Dietary berberine regulates lipid metabolism in muscle and liver of black sea bream (*Acanthopagrus schlegelii*) fed normal or high-lipid diets

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Abstract

The present study investigated the influence of berberine (BBR) supplementation in normal and high-lipid (HL) diets on lipid metabolism and accumulation in black sea bream (*Acanthopagrus schlegelii*). BBR was supplemented at 50 mg/kg to control (Con, 11·1 % crude lipid) and high-lipid (HL, 20·2 % crude lipid) diets and named as ConB and HLB, respectively. After the 8-week feeding trial, fish body length and specific growth rate were significantly reduced by HL diets ($P < 0.05$). Muscle and whole-body crude lipid contents were significantly influenced by both BBR supplementation and dietary lipid level. Fish fed the HLB diet had significantly lower serum TAG, LDL-cholesterol contents and alanine amino-transferase activity compared with the HL group. The HL group presented vast lipid accumulation in the liver, and hypertrophied hepatocytes along with large lipid droplets, and translocation of nuclear to the cell periphery. These abnormalities in black sea bream were alleviated in the HLB group. BBR supplementation in the HL diet significantly down-regulated the hepatic expression levels of acetyl-CoA carboxylase α, sterol regulatory element-binding protein-1, 6-phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase and *pparγ*, whereas the lipoprotein lipase, hormone-sensitive lipase and carnitine palmitoyltransferase 1a expression levels were significantly up-regulated. However, the expression levels of these genes showed opposite trends in muscle (except for *pparγ*). In conclusion, dietary BBR supplementation in the HL diet reduced hepatic lipid accumulation by down-regulating lipogenesis gene expression and up-regulating lipolysis gene expression, and it increased muscle lipid contents with opposite trends of the mechanism observed in the liver.

Key words: *Acanthopagrus schlegelii*: Berberine: Lipid metabolism: Growth performance

Lipids play a crucial role in the intensive aquaculture industry, not only because it provides essential fatty acids but also due to its high energy density. It can impart a 'protein-sparing effect', thereby reducing dietary protein requirement, resulting in feed cost reduction[1]. Studies have demonstrated that increasing dietary lipid level could improve protein efficiency ratio and reduce N discharge in different fish species[2–5]. Thus, the use of energy-dense high-lipid (HL) feeds is prevalent in aquaculture, especially in the farming of marine fish species. However, ingesting excessive lipid generally leads to fatty liver, which is detrimental to liver function. Since the liver is the central organ involved in carbohydrate, lipid and protein metabolism, its impairment eventually causes poor health condition and induces other diseases (e.g. inflammatory reaction and oxidative stress)[6]. Excessive lipid accumulation also poses severe health problems and reduces harvest yields, which is directly responsible for economic losses[7].

Several studies reported that some feed additives could reduce liver lipid deposition and alleviate the adverse effects caused by HL diets, such as bile acids[8,9], L-carnitine[10], fenofibrate[11], choline, betaine[12] and xylooligosaccharides[13]. Berberine (BBR) (18,5,6-dyhydro-9,10-dimethoxybenzo(g)-1,3-benzodioxolo(5,6-a) quinolizinium) is an isoquinoline alkaloid isolated from *Coptis chinensis* (Chinese goldthread), *Hydrastis canadensis* (goldenseal), *Berberis vulgaris* (barberry) and other herbs[14] or manufactured by chemical synthesis. It has been commonly used in Chinese medicine for its anti-inflammatory, antibacterial, antioxidant and antiapoptotic

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features[15]. BBR is also used as an effective medicine in treating fatty liver, obesity, hypercholesterolaemia, hypertension, type 2 diabetes and gastrointestinal infection[16–18]. Generally, BBR presents few side effects and little toxicity[19,20]. In aquaculture, BBR was reported to be an effective immunostimulant for the prevention and treatment of some bacterial diseases[21–23]. Recently, studies on blunt snout bream (*Megalobrama amblyce-phala*)[15,24] and zebrafish (*Danio rerio*)[25] reported the prominent lipid-lowering effect of BBR, but as far as we know, the effects of dietary BBR in marine fish have not been investigated.

Black sea bream (*Acanthopagrus schlegelii*) is a high-value commercially farmed tropical marine fish with high tolerance to environmental changes, desirable growth rate and good meat quality[1,26]. Wang *et al.*[1] reported that black sea bream is prone to store lipid in their liver. Previous studies in black sea bream reported that L-carnitine[27], choline[28] and bile acids[10] supplementation could improve immune and antioxidative abilities and reduce the histopathological changes caused by HL diets. The present study aimed to investigate whether BBR supplementation in HL diet could effectively reduce lipid accumulation in the liver of black sea bream. Other studies have reported that supplementation of BBR in normal lipid level diets could improve the growth performance of fish[21,23,24]. However, BBR shows poor solubility and the absorption rate partly relies on gut microbiota to convert it into its absorbable form[18]. A study in rats observed that the oral bioavailability of BBR was less than 1 %[29], which might be due to the poor aqueous dissolution and solubility[30]. Besides, the absorption of BBR after oral administration has a limit, and low dietary BBR level presents rapid intestinal absorption[31]. Zhou *et al.*[15] reported that an HL diet with 50 mg/kg BBR improved growth performance and attenuated the hepatic abnormalities in blunt snout bream, whereas 100 mg/kg BBR reduced feed palatability and feed intake (FI), thus 50 mg/kg BBR was recommended in fish feed. Supplementation of 50 mg/kg and 100 mg/kg BBR in an HL diet showed similar effects on hepatocytes health in blunt snout bream[32]. Therefore, in the present study, we investigated the supplementation of 50 mg/kg BBR in normal and HL diets on growth performance, liver function and lipid metabolism of black sea bream fingerlings.

Experimental methods

Animal ethics

All the experimental processes followed the Guidance of the Care and Use of Laboratory Animals in China. The Committee on the Ethics of Animal Experiments of Zhejiang University approved the present study.

Diets preparation

The control (Con) and HL diets were formulated as indicated in Table 1, and they were designed as isonitrogenous (42 % crude protein) practical diets. The Con diet contained 11·19 % lipid and the HL diet contained 20·26 % lipid. Fishmeal, wheat gluten meal, soya protein concentrate and fermented soyabean meal were used as the major protein ingredients, while maize oil and fish oil served as the main lipid sources. These main protein

