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Dietary inclusion of *Clostridium butyricum* cultures alleviated impacts of high-carbohydrate diets in largemouth bass (*Micropterus salmoides*)

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Abstract

A 60-d feeding trial was conducted to explore the potential regulatory effects of dietary *Clostridium butyricum* cultures (CBC) supplementation in high-carbohydrate diet (HCD) on carbohydrate utilisation, antioxidant capacity and intestinal microbiota of largemouth bass. Triplicate groups of largemouth bass (average weight 35·03 ± 0·04 g), with a destiny of twenty-eight individuals per tank, were fed low-carbohydrate diet and HCD supplemented with different concentration of CBC (0 %, 0·25 %, 0·50 % and 1·00 %). The results showed that dietary CBC inclusion alleviated the hepatic glycogen accumulation induced by HCD intake. Additionally, the expression of hepatic *ampkα1* and insulin signaling pathway-related genes (*ira, irb, irs, p13kr1* and *akt1*) increased linearly with dietary CBC inclusion, which might be associated with the activation of glycolysis-related genes (*gk, pfkl* and *pk*). Meanwhile, the expression of intestinal SCFA transport-related genes (*ffar3* and *mct1*) was significantly increased with dietary CBC inclusion. In addition, the hepatic antioxidant capacity was improved with dietary CBC supplementation, as evidenced by linear decrease in malondialdehyde concentration and expression of *keap1*, and linear increase in antioxidant enzyme activities (total antioxidative capacity, total superoxide dismutase and catalase) and expression of antioxidant enzyme-related genes (*nrf2, sod1, sod2* and *cat*). The analysis of bacterial 16S rRNA V3–4 region indicated that dietary CBC inclusion significantly reduced the enrichment of *Firmicutes* and potential pathogenic bacteria genus *Mycoplasma* but significantly elevated the relative abundance of *Fusobacteria* and *Cetobacterium*. In summary, dietary CBC inclusion improved carbohydrate utilization, antioxidant capacity and intestinal microbiota of largemouth bass fed HCD.

Keywords: ***Clostridium butyricum*** **cultures: Largemouth bass: Insulin signalling pathway: Antioxidant capacity: Intestinal microbiota**

Carbohydrates are a relatively economical source of energy in aquafeeds, which could reduce the consumption of protein and lipid for energy[1]. Accordingly, carbohydrates are widely used in aquafeeds, although fish has relatively no specific nutritional requirements[2], especially the carbohydrates of starch source, which has good binding and expansion properties and is the pivotal material for the production of puffed feed[3]. However, numerous studies have found that carbohydrates could not be efficiently used by carnivorous fish[4,5] and long-term intake of high-carbohydrate diet (HCD) leads to varying degrees of damage in fish, including growth inhibition, excessive hepatic glycogen accumulation, intestinal microbial disruption and impaired antioxidant capacity[6–9]. Therefore, mitigating the negative effects of HCD in carnivorous fish could be helpful for the extensive application of carbohydrates in feed.

At present, with the ever more advanced research related to probiotics, specific functional probiotics have been demonstrated to partly mitigate the negative effects of HCD in fish[10]. *Clostridium butyricum*, as an anaerobic endospore-forming gram-positive bacterium, has the capacity to actively ferment carbohydrates, and its main ultimate metabolites are SCFA, including butyric, propionic and acetic acids[11,12]. In the past

Abbreviations: AMPK, AMP-activated protein kinase; CAT, catalase; CBC, *Clostridium butyricum* cultures; CFU, colony-forming unit; FI, feed intake; FFAR, free fatty acid receptor; HCD, high-carbohydrate diet; IRS, insulin receptor substrates; HSI, hepatosomatic index; LCD, low-carbohydrate diet; PI3K, phosphatidylinositol 3-kinase; SOD, superoxide dismutase; VSI, viscerosomatic index.

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aquaculture-related studies, *C. butyricum* has been demonstrated to be effective in improving the immune response of tilapia (*Oreochromis niloticus*)[13], common carp (*Cyprinus carpio* L.)[14] and freshwater prawn (*Macrobrachium rosenbergii*)[15]. However, a recent review on mammals declared that SCFA besides their ability to inhibit inflammatory responses also have regulatory effect on glucose metabolism[16]. In past studies, it has been discovered that SCFA have a potential role in mediating the utilisation of glucose in the muscle and liver of mammals through the free fatty acid receptor-2 (FFAR2/GPR43) and free fatty acid receptor-3 (FFAR3/GPR41) pathways[17–19]. However, research on the effect of *C. butyricum* cultures (CBC) on glucose metabolism in fish is lacking.

A previous study showed that butyric acid (the predominant SCFA product of *C. butyricum*) improves insulin sensitivity and increases energy expenditure in mice[20]. Glucose utilisation cannot be achieved without the secretion and regulation of insulin, which are regulated through insulin signalling pathway. In general, through insulin binding to the insulin receptors (IR), phosphatidylinositol 3-kinase (PI3K) is attracted to the insulin receptor substrates (IRS), and thereby AKT is phosphorylated to regulate various metabolic processes such as glycolysis and gluconeogenesis[21]. In addition, the insulin signalling pathway-related genes are also mediated by AMP-activated protein kinase (AMPK)[22], and past studies have reported that SCFA can directly activate the AMPK signalling pathway by increasing the AMP/ATP ratio[17,20]. Therefore, we speculate that investigating the changes in insulin signalling pathways and AMPK may provide a theoretical basis for the improvement of carbohydrate utilisation in largemouth bass by CBC in this study.

In mammals, diabetes triggers cellular damage, lipid peroxidation and insulin resistance as a result of increased free radical production or impaired antioxidant defence[23]. In addition, ecological dysbiosis of gut microbiota is also considered as a symptom of diabetes[24]. Also, carnivorous fish with diabetic features have been found to have impaired antioxidant capacity and gut microbiota at high carbohydrate levels[25]. Previous studies have revealed that the dietary inclusion of *C. butyricum* increased the antioxidant enzyme activities and facilitated the enrichment of beneficial intestinal microbiome in aquatic animals[26,27]. Therefore, CBC as one of the options to solve the negative impacts of HCD has potential research value.

As a typical carnivorous fish, largemouth bass (*Micropterus salmoides*) has been widely cultured in China[28], and it is also a suitable model for glucose metabolism research due to its limited glucose utilisation[9]. Meanwhile, probiotic cultures are characterised by abundant metabolites and active bacteria, which are great choices to be used as functional additives[29]. Therefore, the aim of the present study was to investigate the potential effects of supplementation of CBC in HCD on carbohydrate utilisation, antioxidant capacity and intestinal microbiota of largemouth bass.

Materials and methods

Experimental diets

Previous study in our laboratory has demonstrated that, compared with 5 % inclusion level, 10 % α-starch produced abnormal hepatic glycogen accumulation and further impaired hepatic function of largemouth bass[30]. Therefore, two isonitrogenous (crude protein content, 51 %) and isolipidic (crude lipid content, 11 %) diets formulated with 5 and 10 % α-starch were used as low-carbohydrate diet (LCD) and HCD (Table 1). Another three diets were supplemented with 0·25 % (HCP (0·25)), 0·50 % (HCP (0·50)) and 1·00 % (HCP (1·00)) CBC based on HCD (Table 1). The CBC used in present study was produced with liquid fermentation at 37°C for 20 h under anaerobic condition. The fermentation broth was centrifuged at 5000 × g for 10 min at 4°C, and the slurry was mixed with 1 % sterile water, then freeze-dried to obtain the bacterial powder. The cell quantity of bacterial powder was detected as $5{\cdot}0 \times 10^8$ colony-forming unit (CFU)/g using iron sulfite agar method at 37°C for 24 h under anaerobic condition[31]. Meanwhile, the quantity of *C. butyricum* cells in the experimental diets was examined with the same method as above, and the results were 0·0 CFU/g in LCD and HCD groups, $6{\cdot}2 \times 10^5$ CFU/g in HCP (0·25) group, $1{\cdot}3 \times 10^6$ CFU/g in HCP (0·50) group, and $2{\cdot}5 \times 10^6$ CFU/g in HCP (1·00) group. The experimental diets were prepared with reference to the procedure described previously[30] and then stored at -20°C after manufacture.

Experimental procedure

The present experiment strictly followed the requirement of Animal Care and Use Committee of Shanghai Ocean University and conducted in the constant temperature circulating water system of the joint laboratory of Shanghai Ocean University and Guangdong Evergreen Feed Co., Ltd. Juvenile largemouth bass sourced from our laboratory staff cultured from the larval stage (larval largemouth bass obtained from a commercial hatchery in Guangdong, China) to the specifications required for the present experiment. After that, juvenile largemouth bass (initial weight 35·03 ± 0·04 g) were randomly divided into fifteen reinforced plastic tanks (water volume of 1000 litres) with twenty-eight fish per tank, following anaesthesia with eugenol (1:10 000) (Shanghai Chemical Reagents Co., Ltd) and fed twice a day until apparent satiation using the experimental diet for 60 d, with each experimental diet being randomly assigned to three tanks. About 10 % of the water was replenished daily. All experimental tanks were under natural photoperiod, and water quality was monitored during the feeding trial: water temperature, 27 ± 1°C; pH, 7·2 ± 0·2; dissolved oxygen, 6–7 mg/l; ammonia nitrogen, < 0·05 mg/l.

Sample collection

Before sample collection, all experimental fish were fasted for 24 h, anesthetised with eugenol (1:10 000). The two individuals from each tank were randomly selected and stored at −20°C for body composition analysis. Then, twelve fish of each tank were randomly selected, after body weight and length measurement, blood sample was collected from tail vein, which was maintained at 4°C for 1 h, and then serum was separated by centrifugation at 3000 × g for 10 min at 4°C. Then, six of the above twelve fish were dissected foe viscerosomatic index (VSI) and hepatosomatic index (HSI) calculation, and muscle from lateral surface of fish above lateral line and whole liver were separated for biochemical composition analysis. Livers of the rest six fish were collected for gene expression analysis.

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Table 1. Formulation and proximate composition of experimental diets (% DM)

Ingredients	Experimental diets				
	LCD	HCD	HCP (0·25)	HCP (0·50)	HCP (1·00)
Fishmeal*	35·00	35·00	35·00	35·00	35·00
Fermented soyabean meal*	13·00	13·00	13·00	13·00	13·00
Maize gluten meal*	12·00	12·00	12·00	12·00	12·00
Blood meal*	4·00	4·00	4·00	4·00	4·00
Shrimp meal*	5·00	5·00	5·00	5·00	5·00
Squid viscera meal*	2·00	2·00	2·00	2·00	2·00
Brewer's yeast cultures*	2·00	2·00	2·00	2·00	2·00
C. butyricum cultures	0·00	0·00	0·25	0·50	1·00
Wheat gluten meal*	4·00	4·00	4·00	4·00	4·00
α-starch	5·00	10·00	10·00	10·00	10·00
Lecithin oil	2·00	2·00	2·00	2·00	2·00
Fish oil	2·00	2·00	2·00	2·00	2·00
Soyabean oil	3·00	3·00	3·00	3·00	3·00
Calcium biphosphate	1·00	1·00	1·00	1·00	1·00
Phytase	0·05	0·05	0·05	0·05	0·05
Cellulase	0·10	0·10	0·10	0·10	0·10
Vitamin mixture†	1·20	1·20	1·20	1·20	1·20
Mineral mixture‡	1·00	1·00	1·00	1·00	1·00
Zeolite powder	6·65	1·65	1·4	1·15	0·65
Proximate composition (% DM basis)					
Crude protein	51·23	51·55	51·53	51·51	51·28
Crude lipid	10·31	10·71	10·84	10·67	11·02
Ash	17·07	12·35	11·95	11·69	11·38

LCD, low-carbohydrate diet; HCD, high-carbohydrate diet.

* Supplied by Zhejiang Xinxin Tian'en Aquatic Feed Corporation (Jiaxing, China): fishmeal, crude protein, 71·84 %, crude lipid, 7·66 %; shrimp meal, crude protein, 61·72 %, crude lipid, 11·20 %; fermented soyabean meal, crude protein, 51·67 %, crude lipid, 2·62 %; maize gluten meal, crude protein, 60·41 %, crude lipid, 5·12 %; blood meal, crude protein, 87·06 %, crude lipid, 1·51 %; wheat gluten meal, crude protein, 80·05 %; crude lipid, 1·13 %; brewer's yeast, crude protein, 51·05 %, crude lipid, 0·52 %.

