relative tolerance to carbohydrate also make it possible to add more starch to its feed for saving costs, although excessive carbohydrate still causes disorder of glucose metabolism. Furthermore, adding glucose-lowering substances into the feed to alleviate the metabolic problems caused by the high-carbohydrate feed is thought to be a direct and effective strategy for blunt snout bream. Previous studies in our lab proofed that adding metformin or resveratrol can effectively reduce the blood glucose by regulating the liver glucose metabolism; however, it still causes an increase in liver glycogen and hepatosomatic index (HSI), and may elevate the liver metabolic burden in the long term (Shi et al. 2018; Xu et al. 2017). Therefore, it is very necessary to find a new candidate to reduce blood glucose as well as liver glycogen for fish. BBR has been shown great properties to anti-diabetes in rodents. However, the regulation of BBR on glucose metabolism in fish has only been limited to the study of gut microbiome currently.

Thus, the purpose of this study is to explore hypoglycemic and lipid-lowering effects of dietary BBR supplemented in high-carbohydrate diet of blunt snout bream. And it may help to understand the underlying mechanism of glycolipid metabolism control by BBR and provide some new insights for improving the carbohydrate utilization of aquatic animals.

Materials and methods

Experimental diets

Feed formulation and proximate composition were presented in Table 1. BBR (≥98%) used in this study was purchased from a local company (Spring and Autumn Biotechnology Company, Nanjing, China). The addition amount of BBR in the experimental diet is 50 mg/kg referenced from a previous study (Lu et al. 2016). A total of three isonitrogenous and isolipidic diets were formulated, including normal-carbohydrate diet (NCD, 30% carbohydrate), high-carbohydrate diet (HCD, 43% carbohydrate), and high-carbohydrate diet supplemented with BBR (HCB, 43% carbohydrate + 50 mg/kg BBR). As for the ingredients, fish meal, soybean meal, rapeseed meal, and cottonseed meal served as protein sources. Corn starch was adopted as the main carbohydrate source. Fish oil and soybean oil were applied to meet the lipid demand of this species. The experimental diets were produced by a pellet extruder (MUZL 180, Jiangsu Muyang Group Co., Ltd., Yangzhou, China), dried in a ventilation room for 24 h, and then stored in plastic bags at −20 °C until use. Proximate composition of each diet

Table 1 Formulation and proximate composition of the different experimental diets

	NCD	HCD	HCB
Ingredients (% dry matter)			
Fish meal	5.00	5.00	5.00
Soybean meal	29.0	29.0	29.0
Rapeseed meal	15.0	15.0	15.0
Cottonseed meal	17.0	17.0	17.0
Fish oil	2.00	2.00	2.00
Soybean oil	2.00	2.00	2.00
Corn starch	13.0	27.0	27.0
Microcrystalline cellulose	14.0	0	0
Calcium biphosphate	2.00	2.00	2.00
Premix[a]	1.00	1.00	1.00
Berberine	0	0	0.005
Proximate composition (% dry matter)			
Moisture	7.33	7.38	7.08
Crude lipid	5.09	5.26	5.03
Ash	8.87	9.27	9.34
Crude protein	30.15	30.25	30.42
Crude fiber	16.80	5.96	6.01
Nitrogen-free extract[b]	31.76	41.88	42.12
Energy (MJ/kg)	18.33	18.75	18.72

NCD, diet with 30% carbohydrate; HCD, diet with 43% carbohydrate; HCB, diet with 43% carbohydrate + 50 mg/kg BBR

[a] Premix supplied the following minerals (g/kg of diet) and vitamins (IU or mg/kg of diet): $CuSO_4 \cdot 5H_2O$, 2.0 g; $FeSO_4 \cdot 7H_2O$, 25 g; $ZnSO_4 \cdot 7H_2O$, 22 g; $MnSO_4 \cdot 4H_2O$, 7 g; Na_2SeO_3, 0.04 g; KI, 0.026 g; $CoCl_2 \cdot 6H_2O$, 0.1 g; vitamin A, 900,000 IU; vitamin D, 200,000 IU; vitamin E, 4500 mg; vitamin K_3, 220 mg; vitamin B_1, 320 mg; vitamin B_2, 1090 mg; niacin, 2800 mg; vitamin B_5, 2000 mg; vitamin B_6, 500 mg; vitamin B_{12}, 1.6 mg; vitamin C, 5000 mg; pantothenate, 1000 mg; folic acid, 165 mg; choline, 60,000 mg; inositol, 15,000 mg

[b] Calculated by difference (100 - moisture - crude protein - crude lipid - ash - crude fiber)

was analyzed following the protocol, which consists of moisture, crude lipid, ash, crude protein, crude fiber, nitrogen-free extract, and energy (AOAC 1995).

Fish and the feeding trial

Juvenile blunt snout bream were purchased from the Fish Hatchery of Yangzhou (Jiangsu, China). Before feeding experiment, fish were fed with commercial aquafeed (32% protein, 7% lipid, 33% nitrogen-free extract; Shuaifeng Aquafeed Co., Ltd., Nanjing, China) three times a day to a slight excess of satiety for a week. After acclimation, 288 fish (average initial weight of 20.36 ± 1.44 g) were randomly distributed into 24 floating net cages (1 × 1 × 1.5 m, *L*: *W*: *H*), and each cage houses 12 fish. Fish in each cage were randomly assigned to one of the three experimental diets (each treatment had eight replicates). Fish were hand-fed to an apparent visual satiety three times a day (7:00, 12:00, and 17:00), which lasted for 10 weeks. During feeding trial, the water quality was set up as follows: Temperature varied from 27 to 29 °C; dissolved oxygen was maintained above 5.0 mg/L; pH ranged from 7.3 to 7.6; and total ammonia nitrogen and nitrite were kept < 0.4 and 0.01 mg/L, respectively.