Table 1. Feed formulation and proximate composition of the experimental diets

Ingredients (g/kg)	Con	ConB	HL	HLB
Fishmeal	250·0	250·0	250·0	250·0
Wheat gluten meal	50·0	50·0	50·0	50·0
Soya protein concentrate	80·0	80·0	80·0	80·0
Fermented soyabean meal	150·0	150·0	150·0	150·0
Maize oil	20·0	20·0	100·0	100·0
Fish oil	50·0	50·0	50·0	50·0
Wheat flour	150·0	150·0	150·0	150·0
Alpha starch	40·0	40·0	40·0	40·0
Soya lecthin	20·0	20·0	20·0	20·0
Ca(H$_2$PO$_4$)$_2$·H$_2$O	25·0	25·0	25·0	25·0
Alpha cellulose	98·5	98·45	18·5	18·45
Vitamin premix*	7·5	7·5	7·5	7·5
Mineral premix†	7·5	7·5	7·5	7·5
Y$_2$O$_3$	1·0	1·0	1·0	1·0
Berberine	0·0	0·05	0·0	0·05
Lysine	4·0	4·0	4·0	4·0
Arginine	6·0	6·0	6·0	6·0
Methionine	8·0	8·0	8·0	8·0
Phytase	0·50	0·50	0·50	0·50
Carrageenan	2·0	2·0	2·0	2·0
Carboxymethyl cellulose	30·0	30·0	30·0	30·0
Total	1000·0	1000·0	1000·0	1000·0
Proximate composition (% dry weight)				
Crude protein	42·51	41·71	42·40	41·95
Crude lipid	11·19	11·13	20·26	20·37
Ash	8·77	8·56	8·71	8·78
Carbohydrate	12·69	12·94	12·84	12·71
Gross energy (kJ/g)‡	16·63	16·38	20·19	20·12
Protein:energy ratio (mg/kJ)	25·55	25·45	20·99	20·84

Con, control; ConB, control diet with berberine supplementation; HL, high lipid; HLB, high-lipid diet with berberine supplementation.
* Vitamin premix (mg/kg): α-tocopherol, 80; retinyl acetate, 40; cholecalciferol, 0·1; menadione, 15; niacin, 165; riboflavin, 22; pyridoxine HC1, 40; thiamin mononitrate, 45; D-Ca pantothenate, 102; folic acid, 10; vitamin B$_{12}$, 0·9; inositol, 450; ascorbic acid, 150; Na menadione bisulphate, 15; thiamin, 5; choline chloride, 320 and p-aminobenzoic acid, 50.
† Mineral premix (mg/kg): Na$_2$SiO$_3$, 0·4; CaCO$_3$, 544·9; NaH$_2$PO$_4$·H$_2$O, 200; KH$_2$PO$_4$, 200; MgSO$_4$·7H$_2$O, 10; MnSO$_4$·H$_2$O, 4; CuCl$_2$·2H$_2$O, 2; ZnSO$_4$·7H$_2$O, 12; FeSO$_4$·7H$_2$O, 12; NaCl, 12; KI, 0·1; CoCl$_2$·6H$_2$O, 0·1; Na$_2$MoO$_4$·2H$_2$O, 0·5; AlCl$_3$·6H$_2$O, 1; and KF, 1.
‡ Gross energy = (crude protein × 23·6 + crude lipid × 39·5 + carbohydrate × 17·2)/1000.

and lipid ingredients were provided by the Minghui Feed Company. BBR (purity, HPLC ≥ 98 %) was purchased from the Spring and Autumn Biotechnology Company. BBR was supplemented to Con and HL diets at 50 mg/kg and named as ConB and HLB diets, respectively. After thorough grinding, all the ingredients were mixed and homogenised. Crystalline methionine, arginine and lysine were coated with carrageenan and carboxymethyl cellulose and added to the diet mix. The mixture was subsequently extruded through a feed machine (Modle HKJ-218; HUARUI) as described by Wang *et al.*[1]. The experimental diets were subsequently cooked by steam for 10 min and placed on a clean floor for drying by an electric fan in an air-conditioned room. The diets were stored at −20°C until use, and representative samples were taken for proximate composition analysis.

Fish and feeding trial

Black sea bream fingerlings (mean initial weight 1·47 (SD 0·03) g) were purchased from a local fish farm. The feeding trial was conducted at the Marine Fisheries Research Institute of Zhejiang

Province in Zhoushan, China. Before the feeding trial, all fish were kept in a cement tank (4·5 m × 2·5 m × 1·2 m) for a 14-d acclimatisation period. Then 420 healthy fish were selected and randomly assigned to twelve tanks (400 litres water volume) with thirty-five fish in each tank. Each diet was fed to triplicate groups of fish. Aerated and filtered sea water was provided to each tank at a rate of 2 litres/min during the feeding trial. The water quality parameters were constant throughout the experiment with temperature of 27 (SD 1)°C, pH 8·2 (SD 0·1), dissolved oxygen >5·0 mg/l and salinity 28 (SD 2) g/l. The fish were fed three times (07.00, 12.00 and 16.00 hours) daily to apparent satiation for 8 weeks.

Sample collection

At the end of the feeding trial, all fish were anesthetised (MS-222, 60 mg/l) 24 h after the last feeding. The fish in each tank was counted and weighed, and body length was measured. Blood from twelve fish was obtained from the caudal vein using 1-ml syringe, and pooled blood samples were centrifuged at 10 000 g after a 2-h settlement at 4°C. The fish were subsequently dissected on ice to excise the intraperitoneal fat, liver and dorsal muscle samples. Viscera, intraperitoneal fat and liver were weighed and recorded. Five fish from each tank were taken for whole-body and muscle proximate composition. Triplicate of liver and muscle samples from each treatment for gene expression measurements and all the other samples for further analyses were stored at −80°C. The liver from three fish per treatment was cut into small pieces and fixed in 10 % formalin and 2·5 % glutaraldehyde for histological observation.

Histological observation

The liver samples were stained by Oil Red O following the processes introduced by Volatiana et al.[33]. The slide microphotography was observed using an Olympus CX21 microscope. The transmission electron microscope (TEM) samples were fixed using 1 % OsO₄ for 2 h and desiccated in a graded mixture of alcohol and isoamyl acetate (1:1, v/v) for 30 min. The samples were subsequently kept in isoamyl acetate for 8 h, then washed and dried by liquid CO_2 (Hitachi Model HCP-C critical-point dryer). Gold-palladium was used to coat the desiccated samples (Hitachi Model E-1010 ion sputter), and the samples were viewed in Hitachi H7650 TEM. Morphometry of lipid accumulation, liver cell and nuclear diameters were carried out using Image-Pro Plus 6.0.

Samples analysis

The diets, whole fish and dorsal muscle proximate compositions were determined following the methods of the Association of Official Analytical Chemists[34]. Moisture was analysed by drying sample at 105°C for 24 h, crude lipid was determined by diethyl ether extraction, crude protein was estimated by determining N content following the Kjeldahl N method, and ash content was determined by heating sample at 550°C for 8 h. The liver supernatant was obtained following the method of Wang et al.[35]. The fatty acid synthase (FAS), lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), lipase (LPS), aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities and total cholesterol, TAG, LDL-cholesterol and HDL-cholesterol contents were determined using the diagnostic reagent kits for fish (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions.

Quantitative RT-PCR

The PCR primers of sterol regulatory element-binding protein-1 (srebp-1), acetyl-CoA carboxylase α (accα), fatty acid synthase (fas), glucose 6-phosphate dehydrogenase (g6pd), 6-phosphogluconate dehydrogenase (6pgd), lipoprotein lipase (lpl) pparα, pparγ, carnitine palmitoyltransferase 1a (cpt1a) and hormone-sensitive lipase (hsl) were designed by Primer premier 5.0 (Table 2). The housekeeping gene β-actin was used as the reference gene. The mRNA expression levels of the genes in liver and muscle tissues were assayed by quantitative RT-PCR using the SYBR Premix Ex Taq™ II Kit (Takara, Japan). Triplicate samples of each sample were used for quantitative RT-PCR amplification. The relative mRNA expression levels of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen[36]. The PCR process was performed as follows: 94°C for 30 s, 52°C for 30 s and 72°C for 90 s, followed by 72°C for 10 min. The PCR products were measured by visualised UV transillumination and 1·5 % (w/v) agarose gel electrophoresis.