† Vitamin Premix (mg/kg diet): vitamin B_1, 17·80; vitamin A, 16 000 mg; vitamin B_2, 48; vitamin B_6, 29·52; vitamin B_{12}, 0·24; vitamin C, 800; vitamin D_3, 8000 mg; vitamin E, 160; vitamin K_3, 14·72; choline chloride, 1500; folic acid, 6·40; niacinamide, 79·20; inositol, 320; calcium pantothenate, 73·60; biotin, 0·64.

‡ Mineral Premix (mg/kg diet): I ($Ca(IO_3)_2$), 1·63; Mn ($MnSO_4$), 6·20; Cu ($CuSO_4$), 2·00; Fe ($FeSO_4$), 21·10; se (Na_2SeO_3), 0·18; Co ($CoCl_2$), 0·24; Zn ($ZnSO_4$), 34·40.

The intestines of twelve fish were dissected into hindgut and anterior midgut, with the hindgut used for gene expression analysis and the anterior midgut contents scraped for intestinal microbiota analysis.

Biochemical analysis

The proximate composition, including moisture, crude protein and crude lipid content of whole fish, muscle and liver, as well as ash content of whole fish were analysed. Moisture content was measured by drying the samples in an oven (BPG-9140 A, Shanghai Yiheng Scientific Instrument Co., Ltd) at 105°C to a constant weight, and ash content was determined by burning the samples in a muffle furnace (SX2, Shanghai Laboratory Instrument Works Co., Ltd) at 550°C to a constant weight[32]. Crude protein content was obtained with the Kjeldahl method ($n \times 6{\cdot}25$) by a Kjeldahl apparatus (Kjeltec 2200, Foss Analytical A/S)[32]. Crude lipid content was determined by chloroform-methanol extraction method[33]. Glycogen content was detected with the commercial assay kit provided by Nanjing Jiancheng Bioengineering Institute (A043-1-1) by the potassium hydroxide/anthrone method[34].

The liver samples were homogenised by a tissue homogeniser (Tiss-Basic2, Shanghai Jingxin Industrial Development Co., Ltd) with ice-cold phosphate buffer (1:9, w/v), which were then centrifuged (3500 × ***g***, 10 min, 4°C) to separate the supernatant for antioxidant capacity analysis with commercial assay kits provided by Nanjing Jiancheng Bioengineering Institute (A045-2-2, A007-1-1, A001-3-2, A003-1-1 and A015-2-1). The soluble protein content was assayed with the Coomassie brilliant blue method[35]. The malondialdehyde was measured with thiobarbituric acid-reactive substances method[36]. The activity of total antioxidative capacity was determined by 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^+$)-based methods[37]. The activity of superoxide dismutase (SOD) was detected using xanthine oxidase and the water-soluble tetrazolium (WST-1)[38]. The activity of catalase (CAT) was determined by measuring the variation in the concentration of hydrogen peroxide[39].

RNA extraction and real-time quantitative PCR

Total RNA of liver and hindgut was extracted, respectively, with RNAiso Plus. After RNA quality being tested, it was reverse-transcribed to first-strand cDNA, used for real-time quantification PCR, with the Prime Script™ RT reagent Kit. Real-time quantification PCR was conducted with quantitative thermal cycler (Mastercycler EP Realplex, Eppendorf) with the following procedures: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 57°C for 10 s, 72°C for 20 s and a melting curve analysis. The specific primers used for real-time quantification PCR are shown in Table 2. The relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method[40], and *β-actin* was used as the reference gene.

DNA extraction, amplification and sequencing of gut 16S rRNA genes

The present results showed that the dietary inclusion of 1·0 % CBC had a significant positive effect on carbohydrate utilisation and antioxidant capacity of largemouth bass. Therefore,

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Table 2. Primers used in the present study

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
nrf2	CACCAAAGACAAGCGTAAG	GAAATCATCAACAGGCAGA
keap1	AGACGGCAGGAGATGTTGT	CATGGCTCTGAAGTAGGGG
sod1	TTATTTTGAGCAGGAGGG	TTCTTGTTGTGGGGATTG
sod2	GGTCTCATTCCCCTTCTT	TCGCTCACATTCTCCCAG
cat	TGAATGGCTATGGCTCTC	AATCTGGGTTGGTGGAAG
ampkα1	CACATGAATGCCAAGATTG	GGACCAGCATATAACCTTC
ira	CCCTTGTATCCCTCTCGTTT	CCAATTTCCTGTTCCTCTCC
irb	TGTGTGTCGGAAATAAAACG	TCTGCGAACAGTCAGGTAGC
irs	TAGTGGTGGTGTCAGCGGT	GGAGGTGGAAGTAAAGGAT
pi3kr1	AAGACCTTCCTCATCACGAC	CCTTCCACTACAACACTGCA
akt1	CTTAATTTACCGCCGAAAC	CCTCCTTGACAATCACCAC
gk	GGGTTTTACCTTCTCCTTTC	GGTGGCTACTGTGTCATTCA
pfkl	CTGGCTGAGCTCGTAAAG	GTGCCGCAGAAGTCGTTG
pk	CTCTTTCATCCGCAAAGC	AATTCCCAGGTCACCACG
pepck	GGAAACGGCCAACATTCT	GCCAACCAGCAGTTCTCAT
fbp1	GCGATTGGCGAATTTATC	ACTCTGTGACGGCGGGTT
g6pc	AGAAAGCACAGAAGTGGTG	CTTGGTCTCGGTGTAGAGG
ffar3	ATGGCAGTGAGTGTGGTT	GTGTGCGGCTGAGATTAG
mct1	CATCTTCTTTGGTTTCGC	CCTCTTTCCTTTCTCGGT
β-actin	ATCGCCGCACTGGTTGTTGAC	CCTGTTGGCTTTGGGGTTC

nrf2, nuclear factor erythroid 2-related factor 2; *keap1*, Kelch-like ECH-associated protein 1; *sod1*, superoxide dismutase 1; *sod2*, superoxide dismutase 2; *cat*, catalase; *ampkα1*, AMP-activated protein kinase α 1; *ira*, insulin receptor a; *irb*, insulin receptor b; *irs*, insulin receptor substrate; *pi3kr1*, phosphatidylinositol-3-kinase p85 α; *akt1*, serine/threonine kinase 1; *gk*, glucokinase; *pfkl*, phosphofructokinase liver type; *pk*, pyruvate kinase; *pepck*, phosphoenolpyruvate carboxykinase; *fbp1*, fructose-1,6-bisphosphatase-1; *g6pc*, glucose-6-phosphatase catalytic subunit; *ffar3*, free fatty acid receptor 3; *mct1*, monocarboxylate transporter 1.

considering the logistic and cost limitations, microbiological analysis was performed only in HCD and HCP (1·00) (defined as HCP group in the intestinal microbiota analysis) groups. Bacterial DNA from HCD and HCP groups was extracted with the E.Z.N.A.® Soil DNA kit (Omega). DNA integrity and concentration were determined using 1% agarose gel electrophoresis and NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific), respectively. Then, amplification of the hypervariable region V3–V4 of the bacterial 16S rRNA gene was performed in a PCR thermal cycler, using the 338F/806R primer pair, with the following procedures: 95°C for 3 min, followed by 27 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 45 s, and 72°C for 10 min. After the PCR products were extracted and purified, the paired-end sequencing of the purified products was conducted using the Illumina MiSeq PE300 platform (Shanghai Bio-Pharm Technology Co. Ltd). The raw reads were stored in the NCBI Serial Read Archive (SRA) database with the accession number PRJNA962001.

Bioinformatics analysis

The raw 16S rRNA sequences were demultiplexed and quality-filtered using the Quantitative Insights into Microbial Ecology (QIIME) quality filters[41] and then were processed with FLASH software to merge them[42]. After that, operational taxonomic units according to the 97 % similarity threshold were clustered using UPARSE pipeline and chimeric sequences were removed using UCHIME[43]. The phylogenetic affiliation of each operational taxonomic unit representative 16S rRNA gene sequence was analysed by RDP Classifier based on the 16S rRNA database (Silva 136) according to a confidence threshold of 70 %[44]. The α-diversity analysis and β-diversity analysis were calculated with QIIME and displayed with R software, for evaluating changes in anterior midgut micro-organisms of largemouth bass with dietary supplementation of CBC. Largemouth bass anterior midgut bacterial diversity and operational taxonomic unit abundance in HCD and HCP groups were statistically analysed using Welch's *t* test, with $P < 0{\cdot}05$ as significant. Linear discriminant analysis effect size analysis was used to identify the different abundant taxa between the HCD and HCP groups[45].

Calculation and statistical analysis

The relevant variables were obtained using the following formula:

$$\text{Specific growth rate(SGR, \%/d)} = \text{Ln(final body weight/initial body weight)/experimental days} \times 100;$$

$$\text{Hepatosomatic index(HSI, \%)} = \text{liver weight/final body weight} \times 100;$$

$$\text{Viscerosomatic index(VSI,\%)} = \text{viscera weight/final body weight} \times 100;$$

$$\text{Conditionfactor(CF, g/cm}^3\text{)} = \text{final body weight(g)/length(cm)}^3 \times 100;$$

$$\text{Feed intake(FI, \% body weight/d)} = \text{dry feed consumed/((final body weight + initial body weight)/2)/experimental days;}$$

$$\text{Feed conversion ratio(FCR)} = \text{dry feed consumed/body weight increased;}$$

$$\text{Protein efficiency ratio(PER)} = \text{body weight increased/protein intake;}$$

All statistical assessments were performed after data normality and homoscedasticity tests with SPSS 19.0 for

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Windows. The data of the HCD treatments and the LCD group were analysed by Dunnett's test. All data from HCD treatments were statistically analysed by one-way ANOVA with Duncan's multiple range test on SPSS 19.0 for Windows. In addition, depending on the experimental design and to evaluate possible trends of experimental results, polynomial comparisons (linear and quadratic) were used to detect all the variables and parameters tested in HCD treatments. When linear and quadratic significance regressions were detected simultaneously, linearity, as the first priority, was chosen to describe the observed trend. In the present study, experimental results were presented as mean ± SEM, and the significance was set at $P < 0{\cdot}05$.

Results

Growth performance and feed utilisation

No mortality was observed in all experimental fish in this study. Compared with the LCD group, final body weight and SGR were significantly reduced in the HCD group; however, final body weight and SGR were linearly increased with inclusion of CPC in the HCD ($P < 0{\cdot}05$) (Table 3). In addition, HSI and VSI were significantly increased with increasing levels of carbohydrates ($P < 0{\cdot}05$) (Table 3). With the inclusion of CPC in the HCD, both HSI and VSI were linearly reduced, but VSI was consistently higher than the LCD group ($P < 0{\cdot}05$) (Table 3). However, no statistical difference was produced in the CF ($P > 0{\cdot}05$) (Table 3). The relevant parameters of feed utilisation, FCR and FI, in HCD treatments were observed to be significantly lower than in the LCD group, although FI showed a linear increase with the addition of CBC in the HCD ($P < 0{\cdot}05$) (Table 3). However, PER from all the HCD treatments was significantly higher than that of the LCD group ($P < 0{\cdot}05$).

Body composition analysis

The crude protein and ash contents of whole fish did not show statistical differences in all treatments ($P > 0{\cdot}05$) (Table 4). Compared with the LCD group, the moisture content of whole fish did not produce significant differences in HCD group ($P > 0{\cdot}05$), and the crude lipid content of whole fish in the HCD was significantly increased ($P < 0{\cdot}05$) (Table 4). The moisture content of whole fish increased in a quadratic manner with the addition of dietary CBC, while crude lipid content of whole fish linearly decreased ($P < 0{\cdot}05$) (Table 4). In addition, the moisture and crude protein content of liver and muscle tissues presented no significant differences among all treatments ($P > 0{\cdot}05$) (Table 4). The crude lipid content of liver was significantly decreased in HCD group compared with LCD treatment and increased quadratically with the dietary inclusion of CBC ($P < 0{\cdot}05$) (Table 4). Glycogen content of liver and crude lipid content of muscle had similar trends, and both were enhanced in HCD group compared with LCD group and showed the linear decrease with a dose-dependent manner in HCD treatments ($P < 0{\cdot}05$) (Table 4).

