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RESEARCH PAPER

Effects of *Bacillus subtilis* on hepatic lipid metabolism and oxidative stress response in grass carp (*Ctenopharyngodon idellus*) fed a high-fat diet

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Abstract

Bacillus subtilis is widely used in aquaculture as a probiotic. However, few studies have been conducted to examine the effect of *B. subtilis* on liver lipid metabolism. A total of 135 healthy grass carp (50.24 ± 1.38 g) were randomly divided into three groups: control (Con), high-fat diet (HF), and high-fat diet + *B. subtilis* (HF + *B. subtilis*), and fed for 8 weeks. The results showed that compared with the HF group, the weight gain rate (WGR) significantly increased ($P < 0.05$) and the hepatic lipid content, serum low-density lipoprotein cholesterol (LDL-C), and aspartate aminotransferase (AST) decreased in the group supplemented with *B. subtilis* ($P < 0.05$). Moreover, the hepatic mRNA expression of fatty acid synthase (*FAS*) was significantly down-regulated and the carnitine palmitoyl transferases (*CPT1α1a*) were up-regulated in the HF + *B. subtilis* group compared to the HF group ($P < 0.05$), respectively. Additionally, in the HF + *B. subtilis* group, glutathione (GSH) significantly increased ($P < 0.05$), while hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents significantly decreased compared to the HF group ($P < 0.05$). *B. subtilis* may reduce the hepatic lipid content by inhibiting its synthesis and promoting β-oxidation of fatty acids. *B. subtilis* may also alleviate dyslipidaemia and prevent oxidative damage in the liver caused by the high-fat diet of grass carp. Hence, dietary supplementation with *B. subtilis* shows promise as a therapeutic or preventive tool against fatty liver disease.

Keywords *Bacillus subtilis* · Grass carp · Growth performance · Hepatic lipid metabolism · Oxidative stress

Introduction

Grass carp is one of the most important freshwater species in China that can consume artificial diets and water plants (Du et al. 2006; Kong et al. 2017b). Feeding carp a high-fat diet may lead to fatty liver disease in this species and adversely influence aquaculture production of grass carp (Huang et al. 2018). The literature clearly shows that dietary lipids can bring protein sparing effect and enhance growth. However, high levels of dietary lipids may have a negative effect on fish growth. A study by Du et al. (2005) showed a positive effect only when dietary lipids were below 4% and a negative effect was observed when dietary lipids were higher than 6% in the growth of grass carp. Furthermore, consumption of a high-fat diet may lead to unwanted hepatic fat deposition (Li et al. 2016; Wang et al. 2015), dyslipidemia (Sabzi et al. 2017; Zhang et al. 2018), affect the antioxidative system (Chen et al. 2016; Huang et al. 2018; Ma et al. 2018), and affect normal gut microbiota leading to dysbiosis (Al-Muzafar and Amin 2017; Falcinelli et al. 2017; Tian et al. 2016). The above-mentioned

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effects of consuming a high-fat diet may ultimately impact health and reduce fish harvest yields. Given this, it is of vital importance to find solutions to alleviate the adverse effects of consuming a high-fat diet.

Published studies have shown that probiotics can modify gut microbiota and significantly reduce the risk for fatty liver disease in mammals, improve liver function, and have significant therapeutic effects on fatty liver disease induced by high-fat diet (Al-Muzafar and Amin 2017; Awaisheh et al. 2013; Xu et al. 2012). Recently, two published studies showed that using the probiotic *Lactobacillus rhamnosus* as a feed additive could improve intestinal epithelium structures and induce transcriptional decrease of genes related to triglyceride and cholesterol metabolism, concomitantly decreasing the body triglyceride and cholesterol levels in zebrafish induced by a high-fat diet (Falcinelli et al. 2015, 2017). *B. subtilis*, a gram-positive, spore-forming, non-pathogenic bacterium, is widely used in aquaculture as a probiotic (Ren et al. 2017). *B. subtilis* has been shown to have a positive impact on the size and structure of the microbial community in the intestinal chyme, on the activity of some digestive enzymes, and on the digestibility and weight gain of carp (Zuenko et al. 2017). It has also been shown to improve growth performance and disease resistance in shrimp by enhancing the immune response (Zokaeifar et al. 2012). Our studies also show that *B. subtilis* has a protective effect, specifically on the intestines in grass carp. This protective effect is based on the following knowledge about *B. subtilis*. (1) *B. subtilis* can modulate intestinal microflora and improve digestive enzyme activity and growth performance of grass carp (Wu et al. 2012). (2) *B. subtilis* has a protective effect on the intestinal mucosal structure, which can reduce damage to the intestinal mucosal barrier, and decrease inflammation in grass carp (Huang et al. 2017). (3) *B. subtilis* effectively protects fish against oxidative stress damage (Tang et al. 2018). However, few studies examine the effect of *B. subtilis* on liver lipid metabolism at present.