Statistical analysis

All the results are presented as mean values and standard deviations. Statistical analyses were conducted by IBM SPSS Statistics 20.0 (SPSS). Kolmogorov–Smirnov and Levene's tests were applied to determine normality and homogeneity of all data, respectively. Two-way ANOVA was applied to evaluate the main effects of BBR supplementation, different basal diet and their interactions on each parameter. One-way ANOVA by Duncan's multiple range test was used to determine the significant differences between different treatments, and $P < 0.05$ was considered as significant.

Results

Growth performance, morphological and feed utilisation parameters

The effects of BBR supplementation in normal and HL diets on fish growth performance, morphological and feed utilisation parameters are presented in Table 3. Growth performance was significantly affected by dietary lipid level ($P < 0.05$) but not by BBR supplementation. Significantly lower body length and specific growth rate were found in fish fed the HL diets compared with feeding the Con diets. Besides, fish fed the HLB diet obtained significantly higher viscerosomatic index (VSI) value than that of the other groups. Hepatosomatic index values were significantly increased by HL diets compared with the Con diets. Fish fed the ConB diet had significantly lower FI than that of fish fed the Con diet. Significantly reduced feed efficiency ratio was found in fish fed the HL diets. Besides, a significant interaction between dietary BBR supplementation and dietary lipid level

Table 2. Primers of real-time quantitative PCR for lipid metabolism-related genes and β-actin of black sea bream

Gene	Nucleotide sequence (5′–3′)	Size (bp)	GenBank reference or publication	T_M (°C)
accα	Forward: AGTAGCCTGATTCGTTGGT	154	KX066238	55·4
	Reverse: GATTGAGGAGTCTGTTCGC			55·1
fas	Forward: AAGAGCAGGGAGTGTTCGC	213	KX066240	59·7
	Reverse: TGACGTGGTATTCAGCCGA			57·6
srebp-1	Forward: TGGGGGTAGGAGTGAGTAG	247	KX066235	59·7
	Reverse: GTGAAGGGTCAGTGTTGGA			57·6
6pgd	Forward: GAAGGGCTTGCTGTTTGTT	101	KX066237	55·4
	Reverse: GTGGCCAGGCCTCTATATG			59·7
g6pd	Forward: TCTCGCCAAGAAGAAAATC	297	KX078573	53·2
	Reverse: GCCAGGTAGAACAAACGGT			57·6
cpt1a	Forward: TGCTCCTACACACTATTCCCA	203	KX078572	58·1
	Reverse: CATCTGCTGCTCTATCTCCCG			61·9
pparα	Forward: ACGACGCTTTCCTCTTCCC	183	KX066234	59·7
	Reverse: GCCTCCCCCTGGTTTATTC			59·7
pparγ	Forward: CAGCCGAAGCGGTGAGAGAAGTGAC	158	MT61335	60·3
	Reverse: AGATAAGGGTCCCGTCTTTGTTCAT			63·8
hsl	Forward: AGCAACTAAGCCCTCCCCATC	179	KX066236	61·9
	Reverse: TCTTCACCCAGTCCGACACAC			61·9
lpl	Forward: CTGCTACTCCTCTGCCCA	204	KX078571	59·6
	Reverse: ACATCCCTGTTACCGTCC			57·3
β-actin	Forward: ACCCAGATCATGTTCGAGACC	–	Baowei *et al.*[81]	58·7
	Reverse: ATGAGGTAGTCTGTGAGGTCG			59·7

accα, Acetyl-CoA carboxylase α; *fas*, fatty acid synthase; *srebp-1*, sterol regulatory element-binding protein-1; *6pgd*, 6-phosphogluconate dehydrogenase; *g6pd*, glucose 6-phosphate dehydrogenase; *cpt1a*, carnitine palmitoyltransferase 1a; *hsl*, hormone-sensitive lipase; *lpl*, lipoprotein lipase.

Table 3. Effects of berberine supplementation in normal and high-lipid diets on growth performance, morphological and feed utilisation parameters of black sea bream*
(Mean values and standard deviations; *n* 3)

	Con		HL		ConB		HLB		Two-way ANOVA (*P*)		
Berberine...	0		0		50		50				
Diet...	P42/L11		P42/L20		P42/L11		P42/L20				
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Berberine	Diet	Interaction
Survival (%)	99·05	1·65	93·81	10·12	99·05	1·65	100	0·00	0·231	0·310	0·231
FW (g)	25·63^a	1·45	20·44^b	0·60	24·19^{a,b}	1·76	19·59^b	0·15	0·178	<0·001	0·716
BL (cm)	9·52^a	0·18	8·77^{b,c}	0·13	9·20^{a,b}	0·30	8·60^c	0·07	0·085	0·001	0·536
SGR (%/d)	5·13^a	0·11	4·67^b	0·23	4·90^a	0·15	4·67^b	0·07	0·200	<0·001	0·281
CF (g/cm^3)	2·95	0·03	2·90	0·05	2·96	0·06	2·94	0·03	0·331	0·388	0·522
IFR	2·61^a	0·11	2·44^{a,b}	0·18	2·25^b	0·11	2·56^a	0·02	0·203	0·411	0·031
VSI	8·16^{b,c}	0·1	8·14^{b,c}	0·19	7·85^c	0·24	8·83^a	0·29	0·171	0·005	0·004
HSI	1·34^b	0·11	1·59^a	0·20	1·29^b	0·06	1·59^a	0·20	0·702	0·027	0·987
FI (g/fish per d)	0·55^a	0·02	0·44^{a,b}	0·02	0·41^b	0·03	0·41^b	0·01	0·001	0·014	0·006
FER	0·84^a	0·12	0·58^b	0·13	0·83^a	0·14	0·61^b	0·21	0·956	0·027	0·840
PER	28·47	0·76	30·42	5·57	27·19	2·96	29·79	5·77	0·773	0·500	0·920

Con, control; HL, high lipid; ConB, control diet with berberine supplementation; HLB, high-lipid diet with berberine supplementation; FW, final weight; BL, body length; SGR, specific growth rate; CF, condition factor; IFR, intraperitoneal fat ratio; VSI, viscerosomatic index; HSI, hepatosomatic index; FI, feed intake; FER, feed efficiency ratio; PER, protein efficiency ratio.
^{a,b,c} Mean values in a row with unlike superscript letters were significantly different ($P < 0·05$).
* Survival = 100 × (final fish number/initial fish number); SGR = 100 × (ln final body weight − ln initial body weight)/d; CF = final body weight/final body length^3 × 100; IFR = 100 × (intraperitoneal fat weight/body weight); VSI = 100 × viscera weight/whole body weight; HSI = 100 × (liver weight/body weight); FI = dry diet feed/final fish number/d; FER = wet weight gain/dry diet fed; PER = 100 × weight gain/protein intake.

was observed with intraperitoneal fat ratio, VSI and FI. The survival, condition factor and protein efficiency ratio showed non-significant differences in all groups ($P > 0·05$).