Table 3. Effects of dietary CBC on the growth performance and feed utilisation of largemouth bass

	Experimental diets										Regression (P, R^2)	
	LCD		HCD		HCP (0·25)		HCP (0·50)		HCP (1·00)			
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Linear	Quadratic
IBW (g)	34·70	0·23	35·02	0·01	35·06	0·01	34·94	0·03	35·11	0·14	NA	NA
FBW (g)	119·26	0·85	111·00*	0·48	113·91	1·36	114·58	2·03	115·91	1·66	0·041, 0·356	0·094, 0·409
SGR (%/d)	2·06	0·01	1·92*	0·01	1·96*	0·02	1·98	0·03	1·99	0·02	0·042, 0·353	0·076, 0·437
CF (g/cm^3)	2·02	0·01	2·05	0·02	2·07	0·02	2·06	0·01	2·07	0·01	0·269, 0·121	0·499, 0·143
HSI (%)	2·01	0·10	3·09^{*,a}	0·23	2·58^{a,b}	0·19	2·53^{b}	0·09	2·37^{b}	0·06	0·020, 0·431	0·030, 0·540
VSI (%)	6·43	0·27	7·44^{*,a}	0·08	7·45^{*,a}	0·11	7·18^{*,b}	0·04	7·15^{*,b}	0·03	0·010, 0·498	0·038, 0·518
FCR	0·82	0·01	0·79*	0·01	0·79*	0·01	0·80*	0·01	0·79*	0·01	0·482, 0·051	0·774, 0·055
PER	2·38	0·01	2·45*	0·02	2·46*	0·01	2·44*	0·01	2·45*	0·01	0·982, <0·001	0·888, 0·026
FI (%BW/d)	1·50	0·02	1·37*	0·01	1·39*	0·01	1·41*	0·02	1·42*	0·01	0·048, 0·337	0·094, 0·408

CBC, *Clostridium butyricum* cultures; LCD, low-carbohydrate diet; HCD, high-carbohydrate diet; NA, not applicable; IBW, initial body weight; FBW, final body weight; SGR, specific growth rate; CF, condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index; FCR, feed conversion ratio; PER, protein efficiency ratio; FI, feed intake.
Dunnett's test was conducted for comparing LCD and other dietary groups, and the significantly different values (n 3) within a row were marked '*' at the superscript ($P < 0{\cdot}05$). Duncan's multiple range test was performed in all high-carbohydrate diet groups, and values within a row with a common superscript letter are not significantly different from the other dietary groups ($P > 0{\cdot}05$) but with a different superscript letter are significantly different from the other dietary groups ($P < 0{\cdot}05$).

Table 4. Effect of dietary CBC on the body composition (% wet weight) of largemouth bass

	Experimental diets									Regression (P, R^2)	
	LCD		HCD		HCP (0·25)		HCP (0·50)		HCP (1·00)		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	Linear	Quadratic
Whole fish											
Moisture	71·46	0·43	71·31^{c}	0·12	72·36^{b,c}	0·04	73·72^{*,a}	0·28	72·73^{a,b}	0·082, 0·272	0·003, 0·726
Crude protein	17·67	0·18	17·03	0·20	17·02	0·02	17·36	0·26	17·25	0·270, 0·120	0·456, 0·160
Crude lipid	4·58	0·09	5·39^{*,a}	0·10	4·75^{b}	0·08	3·75^{*,d}	0·11	4·22^{c}	0·013, 0·478	<0·001, 0·871
Ash	3·90	0·10	4·10	0·13	3·87	0·08	3·75	0·06	3·83	0·225, 0·143	0·184, 0·314
Liver											
Moisture	73·64	0·67	75·69	0·73	75·26	0·38	74·00	0·41	73·87	0·064, 0·303	0·154, 0·340
Crude protein	13·28	0·18	12·80	0·61	13·30	0·73	12·07	1·10	13·05	0·992, 0·000	0·890, 0·025
Crude lipid	2·91	0·09	2·42^{*,b}	0·07	2·60^{*,a,b}	0·04	2·68^{a}	0·07	2·65^{a}	0·053, 0·326	0·026, 0·556
Glycogen	8·87	0·43	11·49^{*,a}	0·28	10·23^{a,b}	0·52	9·50^{b}	0·57	9·16^{b}	0·006, 0·552	0·007, 0·670
Muscle											
Moisture	77·51	0·26	77·62	0·24	78·20	0·38	78·34	0·19	78·22	0·167, 0·182	0·094, 0·408
Crude protein	19·88	0·12	19·44	0·10	19·51	0·13	19·43	0·13	19·28	0·211, 0·152	0·366, 0·200
Crude lipid	1·23	0·06	2·04^{*,a}	0·07	1·62^{b}	0·17	1·04^{c}	0·08	1·10^{c}	0·002, 0·623	<0·001, 0·833

CBC, *Clostridium butyricum* cultures; LCD, low-carbohydrate diet; HCD, high-carbohydrate diet.
Dunnett's test was conducted for comparing LCD and other dietary groups, and the significantly different values (n 3) within a row were marked '*' at the superscript ($P < 0·05$). Duncan's multiple range test was performed in all high-carbohydrate diet groups, and values within a row with a common superscript letter are not significantly different from the other dietary groups ($P > 0·05$) but with a different superscript letter are significantly different from the other dietary groups ($P < 0·05$).

Expression of hepatic ampkα1 and insulin pathway-related genes

The relative expression of AMP-activated protein kinase α 1 (*ampkα1*) increased linearly in a dose-dependent manner, but the expression was dramatically decreased in the HCD and HCP (0·25) groups compared with the LCD group ($P < 0·05$) (Fig. 1(a)). The results of insulin receptor a (*ira*) and insulin receptor b (*irb*) expression were observed similar to *ampkα1*, both showing a linear increase in the HCD treatments, and the HCD and HCP (0·25) groups remained statistically different with the LCD group ($P < 0·05$) (Fig. 1(b–c)). Meanwhile, CBC inclusion led to a linear increase of insulin receptor substrate (*irs*) and phosphatidylinositol-3-kinase p85 α (*pi3kr1*) expression in HCD treatments ($P < 0·05$) (Fig. 1(d–e)). The results of comparison with the LCD group indicated that the expression of *irs* in the HCD group and *pi3kr1* in the HCD and HCP (0·25) groups was significantly reduced ($P < 0·05$) (Fig. 1(d–e)). In addition, the variation of serine/threonine kinase 1 (*akt1*) expression was also consistent with a linear increase; however, the expression in all HCD treatments was still significantly lower than in the LCD group ($P < 0·05$) (Fig. 1(f)).

Expression of hepatic glucose metabolism-related genes

The inclusion of CBC in HCD did not significantly influence the relative expression of glucokinase (*gk*), and no significant difference was found between the LCD group and HCP (1·00) group ($P > 0·05$) (Fig. 2(a)). Meanwhile, the relative expression of phosphofructokinase liver type (*pfkl*) reached its peak in a linear manner with dietary inclusion of CBC, while the expression in the HCD group became significantly lower as compared with the LCD group ($P < 0·05$) (Fig. 2(b)). In addition, the relative expression of pyruvate kinase (*pk*) showed the similar linear increase in the HCD treatments ($P < 0·05$) and did not produce a statistical difference with the LCD group ($P > 0·05$) (Fig. 2(c)).

The relative expression of fructose-1,6-bisphosphatase-1 (*fbp1*), glucose-6-phosphatase catalytic subunit (*g6pc*) and phosphoenolpyruvate carboxykinase (*pepck*) did not produce significant differences between the HCD and LCD groups ($P > 0·05$) (Fig. 2(d–f)). With the inclusion of CBC in HCD, the expression of *fbp1* presented a linear increase ($P < 0·05$), while the expression of *g6pc* and *pepck* did not produce statistical differences ($P > 0·05$) (Fig. 2(d–f)).

Expression of intestinal SCFA transport-related genes

The relative expression of intestinal free fatty acid receptor 3 (*ffar3*) in largemouth bass was linearly elevated in the HCD treatments, and the expression was significantly reduced in the HCD group compared with the LCD group ($P < 0·05$) (Fig. 3(a)). Meanwhile, with the dietary addition of CBC, a quadratic rise in the relative expression of monocarboxylate transporter 1 (*mct1*) appeared in the largemouth bass gut ($P < 0·05$), but all HCD treatments did not produce significant differences with the LCD group ($P > 0·05$) (Fig. 3(b)).

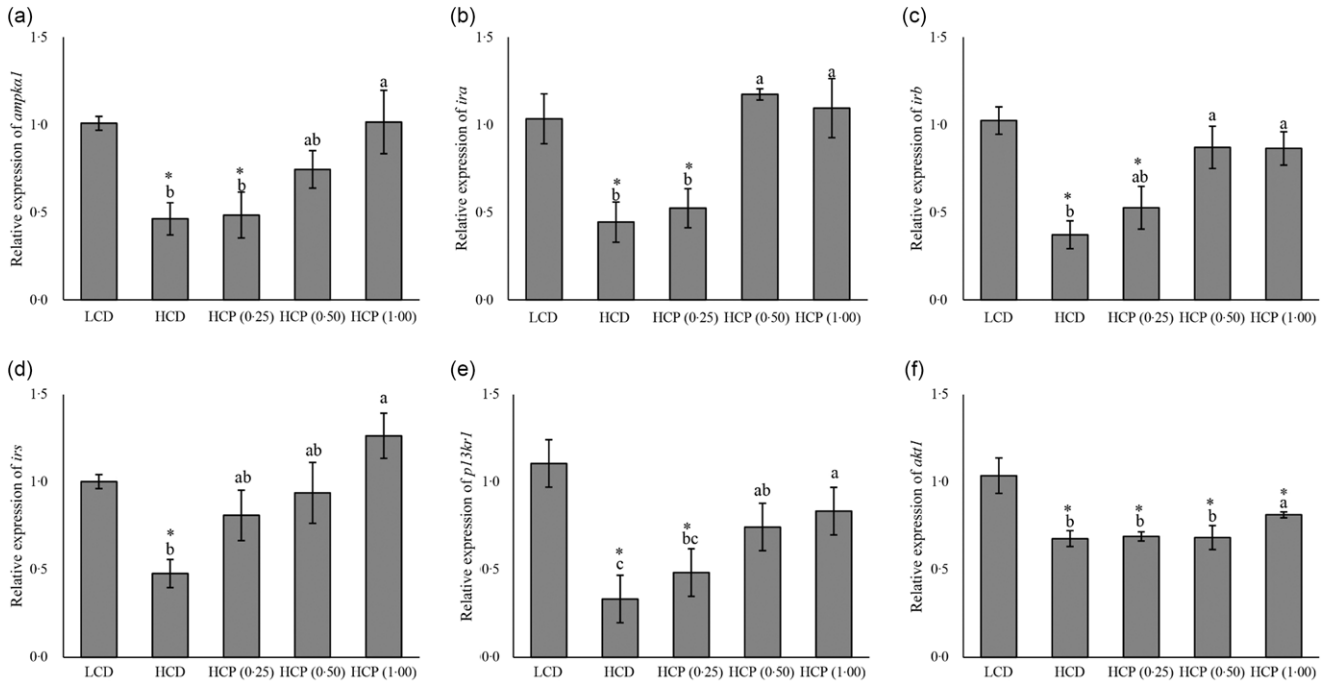

Fig. 1. Relative expression of AMP-activated protein kinase α 1 (*ampkα1*) (a) and insulin signalling pathway-related genes, insulin receptor a (*ira*) (b), insulin receptor b (*irb*) (c), insulin receptor substrate (*irs*) (d), phosphatidylinositol-3-kinase p85 α (*pi3kr1*) (e) and serine/threonine kinase 1 (*akt1*) (f), in liver of largemouth bass fed the experimental diets for 60 d. Dunnett's test was conducted for comparing LCD and other dietary groups, and the significantly different values (mean ± SEM, *n* 3) were marked '*' at the superscript ($P < 0{\cdot}05$). Duncan's multiple range test was performed in all high-carbohydrate diet groups, and values with a different superscript letter are significantly different from the other dietary groups ($P < 0{\cdot}05$). Polynomial comparison results: *ampkα1*, $P_{linear} = 0{\cdot}005$, *R*2 linear = 0·284; *ira*, $P_{linear} = 0{\cdot}007$, *R*2 linear = 0·534; *irb*, $P_{linear} = 0{\cdot}008$, *R*2 linear = 0·677; *irs*, $P_{linear} = 0{\cdot}001$, *R*2 linear = 0·656; *pi3kr1*, $P_{linear} = 0{\cdot}001$, *R*2 linear = 0·675; *akt1*, $P_{linear} = 0{\cdot}010$, *R*2 linear = 0·497. LCD, low-carbohydrate diet; HCD, high-carbohydrate diet.