Sample collection

After the feeding trial, fish were starved for 24 h to empty the intestine, and then all fish were counted and weighed. Sampling was performed using 100 mg/L MS-222 (tricaine methanesulfonate; Sigma, USA) for anesthetization. Blood was rapidly drawn from the caudal vein into 1.5-mL heparinized tubes and centrifuged at 3000 × rpm for 10 min at 4 °C, and the supernatant

plasma was collected and kept at −80 °C. Additionally, liver, muscle, and intraperitoneal fat tissue specimens were taken and immediately frozen in liquid nitrogen, and then kept at −80 °C.

Analytical procedures

Growth parameters The growth parameters obtained in this study were calculated using the following formulas:

$$\text{Feed intake (FI)} = \text{total feed intake (g)}/\text{total fish number}$$
$$\text{Specific growth rate (SGR)} = (\text{Ln}W\text{t}-\text{Ln}W0) \times 100/T$$
$$\text{Feed conversion ratio (FCR)} = \text{total feed intake (g)}/\text{total weight gain (g)}$$
$$\text{Protein efficiency ratio (PER)} = \text{fish weight gain (g)}/\text{total protein fed (g)}$$
$$\text{Hepatosomatic index (HSI)} = \text{liver weight (g)} \times 100/\text{body weight (g)}$$
$$\text{Abdominal fat percentage (AFP)} = \text{abdominal fat weight (g)} \times 100/\text{body weight (g)}$$
$$\text{Nitrogen retention efficiency (NRE)} = [(W\text{t} \times N\text{t})-(W0 \times N0)] \times 100/(N\text{diet} \times \text{feed intake})$$
$$\text{Energy retention efficiency (ERE)} = [(W\text{t} \times E\text{t})-(W0 \times E0)] \times 100/(E\text{diet} \times \text{feed intake})$$

where W_0 is the initial body weight, W_t is the final body weight, T is the feeding period in days, N_0/E_0 and N_t/E_t are the initial and final nitrogen/energy contents in whole body, respectively, and N_{diet}/E_{diet} are the nitrogen/energy contents in diets.

Proximate composition analysis The methods of approximate compositions of diets and fish were as follows: The samples were dried at 105° until constant weight to measure its moisture; crude protein contents (nitrogen × 6.25) were assessed with a Kjeltec analyzer unit; crude lipid was determined by ether extraction using a Soxtec auto extraction unit; ash content was estimated through carbonizing and combusting in a muffle furnace at 550 °C for 2 h; gross energy was measured by a Bomb Calorimeter (PARR 1281; Parr Instrument Company); and crude fiber was determined by fritted glass crucible method using an automatic analyzer (ANKOM A2000i, USA).

Measurements of plasma metabolites and tissue glycogen and lipid contents Plasma glucose levels were determined using the glucose oxidase method described by Asadi et al. (2009). Plasma insulin level was determined by radioimmunoassay kit and used guinea pig anti-insulin antibody (Beijing North Institute of Biotechnology Co., Ltd.). Plasma pyruvate levels were estimated according to the method of Nigam (1962). Levels of advanced glycation products (AGEs) in plasma were measured following the method of Monnier et al. (1986). Plasma triglyceride levels in lipoprotein fractions were evaluated using a colorimetric method (McNamara and Schaefer 1987). Plasma lactate levels were measured by injecting 10 μL of serum into a VITROSDT60II chemical analyzer (Kodak, NY, USA) using a DT pipette. Tissue glycogen levels were determined following the method of Pfleiderer and Bergmeyer (1974). According to the method of Floch (1957), the content of tissue lipid was extracted with a mixture of chloroform:methanol (2:1, V:V).

Oil red O staining Liver samples (n = 3) were fixed in 40 g/kg paraformaldehyde solution for 24 h. The liver was then cut into 20-μm sections and neutral lipid staining with oil red O was performed (Jiang et al. 2018). Under the microscope, the lipid is red and the nucleus is light blue. The Image-Pro Plus 6.0 software was used to measure the red area representing lipid accumulation. Three areas in each sample were randomly selected to obtain the size and number of lipid droplets.

Quantitative RT-PCR Total RNA was extracted from liver tissue with Trizol (Invitrogen, CA, USA) following the manufacturer's instruction. The quantity and purity of the extracted RNA were determined by NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA), in terms of its absorbance at 260 nm and 280 nm. RNA integrity assessment was performed using denatured agarose gel with 1% formaldehyde. cDNA was prepared from 500 ng/μL RNA using a PrimeScript™ RT Master Mix Kit (Cat. No. RR036A, TaKaRa, Co. Ltd., Dalian, China). The resulting cDNA was diluted with DNase/RNase-free water and then worked as a template for quantitative PCR using a TB Green™ Premix Ex Taq™ Kit (Cat. No. RR820A,

Table 2 Nucleotide sequences of primers used to assay gene expressions by real-time quantitative PCR

Target gene	Forward (5′-3′)	Reverse (5′-3′)	*E* (%)	Accession number
pepck	TCAGAGCCATCAACCCAGAG	GTCCATGCCTTCCCAGTAAA	117.88	Gao et al. (2012)
fbpase	TACCCAGATGTCACAGAAT	CACTCATACAACAGCCTCA	104.36	KJ743995.1
g6pase	AACGGTGAGTTGGCCGTTTA	CGCGTGACATCACCGTTTTC	96.38	Gao et al. (2012)
glut2	ACGCACCCGATGTGAAAGT	TTGGACAGCAGCATTGATT	104.27	KC513421.1
pk	GGGCTGGTTAAAGGGTGC	GCGGGTTAGGCTGGEGATA	92.87	Gao et al. (2012)
gs	AGCGATGAGGAAGATGAC	CAAAGGGTAGCAGGTGTA	93.91	Gao et al. (2012)
fas	AGCGAGTACGGTGATGGT	GGATGATGCCTGAGATGG	97.21	KF918747.1
cpt I	GGCGAGACACCCAGAGTA	ACTGGAATGCTTGGAGGA	94.40	Gao et al. (2012)
accα	GCTTACCCAAGTGTCCTC	ATGCCAATCTCATTTCCT	89.16	Gao et al. (2012)
foxo1	GGACTTCAACTTCGACCCCA	ACGTTCCCTGACAAGGGGTT	101.29	Gao et al. (2012)
irs	CGGCGGTACTTCGTGTTA	GCCCGTTTGTTGATGTTG	90.63	Gao et al. (2012)
pi3k	AAGCTCTGCGAGAGAGTTGG	TAGATCCATGTGATTCGGCCA	97.43	Gao et al. (2012)
akt	CTTCCTCCTGTTTGCAGGTGA	ATCAGCTGACACTCTGCGAC	93.88	Gao et al. (2012)
β-actin	AAATTGCCGCACTGGTTGTT	AGGGTCAGGATACCCCTCTT	92.87	AY170122.2