Based on the above rationale and results of our previous studies, we aimed to determine the impact of *B. subtilis* on liver lipid metabolism and oxidative stress of grass carp by evaluating growth performance and morphology, hepatic lipid accumulation and histochemistry, serum biochemical indicators, expression of genes involved in lipid metabolism, and oxidative stress indicators. Our findings can inform solutions for reducing the risk of fatty liver disease in grass carp as well as provide a theoretical basis for the application of *B. subtilis* in aquaculture practice.

Results

Effect of *B. subtilis* on growth performance and morphological parameters

Data on the growth performance and morphological parameters of grass carp are presented in Table 1. After 8 weeks, the SGR and WGR of grass carp in the two groups fed the high-fat diet significantly increased compared to those in the control group ($P<0.05$). The grass carp in the HF + *B. subtilis* group showed the highest WGR ($P<0.05$). Furthermore, the HF group and HF + *B. subtilis* group had a higher FI and lower FCR compared to the control group ($P<0.05$). Lastly, no significant difference was observed in SR, CF, HSI, and VSI among all the experimental groups ($P>0.05$).

Table 1 Effects of the different kinds of feed on growth performance, feed intake and morphological parameters

Item	Con	HF	HF + *B. subtilis*
SR	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
IBW	50.39 ± 1.69	50.15 ± 1.44	50.19 ± 1.89
FBW	83.78 ± 1.92^{a}	92.73 ± 3.22^{b}	95.42 ± 1.85^{b}
WGR	66.30 ± 2.14^{a}	84.90 ± 1.80^{b}	90.19 ± 3.56^{c}
SGR	0.91 ± 0.03^{a}	1.10 ± 0.02^{b}	1.15 ± 0.04^{b}
FI	69.27 ± 3.75^{a}	77.65 ± 2.94^{b}	79.32 ± 1.9^{b}
FCR	2.08 ± 0.14^{b}	1.82 ± 0.05^{a}	1.75 ± 0.04^{a}
CF	1.82 ± 0.12	1.93 ± 0.11	1.87 ± 0.14
HSI	1.92 ± 0.22	1.96 ± 0.27	1.90 ± 0.16
VSI	9.1 ± 0.51	8.95 ± 0.92	9.03 ± 0.73

Values are the mean ± SE ($n=3$ tanks on growth performance and feed intake, and $n=6$ on morphological parameters). Values within the same row with different letters are significantly different ($P<0.05$)

SR survival rate (%) = 100 × (final fish number)/(initial fish number), *IBW* (g · fish^{-1}) initial mean body weight, *FBW* (g · fish^{-1}) final mean body weight, *WGR* weight gain rate (%) = (final mean body weight-initial mean body weight)/initial mean body weight, *SGR* special growth rate (% d^{-1}) = 100 × (ln (final mean body weight) − ln (initial mean body weight))/days, *FI* (g · fish^{-1}) feed intake, *FCR* feed conversion rate = feed intake/weight gain, *CF* condition factor = 100% × (body weight, g)/(body length, cm)^{3}, *HSI* hepatosomatic index = 100% × (liver weight)/(body weight), *VSI* viscera index = 100% × (viscera weight)/(body weight)

Effect of *B. subtilis* on hepatic lipid accumulation in grass carp

Results of oil-red O staining are presented in Fig. 1a. Lipids are red colored and nuclei are blue colored after staining with oil-red O. The images show that the amount and volume of lipid droplets stained by oil-red O were higher in the HF group than those in the Con group, but lower than the HF + *B. subtilis* group. The relative areas of lipid droplets stained with oil-red O (Fig. 1b) were consistent with the results of hepatic lipid accumulation. As shown in Fig. 1c, the hepatic lipid content of the HF + *B. subtilis* group was between the control group and HF group, and was significantly different from the control group and HF group ($P<0.05$).