Whole-body and muscle proximate compositions

The effects of BBR supplementation in normal and HL diets on whole-body and muscle proximate compositions are presented in Table 4. Lipid contents of whole body and muscle were significantly affected by BBR supplementation and dietary lipid level. BBR supplementation in the Con and HL diets did not affect the whole-body and muscle lipid contents significantly. A significant interaction between BBR supplementation and diet was found in whole-body ash content. While whole-body and muscle moisture, protein, and muscle ash contents were not significantly affected by different treatments.

Table 4. Effects of berberine supplementation in normal and high-lipid diets on whole-body and muscle proximate compositions of black sea bream (wet weight %)
(Mean values and standard deviations; n 3)

Sample		Con		HL		ConB		HLB		Two-way ANOVA (P)		
		Berberine... 0		0		50		50				
		Diet... P42/L11		P42/L20		P42/L11		P42/L20				
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Berberine	Diet	Interaction
Whole body	Moisture	68·25	0·57	67·44	0·28	67·96	0·37	66·68	0·89	0·332	0·073	0·654
	Protein	18·02	0·26	17·65	0·64	17·95	0·361	17·43	0·37	0·625	0·160	0·797
	Lipid	8·74[b]	0·57	10·12[a,b]	0·31	9·16[a,b]	1·44	12·71[a]	1·34	0·041	0·005	0·115
	Ash	5·02	0·22	5·24	0·05	5·25	0·05	5·07	0·09	0·695	0·794	0·027
Muscle	Moisture	74·71	1·18	74·48	0·18	74·83	0·47	74·33	1·32	0·470	0·177	0·673
	Protein	21·13	0·89	20·34	0·29	20·67	0·37	20·30	0·83	0·981	0·343	0·934
	Lipid	2·78[b]	0·31	3·99[a,b]	0·07	2·88[b]	0·24	4·55[a]	0·27	0·045	<0·001	0·107
	Ash	1·56	0·08	1·56	0·04	1·53	0·03	1·49	0·06	0·195	0·555	0·644

Con, control; HL, high lipid; ConB, control diet with berberine supplementation; HLB, high-lipid diet with berberine supplementation.
[a,b] Mean values in a row with unlike superscript letters were significantly different (P < 0·05).

Table 5. Effects of berberine supplementation in normal and high-lipid diets on serum biochemical parameters of black sea bream
(Mean values and standard deviations; n 3)

	Con		HL		ConB		HLB		Two-way ANOVA (P)		
Berberine...	0		0		50		50				
Diet...	P42/L11		P42/L20		P42/L11		P42/L20				
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Berberine	Diet	Interaction
TAG (mmol/l)	1·23[b]	0·17	2·27[a]	0·42	1·04[b]	0·12	1·60[b]	0·13	0·048	0·004	0·219
TCH (mmol/l)	9·37	0·87	10·32	0·57	9·12	1·01	9·57	0·29	0·372	0·229	0·652
HDL-cholesterol (mmol/l)	3·15	0·17	2·98	0·32	3·45	0·66	3·00	0·16	0·497	0·203	0·542
LDL-cholesterol (mmol/l)	3·84[b]	0·19	5·21[a]	0·24	3·84[b]	0·03	4·15[b]	0·19	0·015	0·003	0·015
ALT (U/l)	2·82[a,b]	0·36	3·35[a]	0·58	2·18[b]	0·39	2·01[b]	0·73	0·019	0·597	0·314
AST (U/l)	6·75	0·52	6·21	0·93	6·31	1·15	6·57	0·09	0·951	0·826	0·530

Con, control; HL, high lipid; ConB, control diet with berberine supplementation; HLB, high-lipid diet with berberine supplementation; TCH, total cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
[a,b] Mean values in a row with unlike superscript letters were significantly different (P < 0·05).

Serum biochemical parameters

As shown in Table 5, the fish fed the HL diet had significantly higher serum LDL-cholesterol and TAG contents compared with other groups. Serum LDL-cholesterol and TAG contents were affected by BBR supplementation and dietary lipid level significantly. A significant interaction between BBR supplementation and diet lipid level was observed in serum LDL-cholesterol content. Besides, serum ALT was significantly influenced by BBR supplementation. Fish fed the HLB diet resulted in significantly lower serum ALT activity compared with that of the HL group, whereas the total cholesterol and HDL-cholesterol contents, as well as the AST activity showed no statistical differences among different groups.

Activities of hepatic metabolic enzymes

The effects of BBR supplementation in normal and HL diets on hepatic metabolic enzyme activities are shown in Table 6. Hepatic LPL and LPS activities were significantly affected by BBR supplementation, while ALT activity was significantly affected by dietary lipid level. BBR supplementation in the HL diet reduced LPS activity significantly. In addition, hepatic

FAS, HSL and AST activities were not significantly influenced by different treatments.

Liver lipid deposition and ultrastructure

Oil Red O-stained liver pictures are shown in Fig. 1. Fish fed the HL diet had significantly higher hepatic lipid accumulation compared with the other groups. BBR supplementation in Con and HL diets significantly reduced liver lipid accumulation as indicated by the dramatic decrease in the relative areas of the liver that was stained red.

The liver ultrastructure pictures of fish fed the different diets are presented in Fig. 2. Fish fed the Con or ConB diets exhibited normal ultrastructure with round and clear nucleus. Fewer lipid droplets were visible in the liver of ConB group compared with the Con group. However, fish fed the HL diet showed hypertrophied liver cells with significantly larger cell diameter than that of the other treatments. Large lipid droplets were also observed in the cytoplasm along with nuclear translocation to the cell periphery in the liver of the HL group. In contrast, fish fed the HLB diet showed alleviated hepatic lipid accumulation with fewer droplets surrounding the centred nucleus compared with the HL group.

Table 6. Effects of berberine supplementation in normal and high-lipid diets on hepatic metabolic enzymes activities of black sea bream
(Mean values and standard deviations; *n* 3)

	Con		HL		ConB		HLB		Two-way ANOVA (*P*)		
Berberine...	0		0		50		50				
Diet...	P42/L11		P42/L20		P42/L11		P42/L20				
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Berberine	Diet	Interaction
LPL (ng/mg protein)	9·25	0·89	10·10	1·43	12·14	0·51	11·67	1·38	0·030	0·819	0·439
FAS (ng/mg protein)	1·33	0·01	1·51	0·20	1·29	0·42	1·31	0·19	0·534	0·619	0·656
HSL (ng/mg protein)	1·54	0·02	1·62	0·49	1·93	0·39	2·22	0·54	0·155	0·562	0·730
LPS (U/g protein)	0·69[a,b]	0·10	0·92[a]	0·19	0·70[a,b]	0·02	0·47[b]	0·07	0·035	0·931	0·033
ALT (U/g protein)	27·66[a]	7·63	21·32[a,b]	4·48	31·16[a]	2·43	14·37[b]	3·25	0·600	0·008	0·283
AST (U/g protein)	67·61	0·83	49·27	3·18	69·91	3·52	66·44	2·77	0·114	0·087	0·198