pepck, phosphoenolpyruvate carboxykinase 1; *fbpase*, fructose-1,6-biphosphatase; *g6pase*, glucose-6-phosphatase; *glut2*, glucose transporter 2; *pk*, pyruvate kinase; *gs*, glycogen synthase; *fas*, fatty acid synthetase; *cpt I*, carnitine palmitoyl transferase I; *foxo1*, forkhead transcription factor 1; *akt*, protein kinase B; *pi3k*, phosphatidylinositol 3-kinase; *accα*, acetyl-CoA carboxylase; *irs*, insulin receptor substrate

E amplification efficiency

TaKaRa, Co. Ltd.) and a real-time PCR detection system (ABI7300; Applied Biosystems, Foster City, CA, USA). A 20-μL real-time PCR reaction volume was adopted, consisting of 10 μL of TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (2×), 0.4 μL of PCR forward/reverse primer (10 μM) each, 0.4 μL of ROX reference dye II (50×), 2.0 μL of cDNA template, and 6.8 μL of DNase/RNase-free water. The PCR reaction conditions were as follows: 95 °C for 30 s, then followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Melting curves and electrophoresis were applied to judge the specificity of primers. The relative expression of the target gene is calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *β-Action* was used as an endogenous control for normalization of the gene expression levels of target genes. All primers are presented in Table 2.

Statistical analyses

Data in the present study were all analyzed by one-way ANOVA using the SPSS 25.0 statistical software package for Windows (SPSS, Michigan Avenue, Chicago, IL, USA). All data were firstly tested for the homogeneity of variances using the Levene's test, and then analyzed by one-way ANOVA. Tukey's honestly significant difference test was used to distinguish differences among treatments ($P < 0.05$). All data were expressed as mean ± S.E.M (mean standard error).

Results

Growth performance and feed utilization

To assess the effect of BBR on the growth performance of high-carbohydrate-fed fish, NCD, HCD, or HCB were fed to blunt snout bream for 10 weeks, and no mortality was observed during the trial. As was showed in Table 3, final weight, SGR, FI, FCR, PER, NRE, and HSI observed no significant difference among all groups, but the AFP value of fish-fed NCD was significantly lower than those of fish-fed HCD and HCB. HCB-fed fish had a higher ERE than that of NCD-fed fish. For the results of whole-body composition, there were no differences in moisture, ash, and crude protein among all groups, whereas HCB feeding resulted in

Table 3 Growth performance, feed utilization, and whole-body composition of blunt snout bream fed with different experimental diets

	NCD	HCD	HCB	*P* value
Initial weight (g)	20.32 ± 0.59	20.13 ± 0.35	20.63 ± 0.60	0.801
Final weight (g)	128.46 ± 3.82	135.02 ± 4.38	138.90 ± 4.77	0.251
SGR (%/day)	2.63 ± 0.05	2.72 ± 0.05	2.72 ± 0.07	0.478
FI (g/fish)	165.39 ± 4.02	177.56 ± 9.76	178.93 ± 6.97	0.371
FCR	1.18 ± 0.02	1.15 ± 0.01	1.17 ± 0.02	0.567
PER	2.18 ± 0.09	2.16 ± 0.09	2.19 ± 0.11	0.976
NRE (%)	45.17 ± 1.83	44.42 ± 1.86	44.21 ± 1.07	0.936
ERE (%)	25.84 ± 1.05^{a}	27.60 ± 1.15^{ab}	30.52 ± 1.44^{b}	0.041
HSI (%)	1.22 ± 0.07	1.48 ± 0.09	1.42 ± 0.08	0.080
AFP (%)	1.77 ± 0.13^{a}	2.64 ± 0.14^{b}	2.42 ± 0.21^{b}	0.003
Whole-body composition (% wet weight)				
Moisture (%)	73.02 ± 0.13	72.08 ± 0.93	70.58 ± 0.52	0.058
Crude lipid (%)	7.33 ± 0.11^{a}	8.47 ± 0.62^{ab}	9.51 ± 0.29^{b}	0.012
Ash (%)	3.53 ± 0.16	3.36 ± 0.09	3.44 ± 0.08	0.583
Crude protein (%)	20.42 ± 0.18	20.25 ± 0.24	19.96 ± 0.31	0.466
Energy (MJ/kg)	7.12 ± 0.01^{a}	7.71 ± 0.31^{ab}	8.28 ± 0.12^{b}	0.009

Each data represents the average of 4-8 replicates. Mean values with different lowercase letters are significantly different ($P < 0.05$)

NCD, diet with 30% carbohydrate; HCD, diet with 43% carbohydrate; HCB, diet with 43% carbohydrate + 50 mg/kg BBR

SGR specific growth rate; *FI* feed intake; *FCR* feed conversion ratio; *PER* protein efficiency ratio; *NRE* nitrogen retention efficiency; *ERE* energy retention efficiency; *HSI* hepatosomatic index; *AFP* abdominal fat percentage

significant increases in crude lipid and gross energy versus NCD feeding.

Plasma biochemistry parameters

BBR addition significantly reversed the HCD-induced increases in plasma glucose, TG, and TC contents (Table 4). Higher AGE level was detected in HCD-fed fish than in NCD-fed fish, while BBR supplementation tended to reduce this effect. And dietary BBR inclusion was inclined to ameliorate the HCD-induced blood insulin impairment versus the HCD group. In addition, plasma pyruvate level of the HCB group was significantly lower than those of the other groups.