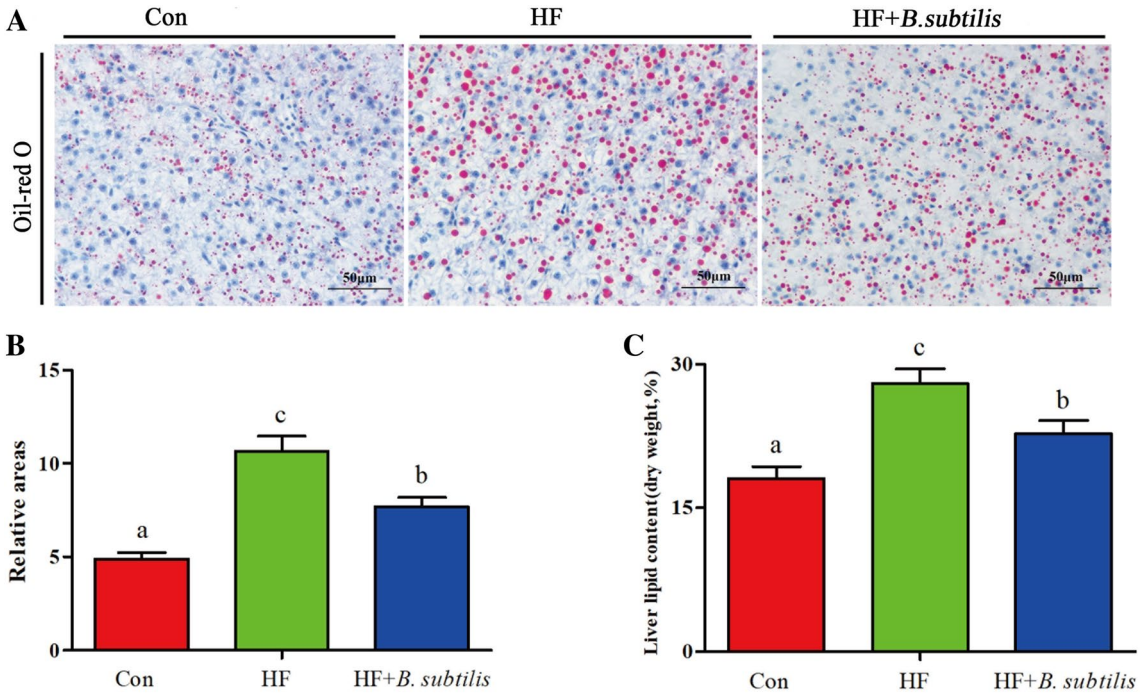

Fig. 1 Effect of *B. subtilis* on hepatic lipid accumulation in grass carp: (**a**) effect of the *B. subtilis* diet and high-fat diet on histochemistry (oil-red O staining) (original magnification × 400, bars 50 μm); (**b**) the relative areas of the lipid droplets in oil-red O stained grass carp were analyzed by Image-Pro Plus 6.0; (**c**) effect of the *B. subtilis* diet and high-fat diet on the lipid content in the liver of grass carp. Values are the mean ± SE ($n = 6$). Different letters indicate significant differences among groups ($P < 0.05$)

Effect of *B. subtilis* on serum biochemistry indicators of grass carp

As shown in Table 2, analysis of key indicators for lipid metabolism showed that the serum CHO and LDL-C levels in the HF group significantly increased compared to the control group ($P < 0.05$). However, in the HF + *B. subtilis* group, the serum CHO and LDL-C levels decreased to nearly the same level as the control group ($P > 0.05$). Moreover, fish fed the high-fat diet with *B. subtilis* had lower serum AST than fish fed the high-fat diet ($P < 0.05$). There was no significant difference in the serum TG, HDL-C content, ALT, TP, and Alb among the control group, HF group, and HF + *B. subtilis* group ($P > 0.05$).

Table 2 Effect of *B. subtilis* on serum biochemistry indicators

Item	Con	HF	HF + *B. subtilis*
CHO (mmol · L^{-1})	5.94 ± 0.26^{a}	6.71 ± 0.22^{b}	6.08 ± 0.19^{ab}
TG (mmol · L^{-1})	5.57 ± 0.34	5.68 ± 0.24	5.58 ± 0.24
HDL-C (mmol · L^{-1})	2.23 ± 0.07	2.50 ± 0.15	2.26 ± 0.11
LDL-C (mmol · L^{-1})	2.63 ± 0.06^{a}	2.98 ± 0.13^{b}	2.60 ± 0.10^{a}
TP (g · L^{-1})	23.52 ± 0.78	22.61 ± 0.97	24.17 ± 0.83
Alb (g · L^{-1})	15.45 ± 0.51	14.73 ± 0.55	14.18 ± 0.49
AST (U · L^{-1})	61.48 ± 2.24^{a}	79.81 ± 4.26^{b}	65.46 ± 2.97^{a}
ALT (U · L^{-1})	7.09 ± 0.27	7.48 ± 0.22	7.31 ± 0.18

Values are the mean ± SE ($n = 6$). Values within the same row with different letters are significantly different ($P < 0.05$)

CHO cholesterol, *TG* plasma total triglyceride, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *TP* total protein, *Alb* albumin, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase

Effect of *B. subtilis* on the expression of genes associated with lipid metabolism

As shown in Fig. 2, the groups displayed differences in relative mRNA expression of lipid metabolism genes and transcription factors. The *L-FABP* was significantly up-regulated in the HF group ($P < 0.05$) with no significant difference in the HF + *B. subtilis* group compared to the control group and HF group ($P > 0.05$), respectively. The *FAS*, *ACCα*, *SCD*, *LPL*, *CPTIα1a*, *SREBP-1c*, *PPARγ* and *PPARα* were significantly down-regulated after fish were fed the high-fat diet for 8 weeks ($P < 0.05$). Moreover, the expression of *FAS* was significantly down-regulated and the expression of *CPTIα1a* was significantly up-regulated in the HF + *B. subtilis* group compared with the HF group ($P < 0.05$). The expression of *LPL* and *PPARα* in the HF + *B. subtilis* group