Con, control; HL, high lipid; ConB, control diet with berberine supplementation; HLB, high-lipid diet with berberine supplementation; LPL, lipoprotein lipase; FAS, fatty acid synthase; HSL, hormone sensitive lipase; LPS, lipase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
[a,b] Mean values in a row with unlike superscript letters were significantly different (*P* < 0·05).
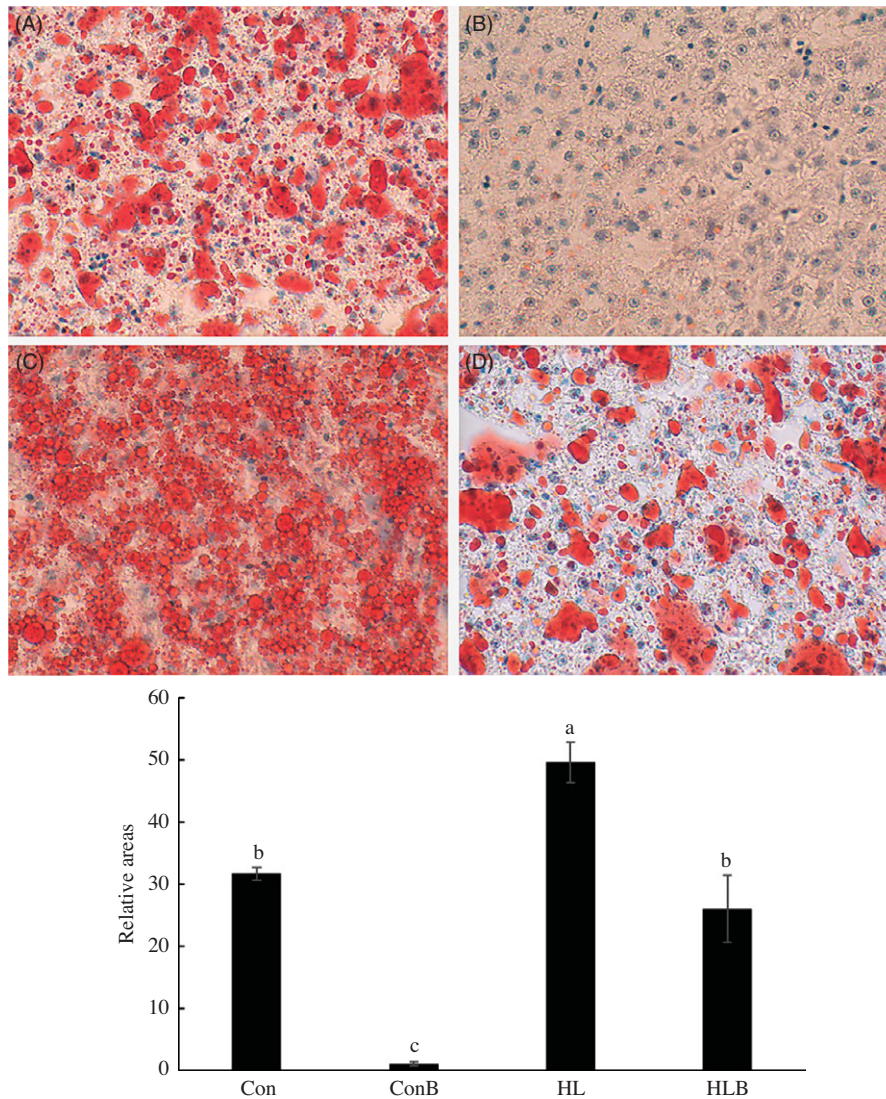

Fig. 1. Oli Red O-stained liver (400×) of black sea bream fed control (A), control + berberine (BBR) (B), high-lipid (C) and high-lipid + BBR (D) diets. Relative areas stained by Oil Red O were determined by Image-Pro Plus 6.0. [a,b,c] Mean values with unlike letters were significantly different (*P* < 0·05). Con, control; ConB, control diet with berberine supplementation; HL, high lipid; HLB, high-lipid diet with berberine supplementation.
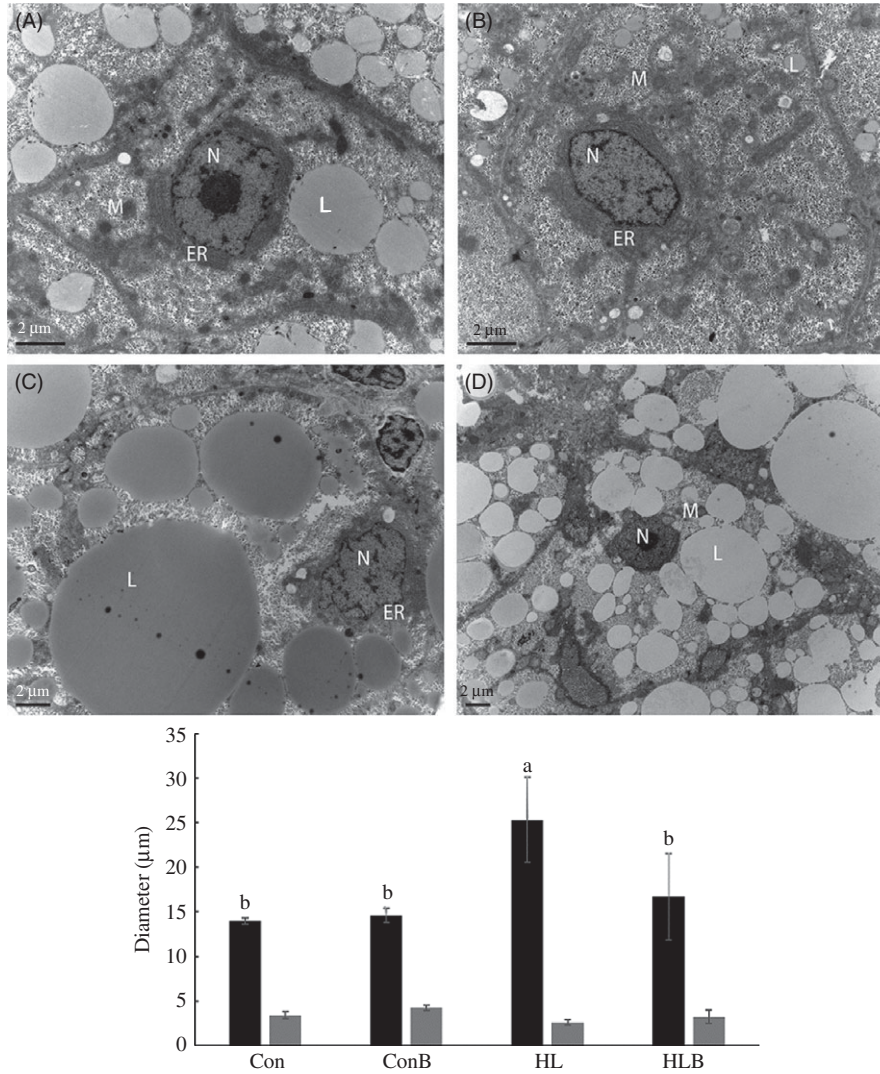
Fig. 2. Transmission electron microscope images of juvenile black sea bream liver fed different diets for 8 weeks. (A) Con; (B) Con + berberine (BBR); (C) HL; (D) HL + BBR. [a,b] Mean values with unlike letters were significantly different ($P < 0.05$). ■, Hepatocyte diameter; ■, Nucleus diameter. N, nucleus; M, mitochondria; ER, endoplasmic reticulum; L, lipid droplet; Con, control; ConB, control diet with berberine supplementation; HL, high lipid; HLB, high-lipid diet with berberine supplementation.