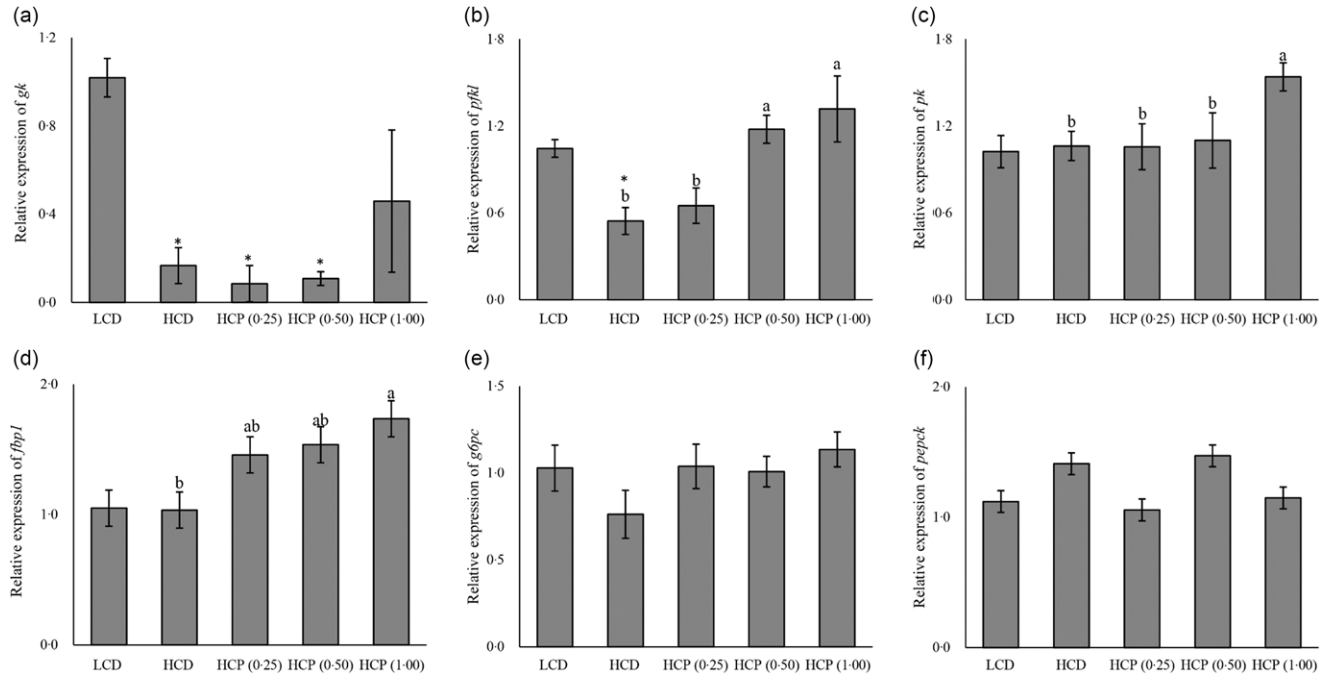

Fig. 2. Relative expression of glycolysis, glucokinase (*gk*) (a), phosphofructokinase liver type (*pfkl*) (b) and pyruvate kinase (*pk*) (c), and gluconeogenesis, fructose-1,6-bisphosphatase-1 (*fbp1*) (d), glucose-6-phosphatase catalytic subunit (*g6pc*) (e) and phosphoenolpyruvate carboxykinase (*pepck*) (f), in liver of largemouth bass fed the experimental diets for 60 d. Dunnett's test was conducted for comparing LCD and other dietary groups, and the significantly different values (mean ± SEM, *n* 3) were marked '*' at the superscript ($P < 0{\cdot}05$). Duncan's multiple range test was performed in all high carbohydrate diet groups, and values with a different superscript letter are significantly different from the other dietary groups ($P < 0{\cdot}05$). Polynomial comparison results: *pfkl*, $P_{linear} = 0{\cdot}002$, *R*2 linear = 0·623; *pk*, $P_{linear} = 0{\cdot}019$, *R*2 linear = 0·438; *fbp1*, $P_{linear} = 0{\cdot}033$, *R*2 linear = 0·379. LCD, low-carbohydrate diet; HCD, high-carbohydrate diet.

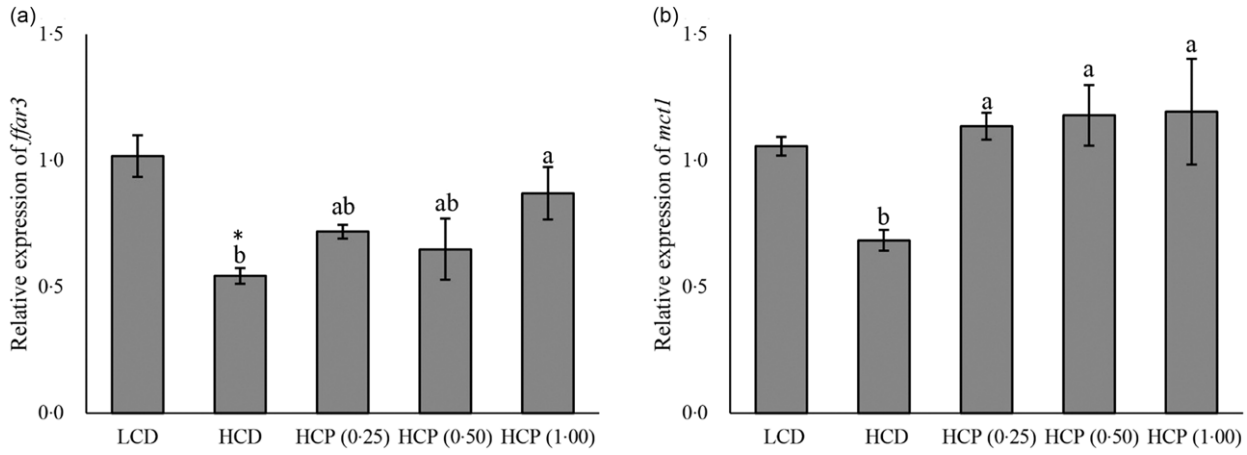

Fig. 3. Relative expression of the SCFA transport-related genes, free fatty acid receptor 3 (*ffar3*) (a) and monocarboxylate transporter 1 (*mct1*) (b), in gut of largemouth bass fed the experimental diets for 60 d. Dunnett's test was conducted for comparing LCD and other dietary groups, and the significantly different values (mean ± SEM, *n* 3) were marked '*' at the superscript ($P < 0·05$). Duncan's multiple range test was performed in all high-carbohydrate diet groups, and values with a different superscript letter are significantly different from the other dietary groups ($P < 0·05$). Polynomial comparison results: *ffar3*, $P_{linear} = 0·024$, *R*2 linear = 0·412; *mct1*, $P_{quadratic} = 0·029$, *R*2 quadratic = 0·546. LCD, low-carbohydrate diet; HCD, high-carbohydrate diet.

Hepatic antioxidant capacity

Largemouth bass hepatic malondialdehyde concentration was significantly higher in the HCD group than LCD group and declined in a linear manner with the addition of CBC ($P < 0·05$) (Table 5). Statistical analysis with the LCD group indicated that antioxidant enzyme activities, including CAT and total superoxide dismutase, were significantly lower in the HCD group ($P < 0·05$) (Table 5). Unlike change in malondialdehyde, both CAT and total superoxide dismutase activities showed a linear increase in all HCD treatments ($P < 0·05$) (Table 5). The variation of total antioxidant capacity was similar to that of antioxidant enzyme activities, presenting lower in HCD group than LCD group and a linear increase with inclusion of CBC in the HCD ($P < 0·05$) (Table 5).

At the transcriptional level, variations of hepatic antioxidant enzyme-related gene expression corresponded to antioxidant enzyme activities, with the relative expression of catalase (*cat*), superoxide dismutase 1 (*sod1*) and superoxide dismutase 2 (*sod2*) being increased linearly among the HCD treatments ($P < 0·05$) (Fig. 4(c–e)). Compared with the LCD group, the relative expressions of *cat* and *sod1* were significantly lower in the HCD and HCP (0·25) groups, while the relative expression of SOD2 still remained remarkably lower than the LCD group with increasing dose of CBC ($P < 0·05$) (Fig. 4(c–e)). Meanwhile, the relative expression of nuclear factor erythroid 2-related factor 2 (*nrf2*) displayed a linear increase in all HCD treatments ($P < 0·05$), but there was no statistical difference between all HCD treatments and the LCD group ($P > 0·05$) (Fig. 4(a)). However, the relative expression of *keap1* decreased linearly with the inclusion of CBC and the expression in the HCD group was significantly higher than that in the LCD group ($P < 0·05$) (Fig. 4(b)).

Microbiota composition and diversity analysis

Alpha diversity analysis revealed that the Chao index, Ace index and Simpson index of intestinal microbiota in juvenile largemouth bass from the HCP group were slightly lower compared with the HCD group, while the Shannon index was slightly higher ($P > 0·05$) (Fig. 5). The results of β-diversity comparisons analysis, including principal coordinate analysis, non-metric multidimensional scaling and hierarchical clustering tree, demonstrated that the intestinal microbiota of largemouth bass in HCP group differed from the HCD group (Fig. 6).

The bar charts showed the dominant intestinal bacterial communities at the phylum and genus levels for the two groups, respectively (Fig. 7). The *Firmicute*, *Fusobacteriota* and *Proteobacteria* were the dominant phylum in HCD and HCP groups (Fig. 7). Meanwhile, the results of community analysis at the genus level revealed that the *Mycoplasma*, *Cetobacterium* and *Achromobacter* were the predominant microbes (Fig. 7). Welch's *t* test showed that the dietary supplementation of CBC significantly increased the relative abundance of *Fusobacteriota* and *Cetobacterium* in the gut of largemouth bass fed the HCD and significantly decreased the relative abundance of *Firmicute* and *Mycoplasma* (Fig. 7).

Venn diagram analysis showed that a total of ten common phyla were detected in the HCD and HCP groups, while five and two unique bacterial phyla were identified in the HCD and HCP groups, respectively (Fig. 8). At the genus level, a total of fifty-two shared genera were identified in the HCD and HCP groups, and 100 and twenty-one unique genera were found in the HCD and HCP groups, respectively (Fig. 8). The relatively abundant genera (>5 %) among the shared genera were *Mycoplasma* (52·17 %), *Cetobacterium* (33·06 %) and *Achromobacter* (12·76 %) (Fig. 8). The unique intestinal genera (>5 %) of largemouth bass in the HCD group were *Exiguobacterium* (5·79 %); meanwhile, the unique intestinal genera (>5 %) in the HCP group were *Geodermatophilus* (24·26 %), *Parviterribacter* (22·06 %), *Blautia* (6·62 %), *Microvirga* (6·62 %), *Eubacterium* (5·88 %) and *Deinococcus* (5·15 %) (Fig. 8).