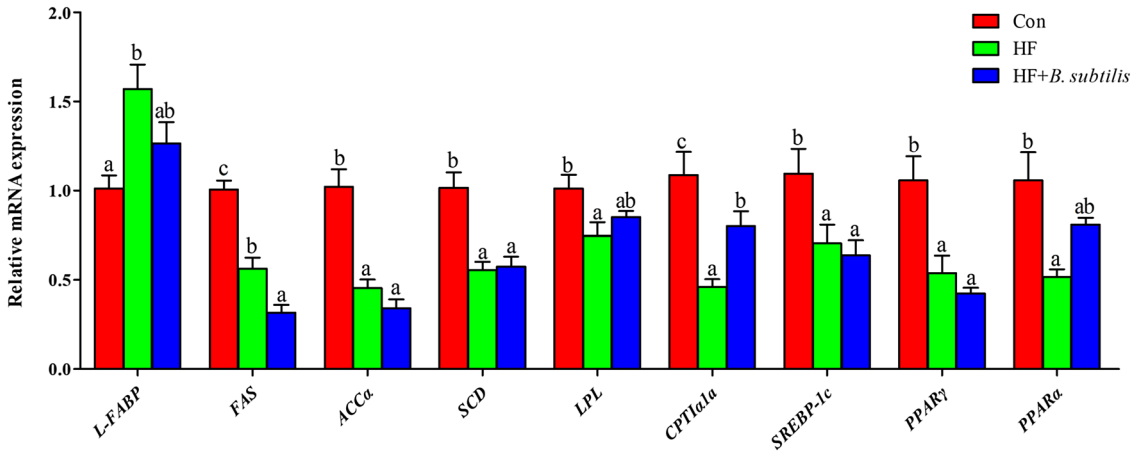

Fig. 2 Effect of *B. subtilis* on lipid metabolism gene and transcription factor mRNA expression in liver of grass carp. Values are the mean ± SE ($n = 6$). Different letters indicate significant differences among groups ($P < 0.05$)

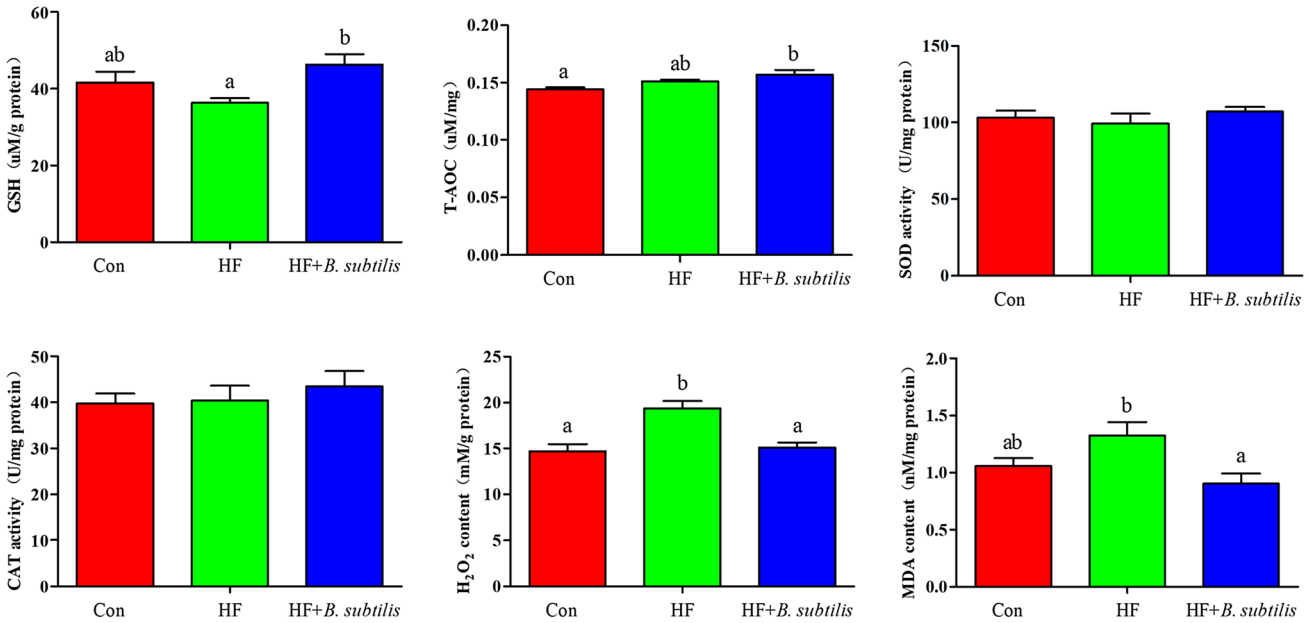

Fig. 3 Effect of *B. subtilis* on MDA and H_2O_2 content and activities of antioxidant enzymes in liver of grass carp. Values are the mean ± SE ($n = 6$). Different letters indicate significant differences among groups ($P < 0.05$)

was regulated to nearly the same level as the control group ($P > 0.05$).

