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Inulin alleviates adverse metabolic syndrome and regulates intestinal microbiota composition in Nile tilapia (*Oreochromis niloticus*) fed with high-carbohydrate diet

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Abstract

A high-carbohydrate diet could achieve a protein-sparing effect, but it may cause negative impacts on the growth condition of fish due to their poor utilisation ability of carbohydrate. How to reduce the adverse effects caused by a high-carbohydrate diet is important for the development of aquaculture. In the present study, we aimed to identify whether inulin could attenuate the metabolic syndrome caused by a high-carbohydrate diet in fish. Nile tilapia (*Oreochromis niloticus*) (1·19 (SD 0·01) g) were supplied with 35 % carbohydrate (CON), 45 % carbohydrate (HC) and 45 % carbohydrate + 5 g/kg inulin (HCI) diets for 10 weeks. The results showed that addition of inulin improved the survival rate when fish were challenged with *Aeromonas hydrophila*, indicating that inulin had an immunostimulatory effect. Compared with the HC group, the HCI group had lower lipid accumulation in liver and the gene expression analyses indicated that addition of inulin down-regulated genes related to lipogenesis and up-regulated genes relevant to β-oxidation significantly ($P < 0·05$). Higher liver glycogen and glucose tolerance were found in the HCI group compared with the HC group ($P < 0·05$). These results indicated that inulin could alleviate the metabolic syndrome induced by a high-carbohydrate diet. Furthermore, addition of inulin to a high-carbohydrate diet changed the intestinal bacterial composition and significantly increased the concentration of acetic acid and propionic acid in fish gut which have the potential to increase pathogen resistance and regulate metabolic characteristics in fish. Collectively, our results demonstrated a possible causal role for the gut microbiome in metabolic improvements induced by inulin in fish.

Key words: **Nile tilapia (*Oreochromis niloticus*): Inulin: Metabolic syndrome: Intestinal microbiota: SCFA**

Carbohydrates as the most abundant and cheapest energy sources for fish are incorporated in aquatic feeds for the potential protein-sparing effect[1–3]. Furthermore, carbohydrates can also act as stabilisers and swelling agents in diets, and appropriate concentration of dietary carbohydrates could reduce pathogen susceptibility and cause lesser environment pollution[4].

In order to produce cost-effective aquafeeds, more and more competitively priced carbohydrates have been incorporated in feed formulation to substitute expensive marine ingredients[5]. However, most fish species are not good at utilising carbohydrate. Excessive levels of carbohydrate in diets may induce metabolic load and stress responses, thus cause negative effects on health and increase the susceptibility to infectious diseases of fish. For instance, it has been found that high-carbohydrate diet depressed feed intake and feed utilisation efficiency, leading to poorer growth performance in gibel carp (*Carassius auratus* var. gibelio)[6]. Furthermore, high levels of carbohydrate in the diet could induce negative effects on metabolic homoeostasis and immune status. It has been reported that excessive carbohydrates could increase the lipid content of fish body in Nile tilapia[7] and cause long-lasting hyperglycaemia in rainbow trout (*Oncorhynchus mykiss*)[8]. Suppressed immune response and reduced antioxidant reactions were also found in largemouth bass (*Micropterus salmoides*) and *Labeo rohita* (*Hamilton*) when they were fed with high-carbohydrate diet[9,10]. How to alleviate the negative impacts caused by high-carbohydrate diet has attracted more and more attention. It has been reported that supplementation of bile acids in high-carbohydrate diet could improve growth performance, alleviate metabolic syndromes and enhance immune ability in largemouth bass[11,12], suggesting that dietary additives supplementation have the potential to alleviate negative effects caused by high-carbohydrate diet.

Prebiotics, which are non-digestible ingredients fermented by intestinal microbiota, have been widely used in aquaculture

Abbreviations: CON, 35 % carbohydrate; HC, 45 % carbohydrate; HCI, 45 % carbohydrate + 5 g/kg inulin; OTU, operational taxonomic unit.