The results of largemouth bass gut microbial community data analysed by linear discriminant analysis effect size from domain

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Table 5. Effect of dietary CBC on the hepatic antioxidant enzyme activities of largemouth bass

	Experimental diets								Regression (P, R^2)	
	LCD		HCD		HCP (0·25)		HCP (0·50)		HCP (1·00)		Linear	Quadratic
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM		
MDA (nmol/mgprot)	4·16	0·51	15·02^{*,a}	1·03	8·83^{*,b}	0·81	4·54^{c}	0·10	4·32^{c}	0·32	0·001, 0·702	<0·001, 0·951
T-AOC (mM)	0·11	0·01	0·07^{*,b}	0·01	0·08^{*,b}	0·01	0·11^{a}	0·01	0·11^{a}	0·01	0·002, 0·635	0·003, 0·726
CAT (U/mgprot)	25·15	1·25	16·41^{*,c}	0·52	21·50^{b,c}	1·68	26·00^{a,b}	0·81	28·09^{a}	2·77	0·001, 0·683	0·001, 0·777
T-SOD (U/mgprot)	410·05	8·90	363·42^{*,b}	13·36	383·67^{b}	10·97	403·99^{a,b}	8·51	411·99^{a}	6·84	0·006, 0·500	0·012, 0·626

CBC, *Clostridium butyricum* cultures; LCD, low-carbohydrate diet; HCD, high-carbohydrate diet; MDA, malondialdehyde; T-AOC, total antioxidant capacity; CAT, catalase; T-SOD, total superoxide dismutase.
Dunnett's test was conducted for comparing LCD and other dietary groups, and the significantly different values (n 3) within a row were marked '*' at the superscript ($P < 0·05$). Duncan's multiple range test was performed in all high-carbohydrate diet groups, and values within a row with a common superscript letter are not significantly different from the other dietary groups ($P > 0·05$) but with a different superscript letter are significantly different from the other dietary groups ($P < 0·05$).

to genus level revealed significant difference in the taxonomic distribution of gut microbial communities between HCD and HCP groups (Fig. 9). The gut of largemouth bass fed the diet with CBC exhibited significant enrichment for genus *Cetobacterium* and *Fusobacteriaceae* (from phylum to family) (Fig. 9). In addition, the HCD group had a higher relative bacterial abundance in gut of largemouth bass, including phylum *Firmicutes*, class *Bacilli*, *Mycoplasma* (from order to genus) (Fig. 9).

Discussion

C. butyricum has the properties of high temperature resistance and fermentation of carbohydrates to produce SCFA, and it is a selectable functional additive for aquafeeds at present[11]. In mammals, *C. butyricum* has been demonstrated to prevent and treat hyperglycemia and associated metabolic dysfunction[46]. Based on the beneficial role of SCFA in regulating energy metabolism[16] and the limited glucose utilisation capacity of largemouth bass[47], the present experiment was conducted to investigate the effect of CBC in largemouth bass fed HCD. Past studies have shown that the effect of *C. butyricum* on growth performance may be related to the level of dietary supplementation in teleosts[48–50]. In the present study, largemouth bass final body weight and SGR produced a linear increase with the inclusion of CBC in HCD, but dietary CBC inclusion did not product statistical effect compared with the HCD group, which may be related to the dose of supplementation. In addition, the elevated HSI and VSI caused by the HCD were alleviated with the addition of CBC. The change in HSI and VSI might indicate that liver problems due to excessive accumulation of hepatic glycogen, such as impaired liver function[51], were partly repaired. A former study has been shown that higher carbohydrate levels lead to alterations in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) feed utilisation, as evidenced by lower FI and FCR and higher PER[8]. Meanwhile, the reduction of FI in high carbohydrate levels was also found in largemouth bass[31]. Feed utilisation variations in carnivorous fish associated with HCD were suggested to be caused by excessive total energy intake and therefore improved protein-sparing effect of dietary carbohydrate[52,53]. The results of the present study showed that HCD with the inclusion of CBC did not have a substantial effect on feed utilisation of largemouth bass, and the influence of different carbohydrate content on feed utilisation was similar to those described above.

In general, excessive accumulation of hepatic glycogen is one of the characteristics of carnivorous fish with diabetes-like features fed HCD[7,54,55]. In the present study, hepatic glycogen content of largemouth bass was significantly elevated in the HCD group, while overaccumulation of glycogen in largemouth bass liver was alleviated with dietary supplementation of CBC. The modulatory effect of *C. butyricum* on fish hepatic glycogen at high dietary carbohydrate levels has not been reported yet, but its metabolite butyric acid has been shown to reduce blood glucose level in type 2 diabetic mice[46]. Therefore, the insulin signalling pathway and AMPK were analysed at the transcription

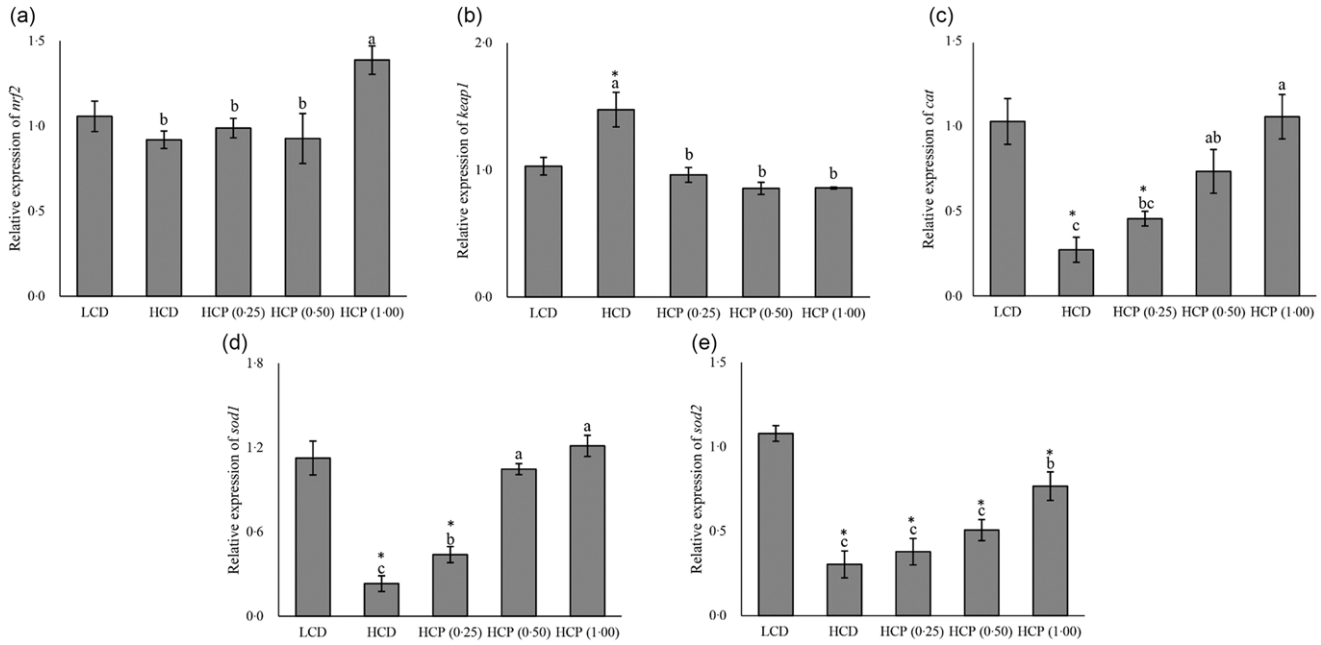

Fig. 4. Relative expression of Nrf2/Keap1 signalling pathway-related genes, nuclear factor erythroid 2-related factor 2 (*nrf2*) (a), Kelch-like ECH-associated protein 1 (*keap1*) (b), catalase (*cat*) (c), superoxide dismutase 1(*sod1*) (d) and superoxide dismutase 2 (*sod2*) (e), in liver of largemouth bass fed the experimental diets for 60 d. Dunnett's test was conducted for comparing LCD and other dietary groups, and the significantly different values (mean ± SEM, *n* 3) were marked '*' at the superscript ($P < 0{\cdot}05$). Duncan's multiple range test was performed in all high-carbohydrate diet groups, and values with a different superscript letter are significantly different from the other dietary groups ($P < 0{\cdot}05$). Polynomial comparison results: *nrf2*, $P_{linear} = 0{\cdot}008$, *R*2 linear = 0·526; *keap1*, $P_{linear} = 0{\cdot}011$, *R*2 linear = 0·491; *cat*, $P_{linear} < 0{\cdot}001$, *R*2 linear = 0·801; *sod1*, $P_{linear} < 0{\cdot}001$, *R*2 linear = 0·838; *sod2*, $P_{linear} < 0{\cdot}001$, *R*2 linear = 0·717. LCD, low-carbohydrate diet; HCD, high-carbohydrate diet.

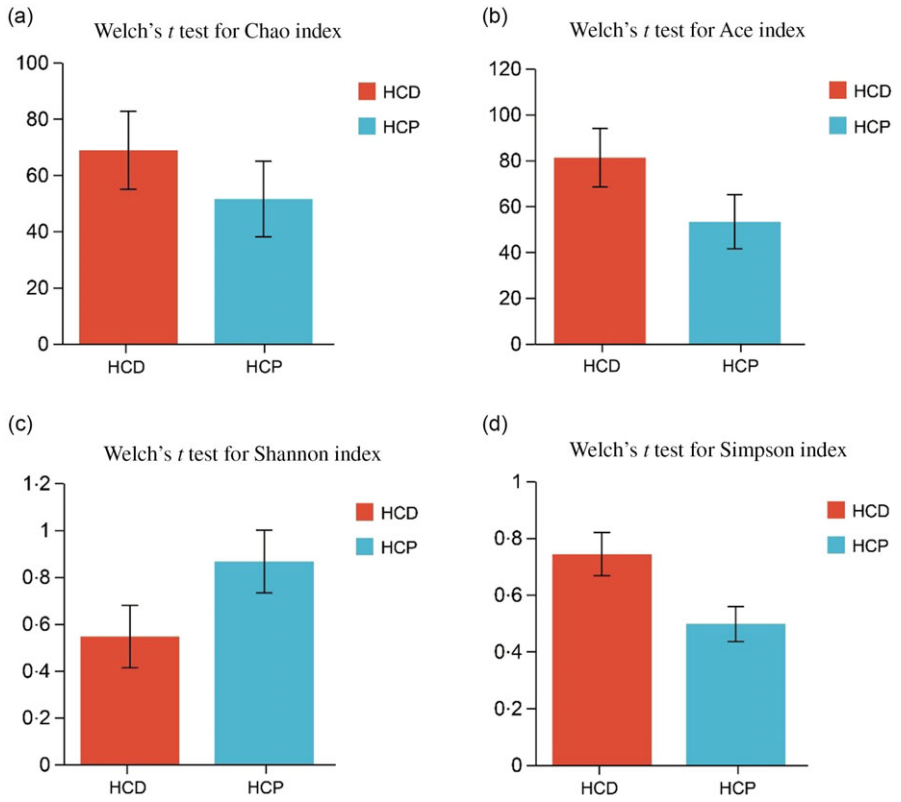

Fig. 5. The α-diversity comparisons analysis, including Chao species richness index (a), Ace species richness index (b), Shannon diversity index (c) and Simpson diversity index (d) of microbial communities in the gut of largemouth bass between the HCD and HCP groups. Values (mean ± SEM) in bars that have no asterisks are not significantly different ($P > 0{\cdot}05$; Welch's *t* test) between HCD and HCP groups (*n* 3). HCD, high-carbohydrate diet.