Effect of *B. subtilis* on the MDA and H_2O_2 contents and activities of antioxidant enzymes

As shown in Fig. 3, in the HF + *B. subtilis* group, GSH showed a significant increase compared to the HF group ($P < 0.05$), and the T-AOC higher than the control group ($P < 0.05$). H_2O_2 and MDA levels in HF + *B. subtilis* group were lower than in the HF group ($P < 0.05$) with no significant difference in the control group.

Discussion

Our results show that feeding a high-fat diet to grass carp for 8 weeks significantly increased SGR, WGR, and FI. This finding is similar to findings from a study conducted in zebrafish fed a high-fat diet (Shimada et al. 2015; Zang et al. 2015) but differs from the results of the study in Atlantic salmon, Japanese seabass, and Jade Perch (Hillestad et al.1998; Xu et al. 2011). The inconsistency across studies may be related to the different feed formula, FI, aquaculture environment, and the species of fish. Many

study authors have reported that some probiotics, such as commercial mixed-species probiotics (Standen et al. 2016), *Lactobacillus acidophilus* (Chelladurai et al. 2012), *Saccharomyces cerevisiae* (Abass et al. 2018), *B. subtilis,* and *Lactobacillus rhamnosus* (Munirasu et al. 2017), are conducive to increasing the growth performance of fish. In the present study, we observed that administration of *B. subtilis* was also beneficial to body weight gain as evidenced by a significant increase on WGR with no significant difference on FI compared to the HF group. In addition, no significant differences were noted for CF, HSI, and VSI among grass carp fed two dietary lipid levels, which is similar to the study results in meagre *Argyrosomus regius* with increased dietary lipid (Chatzifotis et al. 2010).

Lipid content and histochemical analyses of the liver demonstrated that a high-fat diet caused hepatic lipid accumulation and dietary supplementation probiotic *B. subtilis* decreased the hepatic lipid content with one study showing that *Lactobacillus rhamnosus* could decrease the lipid content of zebrafish (Falcinelli et al. 2015). Similar results were also observed in juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858) receiving a diet containing probiotic *Shewanella* spp. strains Pdp11 and Pdp13 significantly decreased numbers of lipid droplets in the liver and modulated the sole intestinal microbiota. This regulation of intestinal flora was mainly manifested as the increased presence of *Shewanella* spp. members and the decreased presence of *Vibrio* spp. members in the microbiota (Banda et al. 2010; Tapia-Paniagua et al. 2014). In our experiments, we only measured the hepatic lipid content of grass carp supplemented with *B. subtilis.* The change of intestinal flora after *B. subtilis* treatment will be a measurement in our follow-up study. Additionally, this observation was also similar to results of treating mammals with fatty liver disease induced by high-fat diet with probiotics (Lee et al. 2006; Ma et al. 2008; Xu et al. 2012).

Once fatty acids enter the liver, they are esterified into TG and then can be stored into the hepatocytes in lipid droplets or enter the blood through TG-rich lipoproteins (Yuan et al. 2016), which can lead to a significant change in CHO and TG in blood (Jung et al. 2013; Rincon-Cervera et al. 2016). Therefore, serum lipid metabolism-related indicators, such as CHO, TG, HDL-C and LDL-C, were evaluated. Our data showed that the content of CHO in the HF group was higher than those of the other two groups. In addition, compared with the HF group, adding *B. subtilis* to the high-fat diet resulted in a decreased LDL-C content. Similar studies confirmed that intestinal probiotics are beneficial for lowering CHO levels in rats (Awaisheh et al. 2013) and treatment with *Bifidobacterium* spp. decreased LDL-C in serum in high-fat diet-induced obese rats (An et al. 2011). This suggests that *B. subtilis* supplementation alleviates dyslipidaemia, which corresponds to results that *B. subtilis* could reduce liver lipid contents in our study. AST and ALT are the best clinical biomarkers of hepatic functions and are mainly distributed in the mitochondria and cytoplasmic water-soluble phase, respectively. Once the liver cells are damaged, a large amount of AST and ALT will enter into the extracellular space and ultimately into circulation (Ozer et al. 2008). Hassaan et al. (2018) observed a significant decrease in AST and ALT in Nile tilapia (*Oreochromis nilotcus*) fed a diet containing *B. subtilis.* We found that the AST in serum decreased in the HF + *B. subtilis* group, similar to results observed with specific probiotic strains in fatty liver disease of mice and rats induced by a high-fat diet (Al-Muzafar and Amin 2017; Xin et al. 2014).