Gene expression

As shown in Fig. 3 and Table 7, BBR supplementation in the HL diet significantly down-regulated hepatic expression levels of *accα*, *srebp-1*, *6pgd*, *g6pd* and *pparγ*. In contrast, hepatic *lpl*, *hsl* and *cpt1a* expression levels were significantly up-regulated by BBR supplementation in the HL diet. However, the hepatic mRNA levels of these genes in the Con and ConB groups showed non-significant differences except for a down-regulation of *pparγ* in CoB group. According to the two-way analysis, BBR supplementation affected the expression levels of *accα*, *6pgd*, *pparγ*, *hsl* and *lpl* in liver significantly. And dietary lipid level affected the expression levels of *fas*, *6pgd*, *pparγ*, *cpt1a*, *pparα*, *hsl* and *lpl* in liver significantly. Significant interactions between BBR supplementation and dietary lipid level were observed for the hepatic *srebp-1*, *6pgd*, *g6pd*, *cpt1a* and *lpl* expression levels.

In the muscle, fish fed the BBR-supplemented diets had significantly higher expression levels of *accα* (4·5-fold increase in ConB group) and *srebp-1* (8·2-fold increase in ConB group), but significantly lower *pparα* expression level in muscle. *Pparγ* expression level was significantly up-regulated in fish fed the ConB diet compared with the Con group, whereas an opposite trend was found in the HL and HLB groups. BBR supplementation in the HL diet up-regulated the expression levels of *fas*, *6pgd* and *g6pd* but down-regulated *lpl*, *hsl* and *cpt1a* expression. Based on the two-way analysis, all these genes (except for *pparγ*) were affected by both dietary lipid level and BBR supplementation significantly, and significant interactions between BBR and dietary lipid level were observed in all these gene expression levels (except for *pparγ*).

Discussion

In the present study, an HL diet reduced growth performance, which could be due to reduced FI observed in fish fed the HL and HLB diets. Feed consumption is directly influenced by
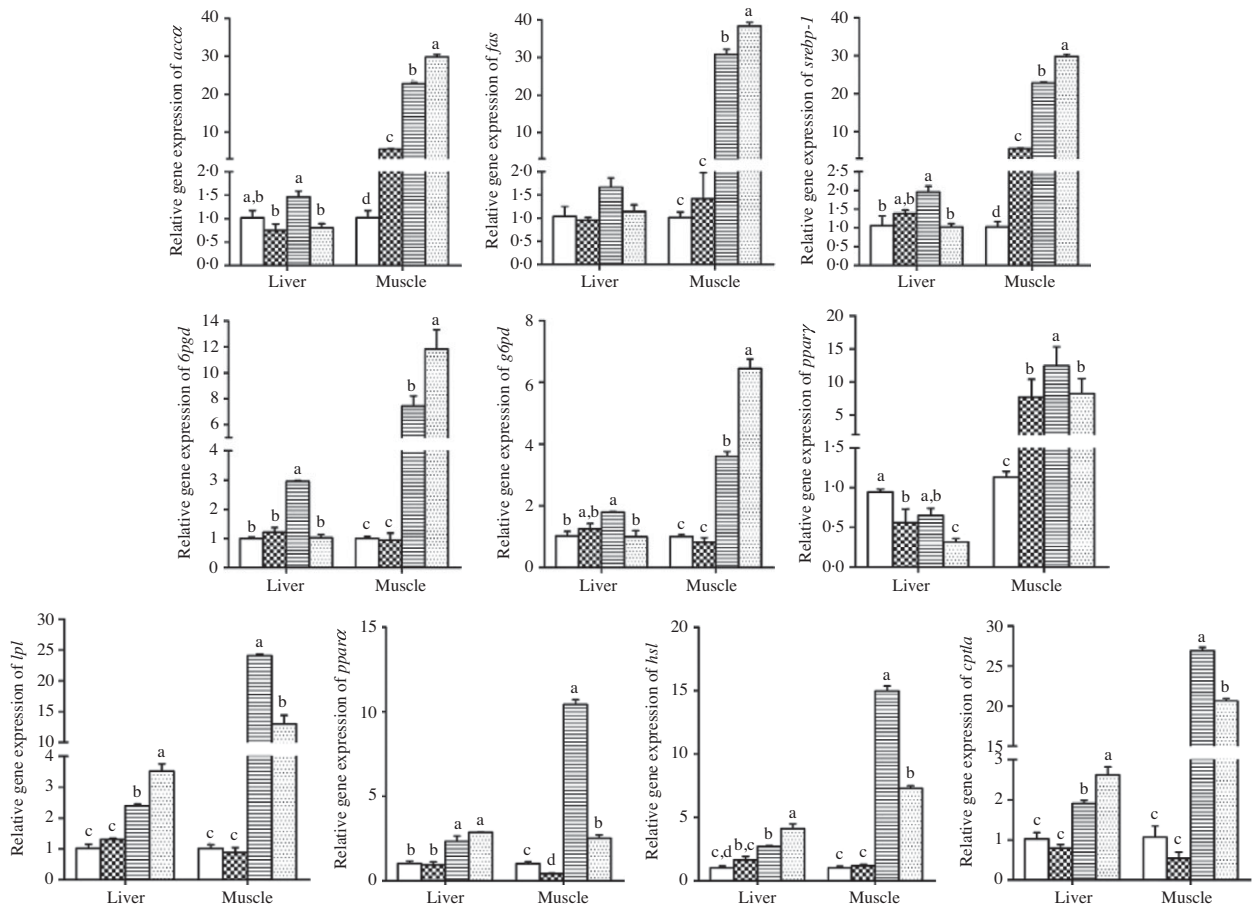
Fig. 3. Results of quantitative real-time PCR analysis carried out for acetyl-CoA carboxylase α (*accα*), fatty acid synthase (*fas*), sterol regulatory element-binding protein-1 (*srebp-1*), 6-phosphogluconate dehydrogenase (*6pgd*), glucose 6-phosphate dehydrogenase (*g6pd*), *pparγ*, lipoprotein lipase (*lpl*), *pparα*, hormone-sensitive lipase (*hsl*), carnitine palmitoyltransferase 1a (*cpt1a*) in the liver and muscle of black sea bream fed different diets for 8 weeks. [a,b,c,d] Mean values with unlike letters were significantly different ($P < 0.05$). ☐, Control; ▨, control diet with berberine supplementation; ▤, high lipid; ▥, high-lipid diet with berberine supplementation.