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as dietary additives[13]. Prebiotics could change gut microbiota composition to improve growth condition and immune ability or reduce risk of diseases[14]. Currently, the most common prebiotics used in aquaculture are oligosaccharides including inulin, fructo-oligosaccharides, short-chain fructo-oligosaccharides, mannan oligosaccharides, *trans*-galacto-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides, arabinoxylo-oligosaccharides and isomalto-oligosaccharides[15].

Inulin is classified as fructans or fructose polymers, and it naturally exists in many plants especially in chicory. In aquaculture, the studies have been conducted to detect the influence of inulin on growth and immune status[16,17], but whether inulin could alleviate high-carbohydrate diet-induced metabolic syndrome remains unknown. A growing body of evidences showed that inulin had potential to alleviate glucose or lipid metabolism disorders in mammals, and inulin was also suggested to have the ability to alter gut microbiota composition and promote the growth of probiotics in mammals. For example, the administration of inulin decreased fasting blood glucose levels and improved insulin resistance which is correlated with the increase of *Anaerovorax* in rats[18]. In addition, inulin could alleviate the lipid accumulation in adipocytes induced by high-fat diet, and it could modulate gut microbiota composition by decreasing abundance of *Roseburia* spp. and *Clostridium* cluster XIVa[19]. Evolutionarily, fish are more primitive than mammals and fish harbour a Proteobacteria-dominated microbiota, which is different from the dominant microbiota in mammals[20], so whether inulin could regulate fish metabolism by regulating gut microbiota remains unknown.

Nile tilapia (*Oreochromis niloticus*) are an economically important fish species and is an ideal fish model for nutritional and metabolic studies due to the fast growth rate, high resistance to diseases and available genomic information[21]. Although Nile tilapia have higher capacity for carbohydrate utilisation, high concentration of carbohydrate in the diet also caused metabolic syndrome in Nile tilapia, including hyperglycaemia and fat deposition[7,22]. In the present study, we hypothesised that inulin could alleviate metabolic syndrome induced by high carbohydrate by regulating intestinal microbiota in Nile tilapia. Fish were fed with three diets and referred as CON (35 % carbohydrate), HC (45 % carbohydrate) and HCI (45 % carbohydrate + 5 g/kg inulin) for 10 weeks. The influence of inulin on Nile tilapia fed with high-carbohydrate diet, including survival rate post *Aeromonas hydrophila* infection, metabolic characteristics and intestinal microbiota, was detected.

Materials and methods

This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University. All experiments were conducted under the Guidance of the Care and Use of Laboratory Animals in China (no. F20190101).

Experimental design

Juvenile Nile tilapia were collected from Shanghai Ocean University (Shanghai, China). During the 2-week acclimatisation period, Nile tilapia were fed on a commercial diet (Tongwei Co. Ltd) at a feeding rate of 3 % body weight. A total of 270 visually healthy fish (1·19 (SD 0·01) g) were divided into three treatments and fed with control diet (35 % carbohydrate, CON), high-carbohydrate diet (45 % carbohydrate, HC) and inulin-addition diet (45 % carbohydrate + inulin (5 g/kg) diet, HCI) (online Supplementary Table S1). Each treatment contained three tanks (thirty fish in each tank). Fish were hand-fed twice daily (09.00 and 18.00 hours) for 10 weeks at a feeding ratio of 4 % body weight. Feeding consumption was adjusted every 2 weeks following a 24-h starvation period and batch weighing. Water temperature was maintained at 27 (SD 1)°C with a natural photoperiodicity (12 h light–12 h dark). During the experimental period, dissolved oxygen and pH were measured daily and kept at 5–7 mg/l and 7·5–8, respectively.

Dietary ingredients (online Supplementary Table S1) were mixed with oil and distilled water to form a paste and then extruded through a Twin-Screw Extruder (TSE65; Yanggong Machine) to produce 2-mm-diameter pellets. The proximate compositions were calculated including crude lipid, crude protein and ash which are showed in online Supplementary Table S1. The prepared diets were dried at room temperature and then stored at −20°C until use.