To explore the molecular mechanisms involved in *B. subtilis* regulation of lipid deposition, the expression of several key genes in the liver was analyzed. We first examined the mRNA expression of *L-FABP,* which is related to fatty acid transport. *L-FABP* is a type of fatty acid binding protein that transports lipids to lipid droplets for storage and to mitochondria for β-oxidation (Schroeder et al. 1998; Venkatachalam et al. 2013). Briskey (2015) observed that probiotics restored expression of *L-FABP* gene to a level similar to that in mice fed with a chow diet, which was similar to our results. The main lipogenic enzymes, such as *FAS*, *ACC*, and *SCD1* (Chen et al. 2013; Song et al. 2016), and important lipogenic factors, such as *SREBP-1c* and *PPARγ*, can regulate hepatic fatty acid synthesis, which in turn, drives hepatic triglyceride synthesis (Eberle et al. 2004; Rosen et al. 1999; Tontonoz et al. 1994). *PPARα* regulates lipid catabolism by inducing the expression of *CPT* (Ji et al. 2011; Kerner and Hoppel 2000; Morash et al. 2008). Our results showed that the high-fat diet caused down-regulation of both the enzymes involved in fatty acid synthesis (*FAS*, *ACCα*, *SCD*) and transcription factors (*PPARγ* and *SREBP-1c*). The down-regulation of these mRNA expressions is a form of adaption of grass carp to a high-fat diet to achieve a dynamic balance between endogenous fatty acids and exogenous fatty acids (Li et al. 2016). Down-regulated *CPTIα1a* mRNA expression suggests that the high-fat diet caused the synthesis and β-oxidation of fatty acids to be reduced. *B. subtil*is down-regulated *FAS* mRNA expression and up-regulated *CPTIα1a* mRNA expression, indicating that adding *B. subtilis* to the diet may decrease the hepatic lipid content by inhibiting the synthesis and promoting the β-oxidation of fatty acids, which is consistent with the result of serum CHO and LDL-C content. Studies by Briskey (2015) and Yoo et al. (2013) showed results similar to ours.

Oxidative stress and altered redox balance play an important role in the pathogenesis of steatosis (Roberto et al. 2011). SOD, CAT, and GSH are important antioxidant enzymes that play an important role in protecting cells from oxidative stress and preventing or repairing oxidative damage. For example, the dismutation of two superoxide radicals to H_2O_2 was

shown to be catalyzed by SOD, whereas H_2O_2 was degraded by CAT (Wang et al. 2009). MDA is a product of lipid oxidative damage and it is used to evaluate lipid peroxidation (Tang et al. 2018; Yang et al. 2010). Our experimental results showed that after feeding grass carp the diet containing *B. subtilis*, their antioxidant capacity was significantly improved and the oxidative stress response was also reduced, which was mainly reflected in the significant improvement of liver GSH, the lower content of MDA and H_2O_2 compared with HF group and the higher T-AOC compared with control group. The supplementation of *B. subtilis* in the diet after 40 days could significantly increase the activity of T-AOC and SOD and significantly decrease the MDA levels in the serum of *Litopenaeus vannamei* (Shen et al. 2010). After feeding the basic diet containing *B. subtilis* for 4 weeks, the activity of T-AOC, SOD, CAT and GSH was significantly increased and the content of MDA was significantly decreased in the liver of grass carp (Li et al. 2012). These results are similar to ours. Consequently, *B. subtilis* effectively enhanced the antioxidant capacity and improved oxidative damage of grass crap fed a high-fat diet.

Conclusions

In conclusion, the present study demonstrated that a high-fat diet intake induced hepatic lipid accumulation, but dietary supplementation of probiotic *B. subtilis* may inhibit the synthesis and promote the β-oxidation of fatty acids in the liver to significantly reduce hepatic lipid accumulation, alleviate dyslipidaemia and liver oxidative damage in grass carp, which contribute to the amelioration of diet-induced fatty liver disease progress. Additionally, *B. subtilis* promotes the growth performance of grass carp, which has a certain guiding significance for aquaculture production.

Materials and methods

Bacterial strains

The bacteria used in this study were isolated from the gut of grass carp. The isolated bacterial strain was identified as *B. subtilis* Ch9 and stored in the Laboratory of Aquatic Animal Medicine, Fisheries College of Huazhong Agricultural University. *B. subtilis* was inoculated onto a Luria–Bertani (LB) agar plate, then incubated for 24 h at 37 °C. A single clone was selected and inoculated into LB broth and cultured in a shaker at 37 °C for 3 days. Bacterial cells were harvested by centrifugation at 3500 rpm for 15 min. The supernatant was discarded. The pellet was re-suspended in sterile phosphate-buffered saline (PBS) to remove metabolic waste from the bacterial solution. It was then diluted.