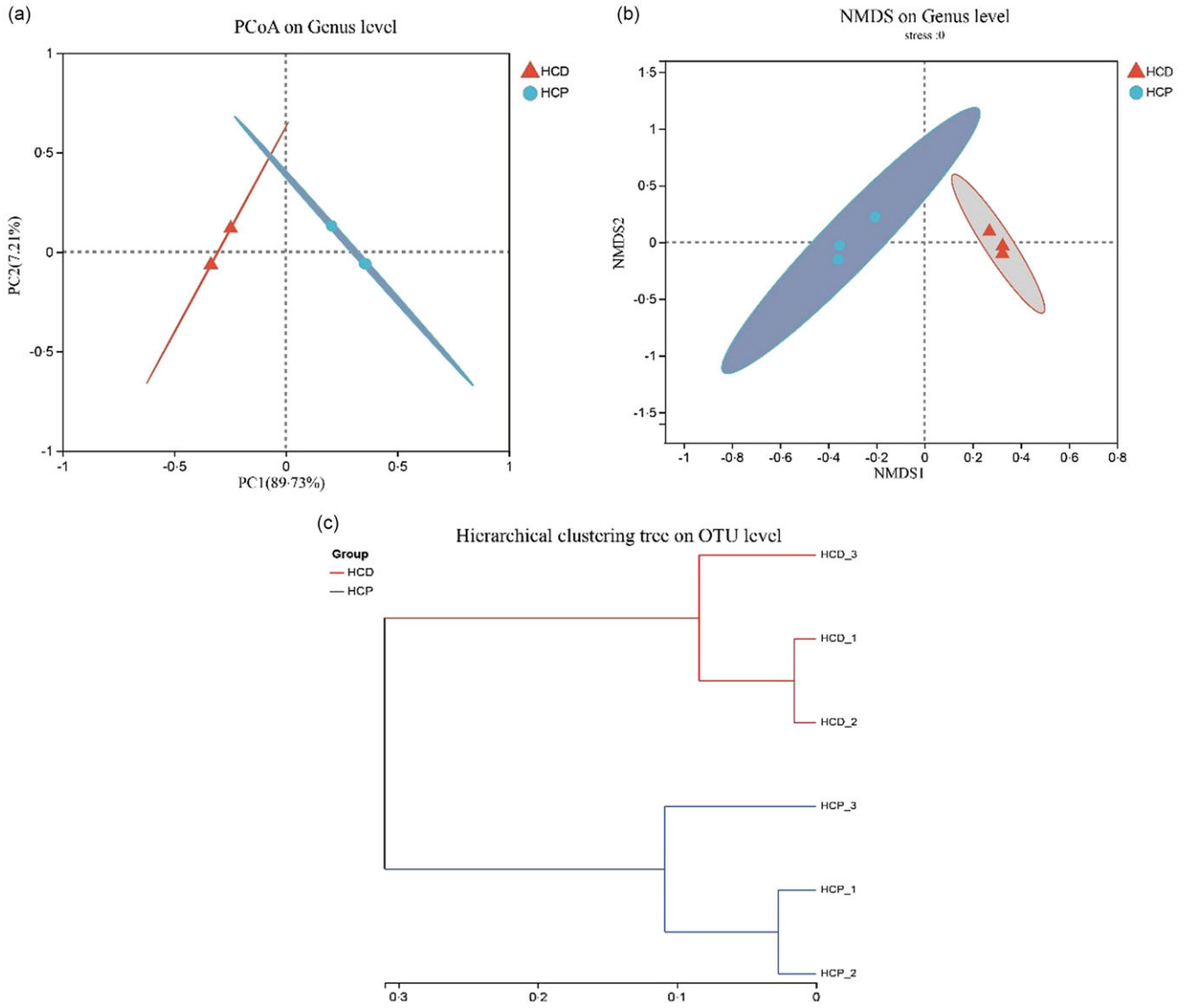

Fig. 6. The β-diversity comparisons analysis, including principal component analysis (PCoA) (a), non-metric multidimensional scaling (NMDS) (b) and hierarchical clustering tree (c) of microbial communities at genus level in the gut of largemouth bass between the HCD and HCP groups. HCD, high-carbohydrate diet. OUT, operational taxonomic units.

level in order to further validate the change in carbohydrate utilisation of largemouth bass after feeding CBC.

The insulin signalling pathway is a key pathway for maintaining glucose homoeostasis through regulating glucose metabolism[21,56]. However, the expression of insulin signalling pathway-related genes are generally inhibited in carnivorous fish fed HCD[57–61]. In the present study, the HCD also inhibited insulin signalling; however, the addition of CBC improved this situation as evidenced by increased expression of *ira*, *irb* and *irs*. This is similar to the results found in butyrate-related studies of chickens[62] and rats[63]. Activation of *ir* and *irs* usually triggers the downstream stimulation of PI3K/AKT1 pathway and thus promotes the role of insulin for regulating metabolism[21]. In the present study, the expression of *pi3kr1* and *akt1* increased linearly with the inclusion of CBC in HCD, which is consistent with the variation tendency of insulin pathway-related genes[64]. In addition, the previous studies have shown that AMPK is always involved in the regulation of glucose metabolism[65], and the phosphorylation and activation of insulin receptor are also associated with AMPK[66]. In the present study, the expression of *ampkα1* of largemouth bass liver was significantly elevated with the inclusion of CBC in HCD, and this result was similar to that in studies of SCFA[67]. The above results demonstrate the activation of insulin signalling pathway and AMPK by CBC in largemouth bass fed HCD.

In general, activation of the insulin signalling pathway and AMPK improves glucose metabolism by promoting glycolysis and inhibiting gluconeogenesis[66,68]. In present study, the inclusion of CBC in HCD significantly enhanced the expression of glycolysis-related genes, such as *pfkl* and *pk*. Similar results were reported from the study correlated with sodium butyrate in type 2 diabetic mellitus[69]. However, in the present study, largemouth bass gluconeogenesis was not significantly inhibited with inclusion of CBC, which has been reported in other

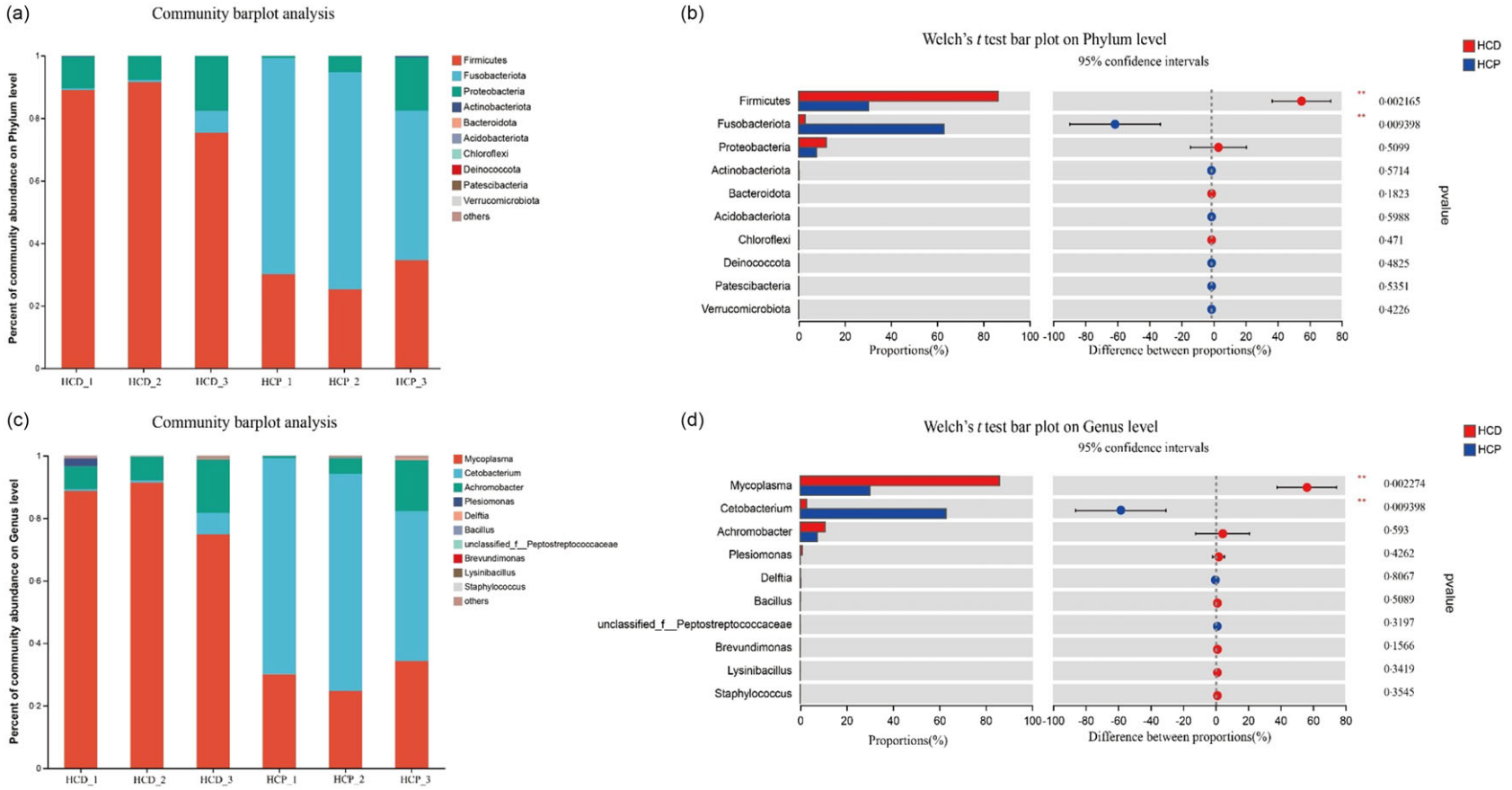

Fig. 7. Relative abundances (%) of intestinal bacteria and comparison of bacterial abundances in the gut of largemouth bass from HCD and HCP groups at the phylum (a, b) and genus (c, d) level, and the phyla and genera with relative abundances lower than 1 % were assigned as 'others'. *0·01 < $P \leq 0{\cdot}05$, **0·001 < $P \leq 0{\cdot}01$ (Welch's *t* test, *n* 3). HCD, high-carbohydrate diet.

Fig. 8. Venn diagram analysis of microbial communities in the gut of largemouth bass between the HCD and HCP groups. The number of microbial communities (a) and overlapping (b) and unique (c, d) bacterial species at phylum level in the gut of largemouth bass were identified (Fig. 8). The number of microbial communities (a) and overlapping (b) and unique (c, d) bacterial species at genus level in the gut of largemouth bass were identified (Fig. 8). HCD, high-carbohydrate diet.

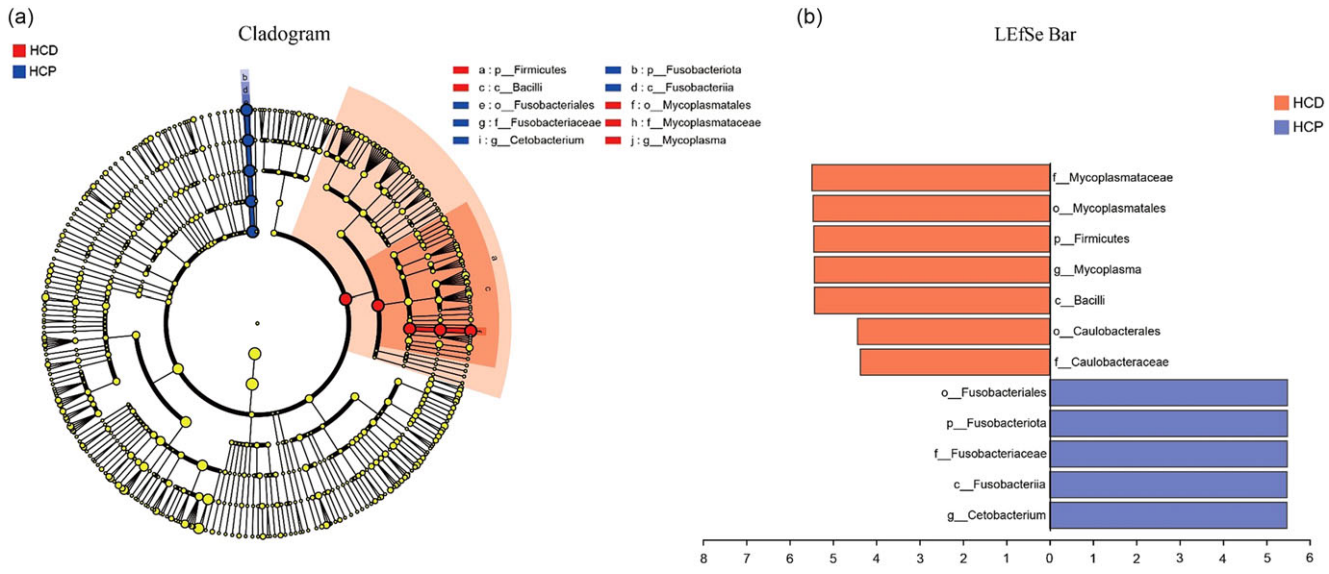

Fig. 9. Cladogram showing the phylogenetic distribution of the bacterial lineages associated with inclusion of CBC in HCD. Taxonomic representation of statistically and biologically consistent differences among intestinal microbiota of largemouth bass between the HCD and HCP groups (a). Differences were represented by the colour for the most abundant class (red indicates HCD group and blue indicates HCP group). Histogram of linear discriminant analysis (LDA) scores for differentially abundant taxon (b). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article. CBC, *Clostridium butyricum* cultures; HCD, high-carbohydrate diet.

largemouth bass studies[9,31]. The potential mechanism for the regulation of gluconeogenesis by CBC in largemouth bass required further investigated. In summary, the activation of glycolysis partially explains dietary CBC inclusion alleviates the hepatic glycogen accumulation of largemouth bass induced by HCD.