Table 7. Two-way ANOVA (*P*) analysis of gene expression of fish fed the experimental diets

Gene	Liver			Muscle		
	BBR	Diet	Interaction	BBR	Diet	Interaction
accα	0.007	0.092	0.174	<0.001	<0.001	0.011
fas	0.103	0.039	0.215	0.001	<0.001	0.002
srebp-1	0.107	0.148	0.006	<0.001	<0.001	<0.001
6pgd	<0.001	<0.001	<0.001	0.034	<0.001	0.003
g6pd	0.111	0.145	0.011	<0.001	<0.001	<0.001
pparγ	0.007	0.030	0.796	0.815	0.039	0.144
cpt1a	0.134	<0.001	0.011	<0.001	<0.001	<0.001
pparα	0.282	<0.001	0.16	<0.001	<0.001	<0.001
hsl	0.003	<0.001	0.142	<0.001	<0.001	<0.001
lpl	0.001	<0.001	0.017	<0.001	<0.001	<0.001

BBR, berberine; *accα*, acetyl-CoA carboxylase α; *fas*, fatty acid synthase; *srebp-1*, sterol regulatory element-binding protein-1; *6pgd*, 6-phosphogluconate dehydrogenase; *g6pd*, glucose 6-phosphate dehydrogenase; *cpt1a*, carnitine palmitoyltransferase 1a; *hsl*, hormone-sensitive lipase; *lpl*, lipoprotein lipase.

dietary energy level[1], and HL diets had been reported to decrease FI and growth performance in giant croaker (*Nibea japonica*)[37], grass carp (*Ctenopharyngodon idella*)[38], blunt snout bream[15] and black sea bream[1]. Furthermore, it was also observed in the present study that BBR supplementation in Con diet caused significant depression of FI, but BBR supplementation in both the Con and HL diets did not affect growth performance significantly. BBR tastes bitter and influences the palatability of diet and was reported to inhibit the FI in blunt snout bream at dietary inclusion level of 100 mg/kg[15].

However, several studies demonstrated that some Chinese herbs have hepatoprotective effects, which are beneficial for growth[39]. Xu et al.[21] observed that supplementation of 50 mg/kg BBR in an HL diet could improve the growth performance of blunt snout bream, as BBR attenuated the adverse effects (hepatocyte apoptosis, suppressed immune function and increased oxidative stress) caused by high dietary lipid. Nevertheless, some other studies in blunt snout bream obtained contradictory results about BBR supplementation and growth performance. Zhou et al.[15] observed that supplementation of 50 mg/kg BBR in the HL (15 % crude lipid) diet significantly improved FI and growth performance, while 100 mg/kg BBR decreased both parameters. Moreover, 50 mg/kg BBR improved the growth performance of fish when supplemented to the normal diet (4·7 % crude lipid) rather than the HL diet (10·1 % crude lipid)[24]. Xu et al.[21] reported that fish fed the normal diet (5·0 % crude lipid) with 50 mg/kg BBR at a 2-week interval mode obtained remarkably better growth performance than other feeding modes (no-supplement, 4-week intervals and continuous feeding). Supplementation of 1, 3, 6 and 9 g/kg BBR in normal diet improved the growth performance of Nile tilapia (*Oreochromis niloticus*), which might be attributed to the promotion of beneficial microflora regulated by BBR[23]. However, zebrafish larvae pretreated with high-cholesterol diet exposed to high dosage of BBR (5 and 25 μM) for 10 d caused a decrease in weight gain[25]. Therefore, the relationship between growth performance and BBR supplementation may vary with fish species, feeding mode, basal diet used, dietary BBR concentration and lipid level. In addition, chronic administration of BBR led to the reduction of weight gain in rats[40] and humans[41], which might be because BBR could improve energy expenditure[42].

HL diets causing excessive lipid deposition in liver have been widely reported in different fish species[15,32,43]. HL diets have been reported to result in cellular swelling, nuclear translocation, lipid droplet accumulation[6,44] and hypertrophied liver cells with larger cell diameter[32,45], which were also observed in the present study. These pathologies might also inhibit lipoprotein secretion and fatty acid oxidation, which then result in a vicious cycle[46]. However, these abnormalities can be mitigated by BBR supplementation. The mitigative effect of BBR against the impact of HL diets was reported in blunt snout bream[15,32] and obese rats[45]. BBR attenuated high-cholesterol diet -induced hepatic steatosis, liver lesion and mitochondrial damage in zebrafish[25]. In mammals, BBR alleviated fatty liver through promoting AMP-dependent protein kinase pathway and improved fatty acid oxidation while suppressing the genes involved in lipogenesis[42]. BBR could also recover mitochondrial function and reduce steatosis caused by high-fat diet[47,48]. In the present study, based on histological observation, BBR supplementation in the normal diet resulted in little lipid accumulation in the liver, whereas considerable hepatic lipid deposition was still observed in the fish fed the HLB diet. This means that the lipid-lowering effect of BBR may vary with dietary lipid and BBR levels.

Cpt1a is a rate-limiting enzyme of fatty acid oxidation which is located in the outer membrane of mitochondria and manages uptake of long-chain fatty acids into mitochondria[49,50]. *Ppara* can bind the promoter region of *cpt1a*, and it also regulates some enzymes involved in fatty acid β-oxidation[49,51]. *Hsl* is the key

rate-limiting enzyme for TAG mobilisation[52]. *Lpl* hydrolyses plasma TAG to release fatty acid for oxidation or storage[53] and highly expressed in tissues that have a high demand for lipid storage or oxidation[54]. It is well known that the imbalance of lipid consumption and oxidation is the main reason for lipid accumulation[55]. In the present study, BBR supplementation in the HL diet up-regulated the hepatic expressions of *lpl*, *cpt1a* and *hsl*, indicating that BBR could improve lipolysis and fatty acid β-oxidation in liver. Consistent with the present study, enhanced fatty acid oxidation by BBR supplementation was also reported in blunt snout bream[15,49,55], obese rats[42] and *in vitro*[16,56].

Accα is a committed enzyme that regulates long-chain fatty acid biosynthesis by producing malonyl-CoA in cytosols. In mammals, *accα* is predominantly expressed in lipogenic tissues, such as liver and adipose[54]. *Srebp-1* is a transcription factor responsible for regulating cholesterol and fatty acid/lipid biosynthesis pathways[54,57]. *Fas* is involved in *de novo* fatty acid synthesis[28]. *G6pd* and *6pgd* participate in NADPH production and are important genes for fatty acids biosynthesis[58]. *Pparγ* regulates lipogenesis and promotes lipid storage by binding to the promoter region of adipogenic genes[51,59], and it is a major metabolic transcription regulator particularly for hepatic lipogenesis[60]. In the present study, BBR supplementation in the HL diet significantly reduced the expression levels of *accα*, *srebp-1*, *6pgd*, *g6pd* and *pparγ* in the liver. Notably, numerous studies have demonstrated that BBR can reduce lipogenesis by down-regulating *pparγ* expression in different tissues[11,29,45,61,62]. Hence, the lipid-lowering effect of BBR was mainly attributed to the fact that it can mitigate lipid accumulation in liver through down-regulating lipogenesis and up-regulating lipolysis pathways, similar to mechanisms reported in mammals[14,42,61].