Tissue glycogen and lipid contents

HCD feeding resulted in significant higher hepatic glycogen and lipid contents than NCD feeding (Table 5), whereas HCB remarkably reversed this effect versus

Table 4 Effect of diets and BBR on plasma biochemistry parameters of blunt snout bream

Plasma	Groups			
	NCD	HCD	HCB	*P* value
Glucose (mmol/L)	5.82 ± 0.79^{a}	7.98 ± 0.37^{b}	5.32 ± 0.45^{a}	0.007
AGEs (ng/mL)	68.54 ± 9.14	85.27 ± 5.94	73.20 ± 4.09	0.194
Insulin (μIU/mL)	9.37 ± 0.99	8.22 ± 1.06	10.61 ± 0.94	0.256
TG (mmol/L)	0.96 ± 0.05^{a}	1.32 ± 0.11^{b}	0.97 ± 0.07^{a}	0.012
TC (mmol/L)	7.05 ± 0.29	8.41 ± 0.64	8.28 ± 0.32	0.086
Pyruvate (μmol/mL)	0.246 ± 0.005^{b}	0.229 ± 0.004^{b}	0.204 ± 0.009^{a}	0.001

Each data represents the average of 5-10 replicates. Mean values with different lowercase letters are significantly different ($P < 0.05$)

NCD, diet with 30% carbohydrate; HCD, diet with 43% carbohydrate; HCB, diet with 43% carbohydrate + 50 mg/kg BBR

AGEs fish advanced glycation end products; *TG* triglyceride; *TC* total cholesterol

Table 5 Tissue glycogen and lipid contents of blunt snout bream fed with different diets

Parameters	NCD	HCD	HCB	*P* value
Tissue glycogen contents (g/kg wet weight)				
Liver	6.60 ± 1.07[a]	21.08 ± 2.49[b]	11.51 ± 1.16[a]	0.002
Muscle	1.08 ± 0.14	0.89 ± 0.07	0.97 ± 0.06	0.749
Intraperitoneal fat	0.82 ± 0.02[a]	0.94 ± 0.05[a]	1.17 ± 0.03[b]	0.015
Tissue lipid contents (g/kg wet weight)				
Liver	9.70 ± 1.45[a]	16.54 ± 0.90[b]	8.70 ± 1.80[a]	0.007
Muscle	2.14 ± 0.15	1.922 ± 0.13	2.35 ± 0.05	0.095
Intraperitoneal fat	57.59 ± 1.23[a]	67.38 ± 2.03[b]	70.37 ± 0.72[b]	0.001

Each data represents the average of 3-4 replicates. Mean values with different lowercase letters are significantly different ($P < 0.05$)
NCD, diet with 30% carbohydrate; HCD, diet with 43% carbohydrate; HCB, diet with 43% carbohydrate + 50 mg/kg BBR

HCD ($P < 0.05$). The glycogen and lipid levels of intraperitoneal fat in NCD-feeding fish were significantly lower than those in HCD-feeding or HCB-feeding fish. However, there were no significant differences in muscular glycogen or lipid content among all groups.

Oil red O staining of the liver

The accumulation of liver lipid was evaluated by oil red O staining (Fig. 1). According to the photomicrographs of the liver tissue, the lipid droplet clusters of fish-fed

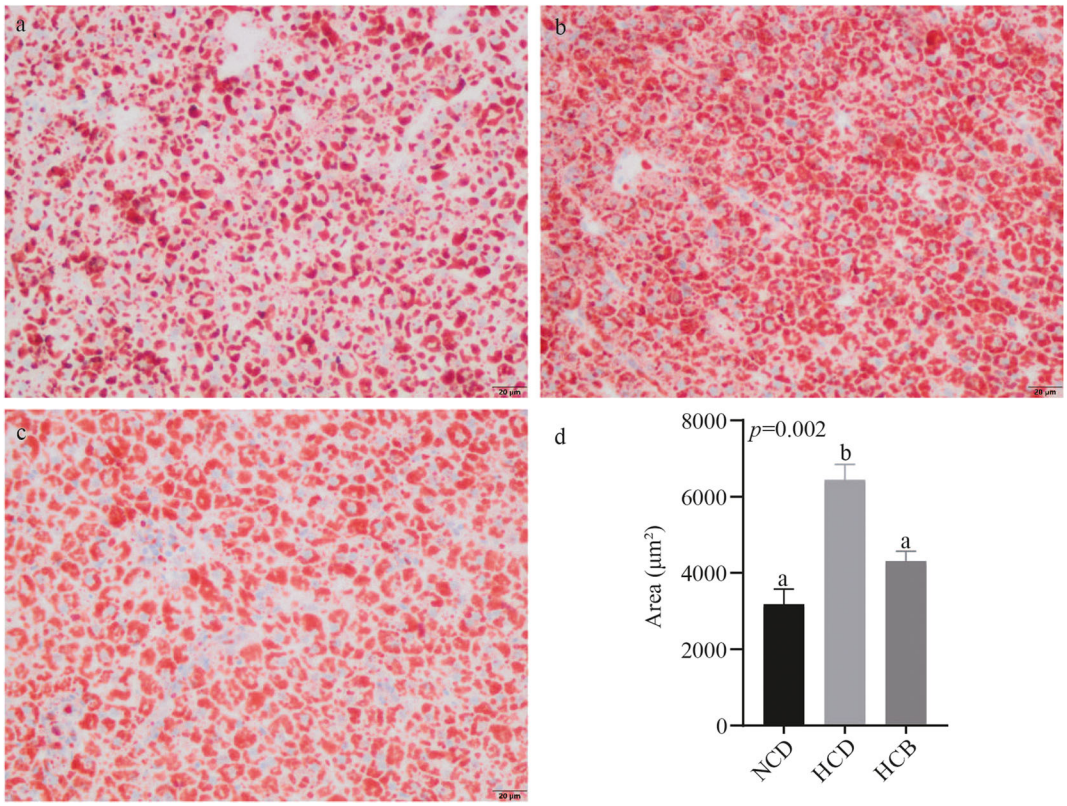

Fig. 1 Hepatic lipid profiles of *Megalobrama amblycephala* fed different experimental diets. **a** Fish-fed NCD, **b** Fish-fed HCD. **c** Fish-fed HCB. **d** The lipid accumulation (red-stained area, oil red O staining). Red point lipid droplet, white point vacuoles, blue point nucleus. Photomicrographs (×400) and scale bar (20 μm). Each data represents the average of 3 replicates. The bars assigned with different lowercase letters are significantly different ($P < 0.05$)

HCD were more than that of fish-fed NCD or HCB (Fig. 1d), whereas the addition of BBR showed an obvious lipid-reducing effect.