A previous study showed that butyric could promote the phosphorylation of AMPK and improve insulin sensitivity in mammals[20,70]. It may imply that the metabolites of *C. butyricum* are the main reason for the above changes. At present, many researches associated with *C. butyricum* on aquaculture have clearly demonstrated that dietary *C. butyricum* inclusion would increase the content of intestinal SCFA in various fish and crustacean[14,49,50,71]. However, there are fewer studies targeting SCFA transport-related genes in *C. butyricum* research on aquatic animals, which is probably a key factor for improving carbohydrate utilisation of largemouth bass in the present study[16]. The *ffar3* and *mct1* are the main vehicles for propionic and butyric acids to enter cells, and *ffar3* is also an effective target for the treatment of type 2 diabetes[72–74]. In the present study, largemouth bass intestinal *ffar3* and *mct1* expression increased linearly in the HCD treatments, which probably indicates that SCFA, especially propionic acid and butyric acid, entered more into the largemouth bass cells with dietary inclusion of CBC. Meanwhile, this is also the potential reason for the improved carbohydrate utilisation of largemouth bass in the present study.

HCD usually cause damage to the antioxidant capacity in largemouth bass[31,75]. It has been reported the beneficial effect of *C. butyricum* on improving antioxidant capacity in mammals[76], fish[48] and shrimps[77]. In the present study, lipid peroxidation products malondialdehyde and total antioxidative capacity showed a linear decrease and increase with inclusion of CBC, respectively, which partly indicates greater antioxidant enzyme activities and reduced reactive oxygen species. Meanwhile, antioxidant enzyme activities, including CAT and total superoxide dismutase, also increased linearly with the inclusion of CBC in the HCD. The above changes were similar to the results in tilapia[48]. In addition, the expression of antioxidant enzyme-related genes, including *cat*, *sod1* and *sod2*, followed similar trends as enzyme activity levels, showing increased linearly in the HCD treatments. The results of enzyme activity and transcript levels partly demonstrate that the inclusion of CBC has a promotive impact on the antioxidant capacity of largemouth bass with HCD treatments. In mammals, the expression of antioxidant enzyme-related genes was identified to be regulated by the Nrf2/Keap1 signalling pathway[78,79]. In the present study, the expression of *nrf2* and *keap1* showed the linear increase and decrease with the dietary inclusion of CBC, respectively. Activation of antioxidant enzyme-related genes through similar variations has also been observed in zebrafish (*Danio rerio*)[80], blunt snout bream (*Megalobrama amblycephala*)[81] and common carp[82]. Therefore, the beneficial role of CBC in the antioxidant capacity of juvenile largemouth bass fed HCD is suggested to be mediated by Nrf2/Keap1signalling pathway.

The formation and establishment of fish intestinal microbiota is a complex process, influenced by feed and water environment[83,84]. In the present study, *Firmicutes*, *Fusobacteria* and *Proteobacteria* were the dominant phyla in the intestine of largemouth bass. It was partly consistent with the results of another high-carbohydrate-related study in largemouth bass, with the dominant phylum *Fusobacteria*, *Tenericutes*, *Firmicutes* and *Proteobacteria*[9]. Past studies in teleosts have identified HCD commonly causing dysbiosis of intestinal

microbiota[25,85]. In general, the inclusion of probiotics in diets would change the diversity or abundance of intestinal microbiota in fish species[13,86,87]. In the present study, the inclusion of CBC significantly reduced the relative abundance of the *Firmicutes* in the intestine of largemouth bass following HCD. Past studies observed the potential relationship between the *Firmicutes* and type 2 diabetes with higher abundance in type 2 diabetics[88,89]. Meanwhile, the abundance of *Firmicutes* was found to have a positive correlation with serum glucose content in grass carp[90]. Therefore, the decreased abundance of *Firmicutes* may have potentially positive consequences for largemouth bass with HCD. *Mycoplasma* is a common bacterium found in the water environment and aquatic animals, and it has been discovered in study of tilapia that infection with *Mycoplasma* led to extensive and marked inflammatory lesions in the gills, liver and spleen[91]. The decreased abundance of *Mycoplasma* (from phylum to genus) probably indicates the reduction of potentially pathogenic bacteria from the largemouth bass intestine. In past studies, *Fusobacteria* has been characterised by the consumption of carbohydrates and peptones to produce butyric acid[92]. Meanwhile, *Cetobacterium* is considered as beneficial intestinal bacteria that favours anaerobic metabolism in fish[93], and acetate-producing *Cetobacterium somerae* has been reported to play a causative role for improving glucose homoeostasis in zebrafish[94]. In the present study, the dietary inclusion of CBC significantly increased the relative abundance of *Fusobacteria* and *Cetobacterium* in the intestine of largemouth bass. This might be potentially linked to the above which mentioned changes in SCFA transport and insulin pathway-related genes.

In conclusion, dietary inclusion of CBC in HCD significantly alleviated glycogen accumulation and improved carbohydrate utilisation in largemouth bass, possibly through activating of AMPK and insulin signalling pathways. Also, dietary inclusion of CBC significantly improved the antioxidant capacity and intestinal microbiota of largemouth bass fed HCD. However, the underlying regulatory mechanisms of SCFA in glucose metabolism needed to be further explored.

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References

1. Stone DA (2003) Dietary carbohydrate utilization by fish. *Rev Fish Sci* **11**, 337–369.
2. National Research Council (2011) *Committee on the Nutrient Requirements of Fish and Shrimp, Nutrient Requirements of Fish and Shrimp*. Washington, DC: National Academies Press.
3. Sørensen M, Nguyen G, Storebakken T, *et al.* (2010) Starch source, screw configuration and injection of steam into the barrel affect the physical quality of extruded fish feed. *Aquacult Res* **41**, 419–432.
4. Kamalam BS, Medale F & Panserat S (2017) Utilisation of dietary carbohydrates in farmed fishes: new insights on influencing factors, biological limitations and future strategies. *Aquaculture* **467**, 3–27.
5. Polakof S, Panserat S, Soengas JL, *et al.* (2012) Glucose metabolism in fish: a review. *J Comp Physiol B* **182**, 1015–1045.
6. Amoah A, Coyle SD, Webster CD, *et al.* (2008) Effects of graded levels of carbohydrate on growth and survival of largemouth bass, *Micropterus salmoides*. *J World Aquacult Soc* **39**, 397–405.
7. Hemre GI, Mommsen TP & Krogdahl Å (2002) Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquacult Nutr* **8**, 175–194.
8. Li S, Li Z, Zhang J, *et al.* (2019) The impacts of dietary carbohydrate levels on growth performance, feed utilization, glycogen accumulation and hepatic glucose metabolism in hybrid grouper (*Epinephelus fuscoguttatus*♀× *E. lanceolatus*♂). *Aquaculture* **512**, 734351.
9. Chen S, Jiang X, Liu N, *et al.* (2022) Effects of dietary berberine hydrochloride inclusion on growth, antioxidant capacity, glucose metabolism and intestinal microbiome of largemouth bass (*Micropterus salmoides*). *Aquaculture* **552**, 738023.
10. Xu R, Ding FF, Zhou NN, *et al.* (2022) *Bacillus amyloliquefaciens* protects Nile tilapia against *Aeromonas hydrophila* infection and alleviates liver inflammation induced by high-carbohydrate diet. *Fish Shellfish Immunol* **127**, 836–842.
11. Tran NT, Li Z, Ma H, *et al.* (2020) *Clostridium butyricum*: a promising probiotic confers positive health benefits in aquatic animals. *Rev Aquacult* **12**, 2573–2589.
12. Cassir N, Benamar S & La Scola B (2016) *Clostridium butyricum*: from beneficial to a new emerging pathogen. *Clin Microbiol Infect* **22**, 37–45.
13. Li H, Zhou Y, Ling H, *et al.* (2019) The effect of dietary supplementation with *Clostridium butyricum* on the growth performance, immunity, intestinal microbiota and disease resistance of tilapia (*Oreochromis niloticus*). *PLOS ONE* **14**, e0223428.
14. Meng X, Wu S, Hu W, *et al.* (2021) *Clostridium butyricum* improves immune responses and remodels the intestinal microbiota of common carp (*Cyprinus carpio* L.). *Aquaculture* **2021**, 735753.
15. Sumon MS, Ahmmed F, Khushi SS, *et al.* (2018) Growth performance, digestive enzyme activity and immune response of *Macrobrachium rosenbergii* fed with probiotic *Clostridium butyricum* incorporated diets. *J King Saud Univ Sci* **30**, 21–28.
16. He J, Zhang P, Shen L, *et al.* (2020) Short-chain fatty acids and their association with signalling pathways in inflammation, glucose and lipid metabolism. *Int J Mol Sci* **21**, 6356.
17. Kondo T, Kishi M, Fushimi T, *et al.* (2009) Acetic acid upregulates the expression of genes for fatty acid oxidation enzymes in liver to suppress body fat accumulation. *J Agric Food Chem* **57**, 5982–5986.
18. Sakakibara S, Yamauchi T, Oshima Y, *et al.* (2006) Acetic acid activates hepatic AMPK and reduces hyperglycemia in diabetic KK-A (y) mice. *Biochem Bioph Res Commun* **344**, 597–604.