In line with the gene expression results, higher hepatic LPS activity was observed in the HL group compared with the Con group, while BBR supplementation significantly reduced the activity. Hepatic LPS hydrolyses TAG-rich LDL to form denser and smaller LDL particles; thus its activity is positively related to hypertriacylglycerolaemia[63]. Accordingly, BBR supplementation had been reported to reduce LPS activity in rats fed the high-fat/sucrose diet[45]. However, the activities of FAS and HSL did not correspond with the gene expression results and showed no significant differences among different groups. This might be because the mRNA level was influenced by time and mRNA stability[51,64]. Similarly, the inconsistent relationship between enzyme activity and mRNA level had been reported in other fish species[51,65].

Conversely, the expression levels of *accα*, *srebp-1*, *6pgd*, *g6pd*, *lpl*, *cpt1a* and *hsl* in the muscle showed the opposite trends with those in the liver of the HL and HLB groups. BBR supplementation in the HL diet up-regulated the expression of *fas* but down-regulated *ppara* expression in the muscle. This means BBR could inhibit lipolysis and promote lipogenesis in muscle, which is in line with the increased crude lipid contents in muscle and whole body of the fish fed the BBR-supplemented diets. In contrast to the present study, BBR reduced malonyl-CoA level and up-regulated fatty acid oxidation genes in muscle of rats[42]. One plausible explanation for the present result is that

the dietary protein level (42 %, DM) was not high enough and black sea bream used both lipid and carbohydrate as energy resources as reported by Wang *et al*.[1], and BBR supplementation might improve the utilisation of carbohydrate. In the present study, the HL diets decreased hepatic ALT activity. It is well known that hepatic ALT and AST activities are related to protein deposition and energy provision, and their activities were negatively associated with dietary lipid and carbohydrate contents[66], which means black sea bream might use carbohydrate and lipid for energy to compensate for protein catabolism[1]. Besides, up-regulated expression levels of hepatic *pparα*, *hsl* and *cpt1a* were found in fish fed the HL diet, indicating improved fatty acid oxidation in HL treatments. Some studies proved that BBR has a hypoglycemic effect through enhancing insulin sensitivity and secretion and stimulating glucose uptake and transport[67–69]. This assumption is supported by the up-regulated *pparγ* level in muscle of ConB group, which is positively related to insulin sensitisation[70]. Glucose can be oxidised for energy, thus inhibits lipid oxidation and improves lipid accumulation[71]. Besides, glucose also provides NADPH or carbon backbones for *de novo* lipogenesis[72]. However, supplementation of BBR in the Con and HL diets showed an opposite trend in regulating *pparγ* expression in muscle. The reason for this is unknown and deserves further investigation. Therefore, BBR supplementation might improve glucose utilisation of muscle, which was shown as improved lipogenesis and restricted lipolysis. Moreover, the lowest hepatic ALT activity was observed in the HLB group, which might be because BBR supplementation improved insulin sensitivity. A study in humans found that hepatic ALT activity is negatively related to insulin sensitivity[73].

Consistently, fish fed the HL diet caused more lipid deposition in the liver, as shown in the higher hepatosomatic index value, which was in line with other studies[1,12]. However, intraperitoneal fat ratio and VSI were not significantly affected by HL treatment, which means that black sea bream tends to accumulate lipid in the liver rather than intraperitoneally. Different fish species have different preferences for organs to store lipid, and HL diet increasing intraperitoneal fat ratio, hepatosomatic index and VSI values were reported in blunt snout bream[12] and common carp (*Cyprinus carpio*)[13]. Interestingly, significant interactions between BBR supplementation and diet were found in VSI and intraperitoneal fat ratio. It seemed that dietary BBR supplementation in the HL diet could improve lipid deposition in intraperitoneal fat and viscera, while the opposite trend was found in that of Con and ConB groups. BBR had been reported to improve energy expenditure and accelerated the oxidation rate of fatty acids[42], thus reduced lipid accumulation in specific tissues when supplemented in the normal diet. On the other hand, BBR was observed to be distributed to all organs with various accumulation levels after oral administration, with the highest distribution in the liver, followed by kidney, muscle and the least accumulation was in fat[18]. Therefore, the diverse contents of BBR in different organs might have resulted in the variation of results. Besides, BBR might stimulate hepatic lipid transport to other tissues, such as mesenteric fat tissue and muscle, which is also supported by the increased lipid contents in the muscle of BBR-supplemented groups. In the present study, hepatic LPL activity was improved by BBR supplementation, which might indicate the increment of lipid importation for storage in the muscle[51]. Notably, BBR can regulate multiple pathways or molecules and reflects a comprehensive effect to treat energy-related metabolic disorders[74]. Hence, the tissue-specific manner of BBR supplementation in different diets might be correlated with a complicated mechanism, which deserves further study.

Feeding fish an HL diet usually causes excessive lipid accumulation and thus impairs health status[12]. Increased plasma TAG, LDL and total cholesterol contents is a significant risk symbol of steatosis, liver dysfunction and regarded as a sign of poor health condition[75,76]. Besides, serum ALT and AST activities are common indicators for liver function and health[1]. In the present study, the HL diet induced higher serum TAG and LDL-cholesterol contents, as well as significantly higher serum ALT activity compared with that of the fish fed the Con diet. These evaluated serum parameters caused by the HL diet are in line with the histological result and some other studies in blunt snout bream[12,13], large yellow croaker (*Pseudosciaena crocea* R.)[43] and black sea bream[1]. Conversely, BBR supplementation in the HL diet reduced these serum parameters significantly, which indicated that impaired lipid metabolism and liver function resulting from the HL diet could be alleviated by BBR supplementation. Similarly, some studies reported that BBR supplementation reduced plasma TAG and total cholesterol contents in blunt snout bream[21], serum TAG and total cholesterol contents in grass carp[77], serum TAG and LDL concentrations in rabbits fed with a high-fat diet[78], plasma total cholesterol and TAG contents, as well as ALT and AST activities in obese rats[42]. Hepatic TAG, total cholesterol and glucose contents were reduced by BBR exposure in zebrafish larvae pretreated with high-cholesterol diet[25]. Previous studies demonstrated that BBR could reduce plasma LDL by increasing LDL receptor gene expression through stabilising LDLR receptor mRNA[15,79], inhibit cholesterol absorption of the intestine[20] and prevent hepatic cholesterol biosynthesis[14]. Moreover, BBR could regulate gut microflora population and structure, which was beneficial for fatty acid oxidation and reducing serum TAG[14]. In contrast, the administration of BBR showed a positive correlation with serum HDL-cholesterol concentration in rats[45,80], which was not observed in the present study.

Conclusion

In conclusion, the HL diet inhibited FI and reduced the growth performance of black sea bream. The high dietary lipid level also caused extensive lipid accumulation in liver and increased serum TAG, LDL-cholesterol contents and ALT activity. BBR supplementation in the HL diet reduced the expression levels of lipogenesis genes and increased lipolysis genes expression, thus alleviating hepatic lipid accumulation and improved the health condition. Besides, BBR supplementation increased lipid deposition in the muscle through the opposite mechanism of that in the liver. The lipid-lowering effect of BBR is affected by dietary BBR and lipid levels. Thus, BBR has the potential to work as a hepatoprotective feed additive to alleviate the harmful effects caused by HL diets in aquaculture.

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