Relative expression of genes related to insulin signaling

As can be seen from Fig. 2, to reveal the molecular mechanism associated with high-carbohydrate diet and BBR supplementation-induced insulin signaling, the relative gene expressions of *insulin receptor substrate* (*irs*), *phosphatidylinositol 3-kinase* (*pi3k*), *protein kinase B* (*akt*), and *forkhead transcription factor 1* (*foxo1*) were detected in the liver of this species using RT-qPCR. BBR supplementation remarkably reversed the HCD-induced suppression of *irs* mRNA expression and the HCD-induced upregulation of *foxo1* mRNA expression. The *pi3k* and *akt* mRNA expressions of HCB-fed fish were significantly higher than those of the other two groups.

Relative expression of glucose metabolism-related genes

As was shown in Fig. 3, there were no differences of *phosphoenolpyruvate carboxykinase* (*pepck*), *glucose-6-phosphatase* (*g6pase*), and *pyruvate kinase* (*pk*) mRNA expressions between the NCD and HCD groups. The *pepck* and *g6pase* mRNA expressions in the liver of HCB-fed fish were significantly lower than those of NCD and HCD-fed fish. HCB feeding resulted in significant increases in *glucose transporter 2* (*glut2*) and *pk* mRNA expressions versus NCD and HCD. HCB remarkably reduced HCD-induced *glycogen*

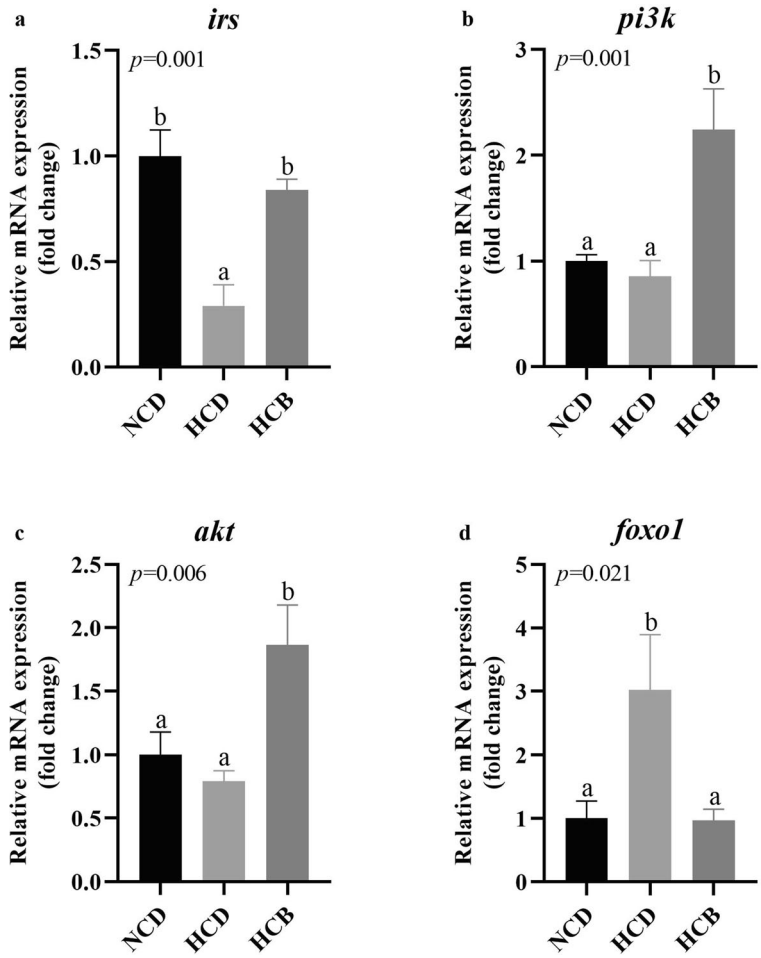

Fig. 2 Relative expressions of insulin signaling-related genes in the liver of blunt snout bream fed with different experimental diets. The mRNA levels of insulin receptor substrate (*irs*) (**a**), phosphatidylinositol 3-kinase (*pi3k*) (**b**), protein kinase B (*akt*) (**c**), and forkhead transcription factor 1 (*foxo1*) (**d**) were evaluated using real-time RT-PCR. Expression values were normalized using *β-action* as an endogenous control. Each data represents the average of 6-7 replicates. The bars assigned with different superscripts are significantly different ($P < 0.05$)