Sample collection

At the end of the trial, all fish were deprived of feed for 12 h before sampling and twenty-one fish were randomly selected from each group (seven fish per tank). Six fish were immediately stored at −80°C to analyse body composition. Fish were euthanised with MS-222 (22 mg/l, tricaine methanesulfonate; Western Chemicals Inc.) and dissected to obtain tissues for biochemical and molecular biological analyses. Gut content was collected and promptly frozen at −80°C for SCFA and intestinal microbial composition analysis.

Growth performance

Weight gain of all fish in each tank was measured, and nine fish per diet (three fish per tank) were collected to detect the viscera index, hepatosomatic index and mesenteric fat index which were calculated according to the formulae:

$$\text{Weight gain rate (\%)} = 100 \times (\text{final fish weight} - \text{initial fish weight})/\text{initial fish weight}$$

$$\text{Feed conversion ratio (\%)} = 100 \times \text{dry feed consumed}/(\text{final fish weight} - \text{initial fish weight})$$

$$\text{Viscera index (\%)} = 100 \times (\text{viscera weight}/\text{body weight})$$

$$\text{Hepatomatic index (\%)} = 100 \times (\text{liver weight}/\text{body weight})$$

$$\text{Mesenteric fat index (\%)} = 100 \times (\text{mesenteric fat weight}/\text{body weight})$$

Biochemical analysis

Six fish from each group were collected for body composition assay. The content of moisture was measured via oven drying to a gravimetric analysis at 105°C for 12 h. Then, whole-dried fish

were grounded by pulverizer (Xulang Machinery Equipment Co. Ltd) and stored in a glass desiccator at room temperature for analysing total protein and total lipid content analyses. Total protein of the whole body was determined by Kjeldahl method using a semi-automatic Kjeldahl System (Foss Tecator, Kjeltec™ 8200). Total lipid in whole fish body and in liver was calculated by a classic methanol–chloroform method[23].

Livers of six fish were collected from each treatment for TAG and glycogen analysis. The contents of TAG and glycogen in sampled tissues were assayed using commercial kits (Jiancheng Biotech Co.). All experiments were conducted according to the relevant kit protocols.

Histochemical staining

Liver samples of three fish per group were embedded in an optimum cutting temperature (OCT) compound (Sakura) at −40°C in a cryostat (Cryostar NX50™; Thermo Fisher Scientific Co. Ltd), and then tissues were cut into 5 μm thickness. Then, 5 μm sections were stained with Oil red O staining solution (O1516; Sigma-Aldrich) overnight. The stained samples were washed with decreasing concentrations of propylene glycol, followed by several rinses with PBS and stored at 80 % glycerol bath. Histology images were observed by a microscope (Nikon, Eclipse, TS100).

RNA extraction and quantitative real-time PCR

Total RNA of six fish per group was extracted from liver by using Tri Pure Reagent (Takara). RNA purity and integrity parameters were measured by Nanodrop 2000 (Thermo Fisher Scientific Co. Ltd) and electrophoresis. A260/A280 of RNA ranged from 1·9 to 2·2 and A230/A260 of RNA was >2. First-strand complementary DNA was synthesised using a FastQuant RT Kit with gDNase (TIANGEN) by S1000™ Thermal Cycler (BioRad). The primers were designed in NCBI for real-time PCR, and the sequences are listed in online Supplementary Table S2. Quantitative real-time PCR (qRT-PCR) includes 2·0 μl of cDNA template, 12·5 μl of 2 × SYBR qPCR Mixture (Aidlab), 2·0 μl of PCR primers (5 μM) and 6·5 μl of nuclease-free water. qRT-PCR was performed in a CFX96 Connect Real-Time System (Bio-Rad) with 95°C for 10 min, forty cycles of 95°C for 5 s and 60°C for 15 s. In order to ensure the specificity of the primers, melt curve of the amplified products was performed at all PCR reactions. The amplification efficiency was between 95 and 105 %, and the correlation coefficient was above 0·98 for each gene. The fold difference in mRNA expression was calculated by $2^{-\Delta\Delta CT}$ method[24].