Diet preparation

Three diets were prepared and used in this study control (basal) diet (4% lipids of the dry matter), high-fat diet (8% lipids of the dry matter), and high-fat diet supplemented with *B. subtilis* (8% lipids of the dry matter). The composition and chemical analyses of the three diets are presented in Table 3. All ingredients were purchased from Hubei Haida Feed Co., Ltd. (Wuhan, China). After ingredients were thoroughly mixed (including *B. subtilis* solution), pellets with a diameter of 2 mm were produced by a granulator in 30 min. After the pellets were air dried, they were stored in a freezer at − 20 °C until use. Before use, the survival of *B. subtilis* was 1×10^7 CFU g^{-1} as determined by the plate counting method.

Experimental procedures

Grass carp were obtained from a commercial freshwater fish farm (Bai Rong Aquaculture Co., Ltd., Hubei Province) and reared in circular polyester tanks. After 2 weeks, 135 healthy grass carp (50.24 ± 1.38) g were randomly divided into one of the three groups: control (fed a basal diet), HF (fed a high-fat diet), and HF + *B. subtilis* (fed a high-fat diet supplemented with *B. subtilis*), with each group in three replicate tanks with a capacity of 300 L (15 fish per tank). All grass carp were fed to apparent satiation twice daily (08:30 and 16:30) for 8 weeks. During the entire experimental period, one-third of the water was replaced daily. The recirculating water temperature was maintained at (25 ± 1) °C, pH at 7.5 ± 0.3, dissolved oxygen at (7 ± 0.45) mg · L^{-1}, ammonia at (0.015 ± 0.002) mg · L^{-1} and nitrate at (0.05 ± 0.008) mg · L^{-1}.

Sampling for analyses

At the end of the 8-week feeding trial, approximately 24 h after the last feeding, the survival rate and body weight gain of fish in every tank were calculated. A total of 24 fish per group were then randomly selected and euthanized (MS-222, 10 mg · L^{-1}). Six fish were measured for their individual body length and body weight to calculate the condition factor (CF). Blood samples were collected from the caudal vein before dissection to measure serum biochemical indicators. The fish were then dissected on ice. The liver weight and visceral mass weight were measured to calculate the hepatosomatic index (HSI) and viscera index (VSI). Six fish were immediately removed using sterile forceps, frozen in liquid nitrogen and stored at − 80 °C (not longer than 2 weeks) for total RNA extraction and antioxidant activities, six fish for determination of

Table 3 Compositions of diets

Experimental diets	Basal diet	High-fat diet	High-fat diet + *B. subtilis*
Ingredients ($g \cdot kg^{-1}$)			
Fish meal	80	80	80
Soybean meal	240	240	240
Rapeseed meal	340	340	340
Wheat flour	250	250	250
Soybean oil	6	46	46
$Ca(H_2PO_4)_2$	20	20	20
Vitamin premix[a]	1	1	1
Mineral premix[b]	3	3	3
NaCl	2	2	2
Choline chloride	2	2	2
Sodium carboxymethyl cellulose	56	16	16
Total	1000	1000	1000
Proximate composition			
Crude protein (% DM)	29.83	29.66	29.61
Crude lipid (% DM)	4.39	8.27	8.29
Moisture (% DM)	10.16	9.95	9.93
Ash (% DM)	8.32	8.12	8.17

[a]Vitamin premix ($mg \cdot kg^{-1}$): vitamin A 6500 IU, vitamin D3 4500 IU, vitamin C 120 mg, vitamin E 25 mg, vitamin K_3 5 mg, vitamin B_1 12.5 mg, vitamin B_2 12.5 mg, vitamin B_6 15.0 mg, vitamin B_{12} 0.025 mg, niacinamide 50 mg, pantothenate 40 mg, inositol 75 mg, folic acid 2.5 mg, biotin 0.08 mg

[b]Mineral premix ($mg \cdot kg^{-1}$): NaCl 1.0, $MgSO_4$ 15.0, $NaH_2PO_4 \cdot 2H_2O$ 25.0, $AlCl_3 \cdot 6H_2O$ 0.06, KH_2PO_4 32.0, $Ca(H_2PO_4)_2 \cdot H_2O$ 20.0, $C_6H_5FeO_7 \cdot 10H_2O$ 2.5, $CaC_6H_{10}CaO_6 \cdot 5H_2O$ 3.5, $ZnSO_4 \cdot 7H_2O$ 0.353, $MnSO_4 \cdot 4H_2O$ 0.162, $CuSO_4 \cdot 5H_2O$ 0.031, $CoCl_2 \cdot 6H_2O$ 0.001, $KIO_3 \cdot 6H_2O$, 0.003, cellulose, 0.39

liver lipid content, and another six fish for histochemical observation.

Hepatic lipid

Live samples were freeze dried for 24 h at − 50 °C to obtain the dried liver sample. The crude lipid content of individual samples was determined using Soxhlet extraction.