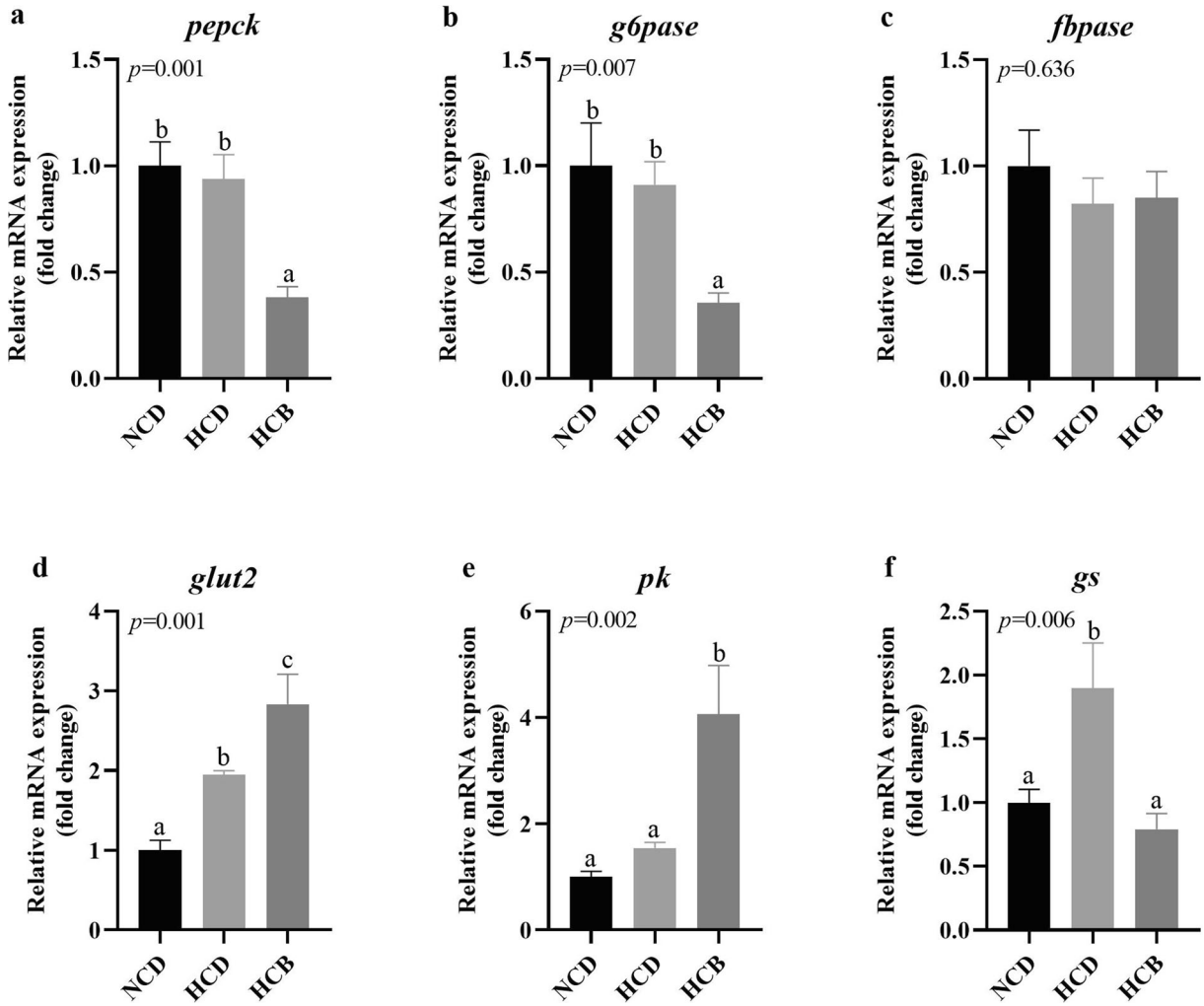

Fig. 3 Relative expressions of glucose metabolism-related genes in the liver of blunt snout bream fed with different experimental diets. The mRNA levels of phosphoenolpyruvate carboxykinase (*pepck*) (**a**), glucose-6-phosphatase (*g6pase*) (**b**), fructose-1,6-biphosphatase (*fbpase*) (**c**), glucose transporter 2 (*glut2*) (**d**), pyruvate kinase (*pk*) (**e**), and glycogen synthase (*gs*) (**f**) were evaluated using real-time RT-PCR. Expression values were normalized using *β-action* as an endogenous control. Each data represents the average of 6-7 replicates. The bars assigned with different superscripts are significantly different ($P < 0.05$)

synthase (*gs*) expression. There was no difference of hepatic *fructose-1,6-biphosphatase* (*fbpase*) mRNA expression among all the treatments.

Relative expression of lipid metabolism-related genes

In Fig. 4, HCD feeding resulted in significantly higher hepatic *fatty acid synthetase* (*fas*) and *acetyl-CoA carboxylase* (*accα*) expressions and a relatively lower hepatic *carnitine palmitoyl transferase I* (*cpt I*) expression than NCD feeding, while HCB remarkably ameliorated this effect versus HCD.

Discussion

The previous studies demonstrated that fish exposed to a high-carbohydrate diet in a long term will suffer from the postprandial hyperglycemia, the metabolic disorder, and be subjected to the impaired health status eventually (Kamalam et al. 2017; Ren et al. 2015; Ren et al. 2013). Although some medicines, such as benfotiamine, metformin, and nicotinamide, can effectively lower blood glucose and improve the glucolipid metabolism, there still exist some side effects, say the increased liver glycogen or lipid content (Shi et al. 2020; Shi et al. 2018; Xu et al. 2017; Xu et al. 2018). As a plant-

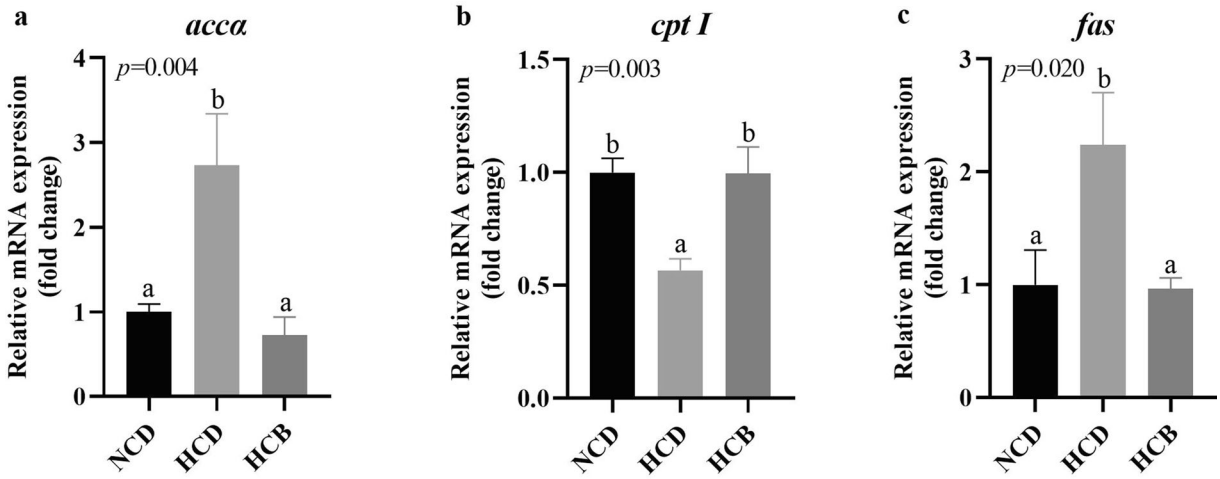

Fig. 4 Relative expressions of lipid metabolism-related genes in the liver of blunt snout bream fed with different experimental diets. The mRNA levels of acetyl-CoA carboxylase α (*accα*) (**a**), carnitine palmitoyl transferase I (*cpt I*) (**b**), and fatty acid synthase (*fas*) (**c**) were evaluated using real-time RT-PCR. Expression values were normalized using *β-action* as an endogenous control. Each data represents the average of 6-7 replicates. The bars assigned with different superscripts are significantly different ($P < 0.05$)