Glucose tolerance test

At the end of the trial, thirty fish from each group (ten fish per tank) were collected for a glucose tolerance test by an i.p. injection of D-glucose (500 mg/kg BW, 20 % in 0·85 % NaCl) (Sigma) after 12 h of fasting. Blood of six fish per group was drawn from the caudal vein of fish at 0, 0·5, 1·5, 3 and 6 h after injection. Blood glucose and inulin were measured using commercial kits (Jiancheng Biotech Co.).

Intestinal microbiota analysis

Gut content (100 mg) was collected from each individual fish of seven fish per treatment for 16S RNA sequencing. Non-template control was involved during DNA extraction and PCR amplification. Total intestinal bacterial DNA was isolated, and the V3–V4 region of the 16S rRNA gene was amplified by PCR using 338F (5′ ACTCCTACGGGAGGCAGCA 3′)/806R (5′ GGACTACHVG GGTWTCTAAT 3′). The quantity of PCR products was detected by a Microplate reader (Bio Tek, Flx800) with a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). PCR products were subjected to Illumina MiSeq/NovaSeq, generating paired-end reads (Shanghai Personal Biotechnology Co. Ltd). Microbiota sequences were available in Sequence Read Archive with the accession number PRJNA612370. The Quantitative Insights Into Microbial Ecology (QIIME, v2) pipeline was performed for quality filter, denoising and chimaera check[25]. All the unique sequences were clustered at 98 % followed by chimaera removing. The non-chimaera sequences were re-clustered at 97 % to generate an operational taxonomic unit (OTU) table. α-Diversity indices including Chao1, Simpson and Shannon indexes were calculated by QIIME. The classification at phylum level of species composition was analysed by QIIME. Principal component analysis and heat map analysis were performed using R software. Fourteen OTU were picked for heat map analysis based on the following: (1) the abundance of these OTU was higher than 1 % at least in one group and (2) the abundance of these OTU was significantly different between CON and HC or HC and HCI.

SCFA analysis

Gut content (200 mg) of three fish per treatment was suspended with 0·5 ml distilled water. 0·4 ml 50 % sulphuric acid was added to the mixture for acidifying. Then, SCFA were extracted with 1 ml diethyl ether. The samples were centrifuged at 12 000 ***g*** for 10 min, and the supernatants were collected for the detection. The analysis was conducted with GC by using GC7900 (TianMei Scientific Instrument). The temperature of the injector port and detector was 180°C. The oven temperature increased from 80 to 155°C at the rate of 5°C/min and hold on 80°C for 2 min. The content of SCFA was measured according to the external standard curve.

Aeromonas hydrophila *challenge trial*

To investigate whether inulin could protect Nile tilapia fed with high-carbohydrate diet from pathogen infection, thirty fish were randomly collected from each group (ten fish per tank) and challenged with *A. hydrophila* which was cultured in a Luria Broth at 37°C for 16 h. *A. hydrophila* was collected and washed with PBS. The quantity of bacteria was counted by a standard dilution and plating method. The bacteria were added to cultural water and diluted to the density of $9{\cdot}2 \times 10^5$ colony-forming units/l. Mortality of Nile tilapia was recorded per d, and the challenge assay lasted for 11 d.

Statistical analysis

Data were presented as mean values with their standard errors. Normal distribution was tested using the Shapiro–Wilk test. All

analyses were performed using GraphPad Prism 8.0 (GraphPad). The significant difference was determined using one-way ANOVA followed by Duncan's test. Differences were statistically significant at $P < 0{\cdot}05$ (*), $P < 0{\cdot}01$ (**) and $P < 0{\cdot}001$ (***).

Results

Addition of inulin in high-carbohydrate diet did not influence the growth performance or body composition of Nile tilapia

After 10-week feeding trial, weight gain showed an increased tendency in the HC group compared with the CON group, but no significant difference has been detected ($P = 0{\cdot}07$). Compared with HC treatment, inulin did not markedly affect weight gain rate (Table 1). High carbohydrate or addition of inulin had no influence on feed conversion ratio or viscera index (Table 1). Hepatosomatic index was significantly higher in the HC group than in CON ($P < 0{\cdot}05$), whereas no obvious difference was found between the HC and HCI groups (Table 1). The body moisture, total lipid and total protein were detected, and no significant difference in body compositions was found among three treatments (Table 1). These results indicated that the supplement of inulin in high-carbohydrate diet had no obvious effect on growth performance and body composition of Nile tilapia.