19. Zadeh-Tahmasebi M, Duca FA, Rasmussen BA, *et al.* (2016) Activation of short and long chain fatty acid sensing machinery in the ileum lowers glucose production *in vivo*. *J Biol Chem* **291**, 8816–8824.
20. Gao Z, Yin J, Zhang J, *et al.* (2009) Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes* **58**, 1509–1517.
21. Titchenell PM, Lazar MA & Birnbaum MJ (2017) Unraveling the regulation of hepatic metabolism by insulin. *Trends Endocr Metab* **28**, 497–505.
22. Zheng T, Yang X, Wu D, *et al.* (2015) Salidroside ameliorates insulin resistance through activation of a mitochondria-associated AMPK/PI3K/Akt/GSK3β pathway. *Br J Pharmacol* **172**, 3284–3301.
23. Maritim AC, Sanders A & Watkins Iii JB (2003) Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol* **17**, 24–38.
24. Singer-Englar T, Barlow G & Mathur R (2019) Obesity, diabetes, and the gut microbiome: an updated review. *Expert Rev Gastroenterol Hepatol* **13**, 3–15.
25. Zhou YL, He GL, Jin T, *et al.* (2021) High dietary starch impairs intestinal health and microbiota of largemouth bass, *Micropterus salmoides*. *Aquaculture* **534**, 736261.
26. Duan Y, Wang Y, Dong H, *et al.* (2018) Changes in the intestine microbial, digestive, and immune-related genes of *Litopenaeus vannamei* in response to dietary probiotic *Clostridium butyricum* supplementation. *Front Microbiol* **9**, 2191.
27. Duan Y, Zhang Y, Dong H, *et al.* (2017) Effect of the dietary probiotic *Clostridium butyricum* on growth, intestine antioxidant capacity and resistance to high temperature stress in kuruma shrimp *Marsupenaeus japonicus*. *J Therm Biol* **66**, 93–100.
28. Ministry of Agriculture and Rural Affairs, China (2021) *China Fishery Statistics Yearbook*. Beijing: China Agriculture Press.
29. Tao J, Wang S, Qiu H, *et al.* (2022) Modulation of growth performance, antioxidant capacity, non-specific immunity and disease resistance in largemouth bass (*Micropterus salmoides*) upon compound probiotic cultures inclusion. *Fish Shellfish Immunol* **127**, 804–812.
30. Liu Y, Liu N, Wang A, *et al.* (2022) Resveratrol inclusion alleviated high-dietary-carbohydrate-induced glycogen deposition and immune response of largemouth bass, *Micropterus salmoides*. *Br J Nutr* **127**, 165–176.
31. Harmon SM, Kautter DA & Peeler JT (1971) Improved medium for enumeration of *Clostridium perfringens*. *Appl Microbiol* **22**, 688–692.
32. AOAC (2003) *Official Methods of Analysis*, 17th ed. Washington, DC: Association of Analytical Communities.
33. Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**, 497–509.
34. Seifter S, Dayton S & Novic B (1950) The estimation of glycogen with the anthrone reagent. *Arch Biochem Biophys* **25**, 191–200.
35. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
36. Buege JA (1978) Microsomal lipid peroxidation. *Method Enzymol* **52**, 302–310.
37. Erel O (2004) A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* **37**, 277–285.
38. Peskin AV & Winterbourn CC (2017) Assay of superoxide dismutase activity in a plate assay using WST-1. *Free Radic Biol Med* **103**, 188–191.
39. Aebi H (1984) Catalase *in vitro*. *Method Enzymol* **105**, 121–126.
40. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.
41. Caporaso JG, Kuczynski J, Stombaugh J, *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**, 335–336.
42. Magoč T & Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**, 2957–2963.
43. Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* **10**, 996–998.
44. Wang Q, Garrity GM, Tiedje JM, *et al.* (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microb* **73**, 5261–5267.
45. Segata N, Izard J, Waldron L, *et al.* (2011) Metagenomic biomarker discovery and explanation. *Genome Biol* **12**, 1–18.
46. Jia L, Li D, Feng N, *et al.* (2017) Anti-diabetic effects of Clostridium butyricum CGMCC0313 1 through promoting the growth of gut butyrate-producing bacteria in type 2 diabetic mice. *Sci Rep* **7**, 7046.
47. Gou S, Chen N, Xu X, *et al.* (2015) Effects of dietary digestible starch levels on growth performance, body composition, and non-specific immunological index of largemouth bass (*Micropterus salmoides*). *J Fish China* **39**, 1499–1510.
48. Zhang M, Dong B, Lai X, *et al.* (2021) Effects of *Clostridium butyricum* on growth, digestive enzyme activity, antioxidant capacity and gut microbiota in farmed tilapia (*Oreochromis niloticus*). *Aquacult Res* **52**, 1573–1584.
49. Li P, Hou D, Zhao H, *et al.* (2022) Dietary *Clostridium butyricum* improves growth performance and resistance to ammonia stress in yellow catfish (*Pelteobagrus fulvidraco*). *Aquacult Nutr* **2022**, 11.
50. Meng X, Cai H, Li H, *et al.* (2023) *Clostridium butyricum*-fermented Chinese herbal medicine enhances the immunity by modulating the intestinal microflora of largemouth bass (*Micropterus salmoides*). *Aquaculture* **562**, 738768.
51. Xu X, Chen N, Liu Z, *et al.* (2016) Effects of dietary starch sources and levels on liver histology in largemouth bass, Micropterus salmoides. *J Shanghai Ocean Univ* **25**, 61–70.
52. Stone DAJ, Allan GL & Anderson AA (2003) Carbohydrate utilization by juvenile silver perch, *Bidyanus bidyanus* (Mitchell). III. The protein-sparing effect of wheat starch-based carbohydrates. *Aquacult Res* **34**, 123–134.
53. Li S, Sang C, Turchini GM, *et al.* (2020) Starch in aquafeeds: the benefits of a high amylose to amylopectin ratio and resistant starch content in diets for the carnivorous fish, largemouth bass (*Micropterus salmoides*). *Br J Nutr* **124**, 1145–1155.
54. Li S, Sang C, Zhang J, *et al.* (2018) Effects of acute hyperglycemia stress on plasma glucose, glycogen content, and expressions of glycogen synthase and phosphorylase in hybrid grouper (*Epinephelus fuscoguttatus*♀× *E. lanceolatus*♂). *Fish Physiol Biochem* **44**, 1185–1196.
55. Wang J, Li X, Han T, *et al.* (2016) Effects of different dietary carbohydrate levels on growth, feed utilization and body composition of juvenile grouper *Epinephelus akaara*. *Aquaculture* **459**, 143–147.
56. Beale EG (2013) Insulin signaling and insulin resistance. *J Invest Med* **61**, 11–14.
57. Capilla E, Médale F, Navarro I, *et al.* (2003) Muscle insulin binding and plasma levels in relation to liver glucokinase activity, glucose metabolism and dietary carbohydrates in rainbow trout. *Regul Pept* **110**, 123–132.
58. Chen P, Wu X, Gu X, *et al.* (2021) FoxO1 in *Micropterus salmoides*: molecular characterization and its roles in glucose metabolism by glucose or insulin-glucose loading. *Gen Comp Endocr* **310**, 113811.

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59. Zhong L, Liu H, Zhang H, *et al.* (2022) High starch in diet leads to disruption of hepatic glycogen metabolism and liver fibrosis in largemouth bass (*Micropterus salmoides*), which is mediated by the PI3K/Akt signaling pathway. *Front Physiol* **13**, 880513.
60. Deng K, Pan M, Liu J, *et al.* (2018) Chronic stress of high dietary carbohydrate level causes inflammation and influences glucose transport through SOCS3 in Japanese flounder *Paralichthys olivaceus*. *Sci Rep* **8**, 1–13.
61. Polakof S, Moon TW, Aguirre P, *et al.* (2010) Effects of insulin infusion on glucose homeostasis and glucose metabolism in rainbow trout fed a high-carbohydrate diet. *J Exp Biol* **213**, 4151–4157.
62. Mátis G, Kulcsár A, Turowski V, *et al.* (2015) Effects of oral butyrate application on insulin signaling in various tissues of chickens. *Domest Anim Endocr* **50**, 26–31.
63. Aguilar EC, da Silva JF, Navia-Pelaez JM, *et al.* (2018) Sodium butyrate modulates adipocyte expansion, adipogenesis, and insulin receptor signaling by upregulation of PPAR-γ in obese Apo E knockout mice. *Nutrition* **47**, 75–82.
64. Zhou YJ, Xu N, Zhang XC, *et al.* (2021) Chrysin improves glucose and lipid metabolism disorders by regulating the AMPK/PI3K/AKT signaling pathway in insulin-resistant HepG2 cells and HFD/STZ-induced C57BL/6J mice. *J Agric Food Chem* **69**, 5618–5627.
65. O'Neill HM, Maarbjerg SJ, Crane JD, *et al.* (2011) AMP-activated protein kinase (AMPK) $\beta1\beta2$ muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. *Proc Natl Acad Sci USA* **108**, 16092–16097.
66. Chopra I, Li HF, Wang H, *et al.* (2012) Phosphorylation of the insulin receptor by AMP-activated protein kinase (AMPK) promotes ligand-independent activation of the insulin signalling pathway in rodent muscle. *Diabetologia* **55**, 783–794.
67. Yoshida H, Ishii M & Akagawa M (2019) Propionate suppresses hepatic gluconeogenesis via GPR43/AMPK signaling pathway. *Arch Biochem Biophys* **672**, 108057.
68. Shi HJ, Liu WB, Xu C, *et al.* (2020) Transcriptional regulation of the AMP-activated protein kinase and glycolipid metabolism-related genes by insulin and glucagon in blunt snout bream (*Megalobrama amblycephala*): a comparative study. *Aquaculture* **515**, 734553.
69. Zhang WQ, Zhao TT, Gui DK, *et al.* (2019) Sodium butyrate improves liver glycogen metabolism in type 2 diabetes mellitus. *J Agric Food Chem* **67**, 7694–7705.
70. Hong J, Jia Y, Pan S, *et al.* (2016) Butyrate alleviates high fat diet-induced obesity through activation of adiponectin-mediated pathway and stimulation of mitochondrial function in the skeletal muscle of mice. *Oncotarget* **7**, 56071.
71. Duan Y, Zhang Y, Dong H, *et al.* (2017) Effect of dietary *Clostridium butyricum* on growth, intestine health status and resistance to ammonia stress in Pacific white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol* **65**, 25–33.
72. Milligan G, Stoddart LA & Smith NJ (2009) Agonism and allosterism: the pharmacology of the free fatty acid receptors FFA2 and FFA3. *Br J Pharmacol* **158**, 146–153.
73. Brown AJ, Jupe S & Briscoe CP (2005) A family of fatty acid binding receptors. *DNA Cell Biol* **24**, 54–61.
74. Yao Y, Cai X, Fei W, *et al.* (2022) The role of short-chain fatty acids in immunity, inflammation and metabolism. *Crit Rev Food Sci* **62**, 1–12.
75. Ma HJ, Mou MM, Pu DC, *et al.* (2019) Effect of dietary starch level on growth, metabolism enzyme and oxidative status of juvenile largemouth bass, *Micropterus salmoides*. *Aquaculture* **498**, 482–487.
76. Li H, Shang Z, Liu X, *et al.* (2021) *Clostridium butyricum* alleviates enterotoxigenic *Escherichia coli* K88-induced oxidative damage through regulating the p62-Keap1-Nrf2 signaling pathway and remodeling the cecal microbial community. *Front Immunol* **12**, 771826.
77. Wangari MR, Gao Q, Sun C, *et al.* (2021) Effect of dietary *Clostridium butyricum* and different feeding patterns on growth performance, antioxidant and immune capacity in freshwater prawn (*Macrobrachium rosenbergii*). *Aquacult Res* **52**, 12–22.
78. Lee JM, Calkins MJ, Chan K, *et al.* (2003) Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. *J Biol Chem* **278**, 12029–12038.
79. Reisman SA, Yeager RL, Yamamoto M, *et al.* (2009) Increased Nrf2 activation in livers from Keap1-knockdown mice increases expression of cytoprotective genes that detoxify electrophiles more than those that detoxify reactive oxygen species. *Toxicol Sci* **108**, 35–47.
80. Timme-Laragy AR, Van Tiem LA, Linney EA, *et al.* (2009) Antioxidant responses and NRF2 in synergistic developmental toxicity of PAHs in zebrafish. *Toxicol Sci* **109**, 217–227.
81. Liang H, Mokrani A, Ji K, *et al.* (2018) Dietary leucine modulates growth performance, Nrf2 antioxidant signaling pathway and immune response of juvenile blunt snout bream (*Megalobrama amblycephala*). *Fish Shellfish Immunol* **73**, 57–65.
82. Shang X, Yu P, Yin Y, *et al.* (2021) Effect of selenium-rich *Bacillus subtilis* against mercury-induced intestinal damage repair and oxidative stress in common carp. *Comp Biochem Phys C* **239**, 108851.
83. Huang H, Zhou P, Chen P, *et al.* (2020) Alteration of the gut microbiome and immune factors of grass carp infected with *Aeromonas veronii* and screening of an antagonistic bacterial strain (*Streptomyces flavotricini*). *Microb Pathog* **143**, 104092.
84. Nayak SK (2010) Role of gastrointestinal microbiota in fish. *Aquacult Res* **41**, 1553–1573.
85. Huang X, Zhong L, Kang Q, *et al.* (2021) A high starch diet alters the composition of the intestinal microbiota of largemouth bass *Micropterus salmoides*, which may be associated with the development of enteritis. *Front Microbiol* **12**, 696588.
86. Liu H, Li J, Guo X, *et al.* (2018) Yeast culture dietary supplementation modulates gut microbiota, growth and biochemical parameters of grass carp. *Microb Biotechnol* **11**, 551–565.
87. Guimarães MC, da Silva Guimarães AIC, Natori MM, *et al.* (2021) Oral administration of *Bacillus subtilis* and *Lactobacillus plantarum* modulates the gut microbiota and increases the amylase activity of Nile tilapia (*Oreochromis niloticus*). *Aquacult Int* **29**, 91–104.
88. Remely M, Hippe B, Zanner J, *et al.* (2016) Gut microbiota of obese, type 2 diabetic individuals is enriched in Faecalibacterium prausnitzii, Akkermansia muciniphila and Peptostreptococcus anaerobius after weight loss. *Endocr Metab Immune* **16**, 99–106.
89. Hung WC, Hung WW, Tsai HJ, *et al.* (2021) The association of targeted gut microbiota with body composition in type 2 diabetes mellitus. *Int J Med Sci* **18**, 511.
90. Pan H, Li Z, Xie J, *et al.* (2019) Berberine influences blood glucose via modulating the gut microbiome in grass carp. *Front Microbiol* **10**, 1066.
91. Ahmed HA & El-Hady MA (2008) Antigenic variations and pathogenicity between *Mycoplasma* spp isolated from fishes and shellfishes. *In 8th International Symposium on Tilapia Aquaculture*. Cairo International Convention Center.

92. Arellano M, Jomard P, El Kaddouri S, *et al.* (2000) Routine analysis of short-chain fatty acids for anaerobic bacteria identification using capillary electrophoresis and indirect ultraviolet detection. *J Chromatogr B* **741**, 89–100.
93. Zhang Z, Fan Z, Yi M, *et al.* (2022) Characterization of the core gut microbiota of Nile tilapia (*Oreochromis niloticus*): indication of a putative novel *Cetobacterium* species and analysis of its potential function on nutrition. *Arch Microbiol* **204**, 690.
94. Wang A, Zhang Z, Ding Q, *et al.* (2021) Intestinal *Cetobacterium* and acetate modify glucose homeostasis via parasympathetic activation in zebrafish. *Gut Microbes* **13**, 1–15.