origin bioactive substance, BBR has been shown an ability to reduce glucose level with almost less side effects (Na et al. 2012). Therefore, this study aimed to explore the role and mechanism of BBR applied in high-carbohydrate diet-fed blunt snout bream, from the aspects of growth, body composition, plasma biochemical indicators, and glucolipid metabolism processes.

We first determined whether BBR supplementation imposes any effects on the growth performance of HCD-fed fish. We found that either dietary carbohydrate levels or the addition of BBR revealed no significant differences of SGR, FCR, and FI among all groups. The reason might be that blunt snout bream has better utilization of carbohydrates than most carnivorous and omnivorous species (Li et al. 2013; Ren et al. 2013; Shi et al. 2018), and in a certain range, BBR inclusion does not impair growth. It was also reported that the addition of BBR did not affect the body weight gain and fatness of *Ctenopharyngodon idellus* compared with the control group (Pan et al. 2019). In addition, HCD feeding significantly enhanced AFP level compared with NCD feeding, while BBR supplemented in HCD did not change this effect. According to previous research, high-carbohydrates diet could increase the synthesis of glycogen and fat (Li et al. 2019; Prisingkorn et al. 2017; Ren et al. 2011; Wang et al. 2016). And the abdomen is the main part of fat deposition, which is easy to cause the increase of AFP, and can lead to an increase in whole-body lipid. With the addition of BBR, the ERE, whole-body lipid, and gross energy in the HCB group increased significantly versus the NCD group. The reason might be related to BBR's ability to inhibit gluconeogenesis pathway, which in turn improves energy retention efficiency (especially fat). The latest research demonstrated that BBR also could enrich the intestinal flora of fish and increase beneficial microorganisms, so that these effects might promote the absorption of nutrients and enhance the ERE in fish whole body (Pan et al. 2019).

For the evaluation of fish physiological health conditions, plasma indicators are considered reliable parameters (Habte Tsion et al. 2016). After 10 weeks' feeding trial, HCD resulted in higher levels of plasma glucose, AGEs, TG, and TC contents compared with NCD (Table 4). According to previous research in fish, long-term excessive carbohydrate intake does cause a symptom of hyperglycemia (Hemre et al. 2002; Peres and Oliva Teles 1999; Wilson 1994), and increase lipogenesis (Zhou et al. 2017), which might lead to the enhanced levels of glucose, AGEs, TG, and TC in blood. Furthermore, in this study, BBR supplementation significantly reduced the levels of glucose, TG, and pyruvate in plasma versus HCD, and insulin secretion tended to be induced by BBR although no significant difference was observed. According to previous reports, these phenomenons might be explained by the following facts: (1) BBR can directly inhibit gluconeogenesis to lower blood glucose, and does not completely depend on the action of insulin (Xia et al. 2011); (2) BBR may reduce blood glucose TG by adjusting the intestinal

microbial flora (Pan et al. 2019) or downregulating stearoyl-CoA desaturase in blunt snout bream (Zhou et al. 2019); (3) BBR may accelerate glycolysis and promote the use of pyruvate (Yin et al. 2008). Regarding the change of TC level, HCD tended to increase TC content compared to NCD, and BBR inclusion had a slight TC-lowering effect (Table 4). These phenomena might be explained that the additive amount of BBR in this study was not enough to lower plasma cholesterol.

In terms of the roles in reducing tissue glycogen and lipid content, BBR has been applied to treat fatty liver and obesity in humans (Lee et al. 2006). In accordance with our results, it was also confirmed that BBR could reduce the accumulation of lipid and glycogen in the liver caused by HCD feeding in blunt snout bream. Oil red O staining of liver tissue demonstrated that BBR addition can reduce lipid droplet in hepatocyte (Fig. 1). It is generally believed that fat is the main form of energy storage, and fish lipid is generally deposited in the peritoneum, mesentery, and muscle tissues besides the liver (Tessari et al. 2009). In this study, BBR promoted the transfer of liver lipid/glycogen to peripheral tissues storing as lipid (abdominal fat and muscle) and enhanced the ERE in fish whole body (Table 3), and alleviated liver injury caused by fatty liver. This effect may be related to the increase of BBR in plasma VLDL level, the promotion of lipoprotein secretion in the liver, and the increase of liver fat output (Lu et al. 2013; Richard et al. 2006; Zhou et al. 2019). Therefore, the accumulation of peripheric fat leads to an increase in lipid in the fish body (Table 3). Hepatic glucose production accounts for more than 90% of endogenous glucose production, and mostly comes from glycogenolysis and gluconeogenesis (Petersen et al. 2017). For liver glycogen, BBR may reduce its content by increasing the expression of PPAR-α and PPAR-δ proteins, and reduce the pathological process of liver to regulate the metabolism of glycolipids (Zhou et al. 2008). Of course, the decrease in glycogen content in the liver may be also related to the decreased expression of *gs*. *Gs* is one of the important regulating enzymes for glycogen synthesis, and the reduced expression of *gs* mRNA in the HCB group may also explain this phenomenon (Fig. 3). Surprisingly, the addition of BBR increased the glycogen content in intraperitoneal fat. This may be because BBR is mainly enriched in the liver, and then liver glycogen synthesis is inhibited, resulting in more glucose accumulation in intraperitoneal fat (Liu et al. 2010; Yan et al. 2015).