Addition of inulin in high-carbohydrate diet improved pathogen resistance of Nile tilapia

The results of challenge assay indicated that both the CON and HC groups had similar cumulative mortality, but addition of inulin in high-carbohydrate diet protected the fish against pathogen infection as evidenced by higher survival rate (Fig. 1(a)). The expression level of immune-related genes was detected in liver, and the results indicated that the HC group had significantly lower expression levels of transforming growth factor *β* and *il-10* compared with the CON group ($P < 0{\cdot}01$), while HCI treatment up-regulated these genes markedly ($P < 0{\cdot}01$) (Fig. 1(b) and (c)). Furthermore, high-carbohydrate diet significantly up-regulated the expression of pro-inflammatory factors, including *tnf-α* and cyclo-oxygenase-2 ($P < 0{\cdot}01$), but these genes were down-regulated in HCI diet (Fig. 1(d) and (e)). Expression level of *il-8* was not affected by excessive carbohydrate, but it was lower in inulin-addition diet ($P < 0{\cdot}01$, Fig. 1(f)). These results suggested that the supplement of inulin in high-carbohydrate diet could improve pathogen resistance of fish which may be caused by the up-regulation of anti-inflammatory factors and down-regulation of pro-inflammatory factors.

Addition of inulin in high-carbohydrate diet alleviated lipid accumulation in liver of Nile tilapia

In order to investigate whether addition of inulin could alleviate the adverse effects caused by high-carbohydrate diet, the content of mesenteric fat and the total lipid in liver was measured in these three groups. High-carbohydrate diet distinctly increased the mesenteric fat index compared with the control group ($P < 0{\cdot}05$), but no significant difference was found between HC and HCI treatments (Fig. 2(a)). The content of lipid in liver was measured, and the results indicated that the HC group had a higher tendency of total lipid compared with the CON group ($P > 0{\cdot}05$), while addition of inulin to high-carbohydrate diet significantly decreased the content of this parameter ($P < 0{\cdot}05$, Fig. 2(b)). Compared with the control diet, the level of TAG in liver was significantly increased in high-carbohydrate diet ($P < 0{\cdot}001$), but inulin obviously reduced its level ($P < 0{\cdot}05$, Fig. 2(c)). These results were further confirmed by oil red staining (Fig. 2(d)). Lipid droplets accumulation was significantly reduced in the HCI group compared with the HC group, suggesting that inulin could alleviate hepatic steatosis caused by high-carbohydrate diet.

Addition of inulin in high-carbohydrate diet influenced the expression level of genes related to lipogenesis and β-oxidation in liver of Nile tilapia

Considering the effects of inulin on hepatic lipid accumulation, the expression level of genes related to lipid metabolism was detected. Compared with CON, HC had markedly higher gene expression level of sterol-regulatory element binding proteins, which plays an important role in up-regulating lipogenic ($P < 0{\cdot}001$), but the expression level of this gene was down-regulated in the HCI group ($P < 0{\cdot}01$, Fig. 3(a)). The similar trend was also found for the expression level of ATP citrate lyase, which encodes a key enzyme of

Table 1. Influence of high carbohydrate and a supplement of inulin on growth condition and body composition of Nile tilapia (Mean values with their standard errors)

	Experimental diets					
	CON		HC		HCI	
	Mean	SEM	Mean	SEM	Mean	SEM
Initial weight (g)	1·19[a]	0·01	1·19[a]	0·02	1·19[a]	0·01
Final weight (g)	12·64[a]	0·57	14·15[a]	0·3	13·73[a]	0·23
Weight gain rate (%)	963·05[a]	47·84	1094·21[a]	25·29	1054·5[a]	19·43
Feed conversion ratio (%)	0·84[a]	0·01	0·88[a]	0·05	0·88[a]	0·02
Viscera index (%)	14·03[a]	0·68	14·55[a]	1·36	14·71[a]	1·35
Hepatosomatic index (%)	4·2[a]	0·34	4·85[b]	0·67	5·00[b]	0·56
Moisture (%)	72·02[a]	0·37	71·06[a]	1·14	70·32[a]	0·34
Total lipid (%)	21·33[a]	2·9	22·8[a]	4·77	23·82[a]	3·26
Total protein (%)	52·84[a]	2·54	52·58[a]	2·0	52·45[a]	1·46