Histochemical observation

Histochemical analysis was performed as described by Song et al. (2016) with slight modifications. Briefly, histochemical observations were carried out by oil-red O staining. First, the liver samples were fixed with paraformaldehyde, dehydrated with sucrose, embedded in optimal cutting temperature compound (OCT), sliced (thickness 8 μm) using a cryostat microtome, and stained with oil-red O. Photos were then taken under a microscope (400×). Ten fields of view were randomly selected from each sample to calculate the relative area of lipid droplets in oil-red-O stained liver tissue.

Serum biochemical analysis

The CHO, TG, HDL-C, LDL-C, Alb, TP, AST, and ALT contents were determined using an automatic biochemical analyser (Selectra-xl, the Netherlands) at the Fisheries College of Huazhong Agricultural University (Wuhan, Hubei, China). All kits were purchased from Biosino Bio-Technology and Science, Inc.

Real-time polymerase chain reaction analysis

TriPure Reagent Kit (Aidlab, RN0102) was used according to its instructions to extract RNA from the liver tissue of grass carp. The quality and quantity of RNA were then assessed via agarose gel (1%) electrophoresis and spectrophotometric (A260:280 nm ratio) analysis, respectively. RNA was reverse transcribed into cDNA using a TRUE script 1st Strand cDNA Synthesis Kit with gDNA Eraser (Aidlab, PC5402). β-Actin is a commonly used reference gene to normalize cDNA loading in grass carp in our laboratory (Kong et al. 2017a, b; Tang et al. 2018) and was used in this study. Specific primers were designed according to the published sequences of grass carp and are presented in Table 4. Real-time quantitative PCR was performed on a Roche LightCycler 480 real-time PCR instrument. The total volume of the reaction was 20 μL and included 2 μL cDNA, 0.5 μL each primer, 7 μL aqueous diethylpyrocarbonate (DEPC), and 10 μL 2×SYBR Green qPCR Mix (Aidlab, PC5902). The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Oxidative stress in liver

The total antioxidant capacity (T-AOC), the malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentration, the superoxide dismutase (SOD) and catalase (CAT) activity, and glutathione (GSH) levels were determined using commercial kits provided by Nanjing Jiancheng Bioengineering Institute (China). Kits were used according to manufacturer instructions.

Statistical analyses

One-way analysis of variance was used to analyze the data. All results are expressed as the mean ± SE (standard error

Table 4 Primers used for real-time PCR analysis

Gene	GenBank accession no.	Primers	Sequence (5′ to 3′)	Annealing temperature
L-FABP	EU220990.1	L-FABP-F	GGGAAAACCATCACTAACTC	58 °C
		L-FABP-R	TCAGGGTCTCAACCATCTC	
FAS	HM802556.1	FAS -F	GTCCACAGGGTGTCGTTC	58 °C
		FAS -R	GAGGTCTTGGGCTCTTTATT	
ACCα	GU908475	ACCα-F	AGTATCGCAGTGGCATCA	58 °C
		ACCα-R	TGTCCCCTTTGTTTTCCT	
SCD	AJ243835	SCD-F	GTTTGTGCCCTGGTTCTT	58 °C
		SCD-R	GGGGTTAATGGTGCTGTC	
LPL	FJ436077	LPL-F	AGCCCTGTATGAACGAGA	58 °C
		LPL-R	CACATCCTTGCCCACTAG	
CPTIα1a	KJ816747	CPTIα1a-F	TTTACGACGGACGGTTGC	58 °C
		CPTIα1a-R	GCTTGTTCTTCCCACGACT	
SREBP-1c	GU339498	SREBP-1c-F	GGATTGAGGTGAGCCGACAT	58 °C
		SREBP-1c-R	TGAGGAAAGCCATTGACTACATT	
PPARγ	GQ220296	PPARγ-F	AATGCACCTTTCGTTATCC	58 °C
		PPARγ-R	GAGCGTCACTTGGTCGTTC	
PPARα	FJ595500	PPARα-F	TGTCAATACTGCCGTTTCC	58 °C
		PPARα-R	GACTGGTGCTCCTCTTTCC	
β-actin	M25013	β-actin-F	CCTTCTTGGGTATGGAGTCTTG	T
		β-actin-R	AGAGTATTTACGCTCAGGTGGG	

L-FABP liver-type fatty acid-binding protein, *FAS* fatty acid synthase, *ACCα* acetyl-CoA carboxylase alpha, *SCD* stearoyl-CoA desaturase, *LPL* lipoprotein lipase, *CPTIα1a* carnitine palmitoyl transferases, *SREBP-1c* sterol-regulatory element binding proteins, *PPARγ* peroxisome proliferator activated receptor gamma, *PPARα* peroxisome proliferator activated receptor alpha, *β-actin* beta-actin

of the mean). Multiple comparisons were performed using the Duncan multiple range test among the groups. Statistical significance was $P < 0.05$. All statistical analyses were performed using SPSS 22.0.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethics statement All applicable international, national, and institutional guidelines for the care and use of animals were followed by the authors.

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