In the regulation of glycolipid metabolism, the insulin signaling IRS/ PI3K/AKT is a major signaling pathway (Kamalam et al. 2017; Nandipati et al. 2017). After insulin binds to a receptor in its target cell, a signal cascade is triggered. Activated insulin receptor promotes tyrosine phosphorylation of IRS, thereby activating PI3K and AKT and downstream glucose and lipid metabolism (Zhang et al. 2018). In this study, the high-carbohydrate diet downregulated *irs* expression significantly versus NCD (Fig. 2). *Pi3k* and *akt* expressions also decreased, although the difference was not significant. This phenomenon was similar with the results in the oral glucose test of blunt snout bream (Pan et al. 2017). Moreover, high level of carbohydrates can also reduce IRS expression by stimulating S6K1 expression, leading to the phosphorylation or degradation of IRS1, which limits the transmission of insulin signal from insulin receptor to PI3K, thus weakening the effect of insulin (Manning 2004). However, the addition of BBR could significantly increase the expression of *irs*, *pi3k*, and *akt* (Fig. 2). Liu et al. (2018) reported that BBR prevents LPS-induced TLR4/TNF-α activation and enhances IRS expression in the liver. Thereafter, IRS could activate and stimulate PI3K and AKT activity, and then further inhibits its downstream targets, such as *foxo1*. Furthermore, AKT could phosphorylate FOXO1 and inhibit its expression to inhibit gluconeogenesis (Datta et al. 1999; Kaestner et al. 2000; Kops and Burgering 1999), and promote glycolysis (Zhang et al. 2006). This is also one of the main ways for BBR to lower plasma glucose.

Regarding glycometabolism, the expressions of *glut2* and *gs* in the HCD group were significantly upregulated compared with the NCD group, and the expressions of *pepck*, *g6pase*, and *fbpase* in the HCD group had no effect. This indicated that long-term high-carbohydrate intake promotes liver glycogen synthesis but has no significant effect on gluconeogenesis. Glucose in the blood moves into the liver mainly through *glut2*, and long-term HCD feeding upregulated *glut2* to promote liver uptake of glucose, which enhanced glycogen synthesis by *gs* upregulation (Enes et al. 2009). *Pepck*, *g6pase*, and *fbpase*, the key genes for gluconeogenesis, were not significantly changed in the HCD group versus the NCD group. This phenomenon could be caused by insufficient insulin secretion (Table 4), which was also observed in rainbow trout (Panserat et al. 2000; Zhou et al. 2016). However, BBR adding could reduce the expressions of *pepck*, *g6pase*, and *gs*, and increase the

transcriptional levels of *glut2* and *pk* (Fig. 3). This indicated that BBR can inhibit liver glycogen synthesis and gluconeogenesis and promote glucose transport and glycolysis, which may also be the cause of lower plasma glucose and reduced liver glycogen contents observed in the HCB group (Tables 4 and 5). It was also supported by a previous study that upregulated hepatic AKT can result in the upregulation of liver *Glut2*, a downstream substrate of AKT, which in turn promotes glucose transport (Giorgino et al. 2000). With regard to the *pk* expression in the HCB group, BBR may enhance the expression of *pk* by demethylating PK promoter and increasing the level of acetylation of histones H3 and H4 of PK (Zhang et al. 2015). BBR may also reduce ATP levels by inhibiting mitochondrial function of hepatocytes or increase the expression of *hnf-4α* mRNA, thereby inhibiting the expressions of *pepck* and *g6pase* (Wang et al. 2008; Xia et al. 2011). The results show that BBR not only promotes the uptake and decomposition of liver glucose, but also inhibits the production of liver glucose.

Compared to mammals, fish are more susceptible to fatty liver because the liver is the main site of fat metabolism for fish (Hemre et al. 2002). In accordance with our results, liver *cpt I* mRNA expression was significantly downregulated in the HCD group, while *accα* and *fas* were opposite. And these changes would increase liver fat synthesis and reduce lipolysis, eventually leading to liver lipid accumulation (Shi et al. 2018; Xu et al. 2017; Xu et al. 2018). *Cpt I* is considered to be a key regulator of mitochondrial β-oxidation (Kerner and Hoppel 2000). The study of Lu et al. (2016) demonstrated BBR could upregulate the expression of *cpt I* by activating *pparα*, thereby regulating mitochondrial β-oxidation. *Accα* and *fas* are two key enzymes closely related to liver fatty acid synthesis (Qian et al. 2015). The plasma TG-lowering and liver lipid-declining effects of BBR might be partially modulated by downregulating gene expressions of *fas* and *accα* through activation of the AMPK signaling pathway (Cao et al. 2013). This can explain why the hepatic lipid content of the HCB group was decreased.

Conclusion

BBR inclusion in HCD could attenuate hyperglycemia of blunt snout bream, mainly through promoting liver's glycolysis and insulin signaling, while inhibiting liver's glycogen synthesis and gluconeogenesis. In addition, BBR could ameliorate the metabolic burden of the liver by inhibiting fat synthesis and promoting lipid decomposition, and then enhance fat uptake in peripheral tissues.

Code availability Not applicable.

Authors contribution Chang He: writing—original draft, writing—reviewing and editing, software, data curation, investigation, and resources.
Xiaoyan Jia: software, conceptualization, and data curation.
Li Zhang: supervision and conceptualization.
Fan Gao: writing—reviewing and editing.
Weibo Jiang: investigation and resources.
Chuang Wen: investigation and resources.
Cheng Chi: software and methodology.
Xiangfei Li: conceptualization.
Guangzhen Jiang: conceptualization.
Haifeng Mi: feed formulation and consultation.
Wenbin Liu: funding acquisition and conceptualization.
Dingdong Zhang: funding acquisition, project administration, supervision, and writing—reviewing and editing.

Funding This study was supported by the National Natural Science Foundation of China (No. 31972801) and the earmarked fund for China Agriculture Research System (CARS-45-14).Data availabilityThe data sets supporting the results of this article are included within the article and its additional files,

Declarations

Ethics approval and consent to participate All experimental procedures for animal care and handling in this study were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Permit number: IACUC2020174).

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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