CON, fish fed with 35 % starch; HC, fish fed with 45 % starch; HCI, fish fed with 45 % starch supplemented with 5 g/kg inulin.
[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0{\cdot}05$).

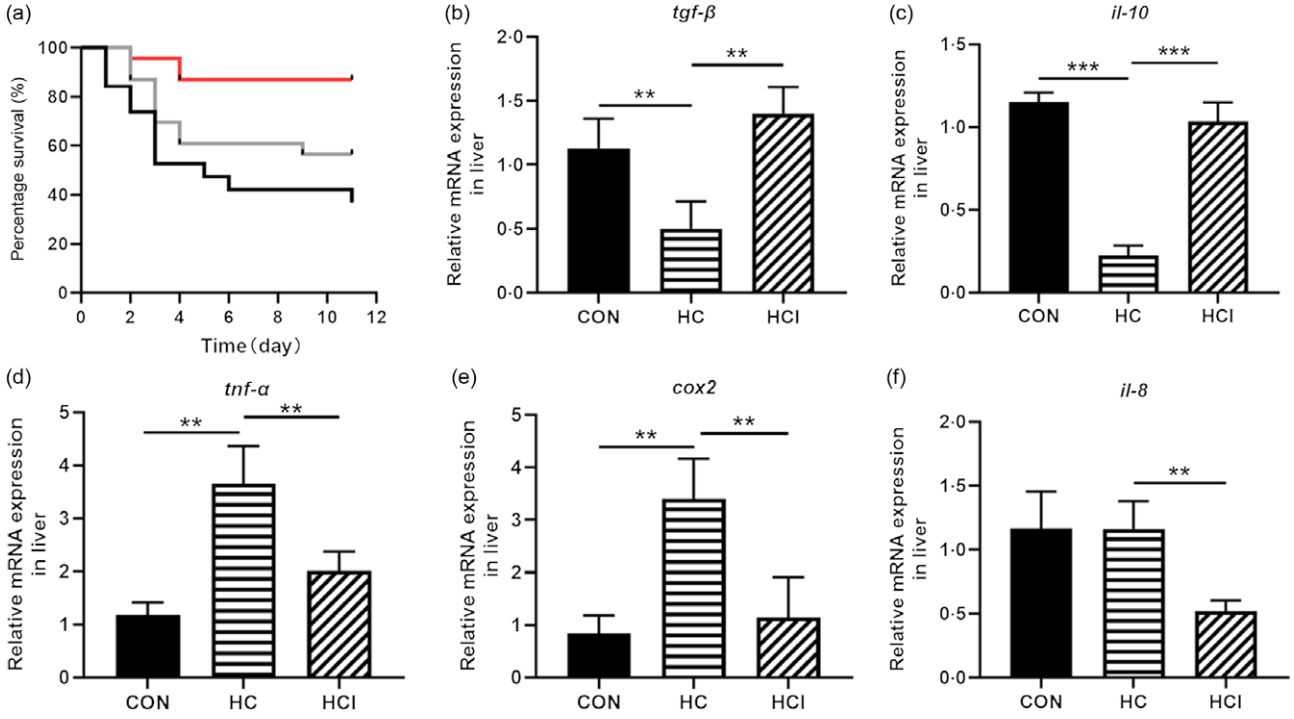

Fig. 1. Inulin improved pathogen resistance of Nile tilapia by increasing anti-inflammatory factors and decreasing pro-inflammatory factors. (a) Survival rate of Nile tilapia after *Aeromonas hydrophila* infection. ▬, Fish fed with 35 % starch (CON); ▬, fish fed with 45 % starch (HC); ▬, fish fed with 45 % starch supplemented with 5 g/kg inulin (HCl). The gene expression of transforming growth factor-*β* (*tgf-β*) (b), *il-10* (c), *tnf-α* (d), cyclo-oxygenase-2 (*cox2*) (e) and *il-8* (f). Data are mean values with their standard errors. Mean values were significantly different: ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$ (one-way ANOVA).

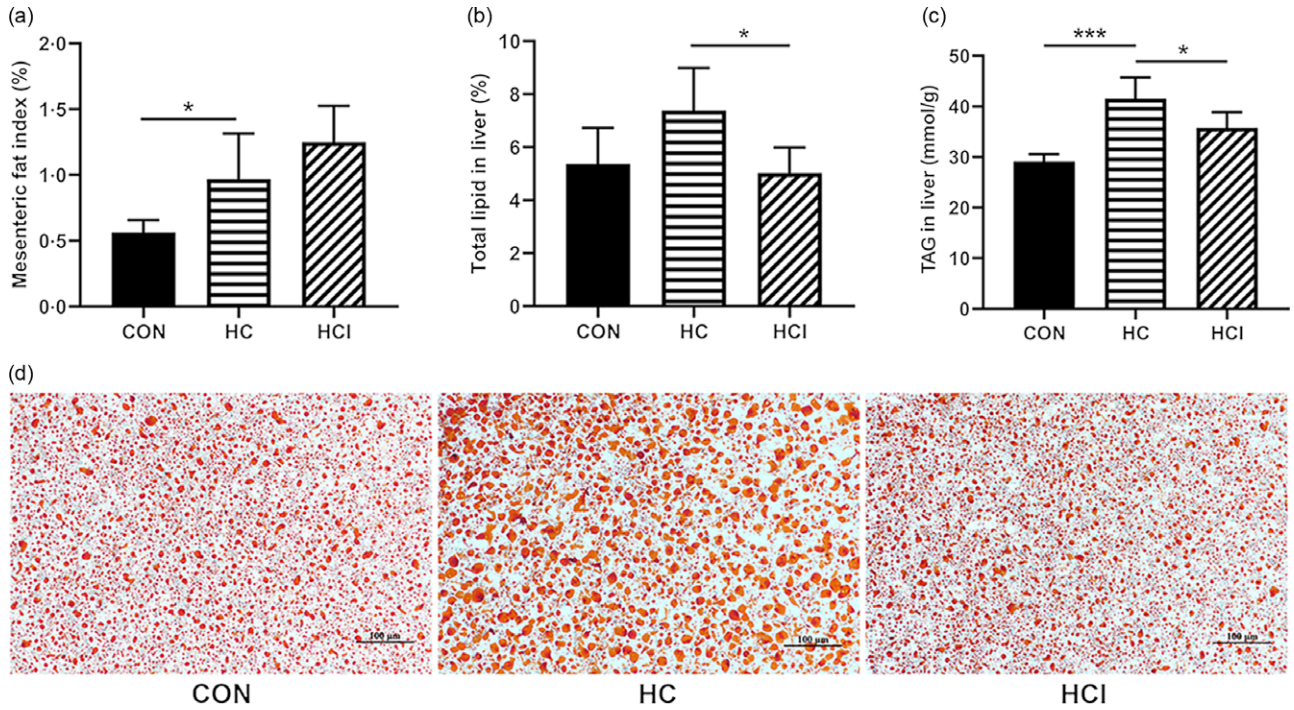

Fig. 2. Inulin alleviated lipid accumulation in liver induced by high-carbohydrate diet. (a) Abdominal adipose factor; (b) total lipid; (c) TAG contents in liver; (d) liver histology images with oil red O staining. The scale bar is 100 μm. Data are mean values with their standard errors. Mean values were significantly different: * $P < 0{\cdot}05$, *** $P < 0{\cdot}001$ (one-way ANOVA). CON, fish fed with 35 % starch; HC, fish fed with 45 % starch; HCl, fish fed with 45 % starch supplemented with 5 g/kg inulin.

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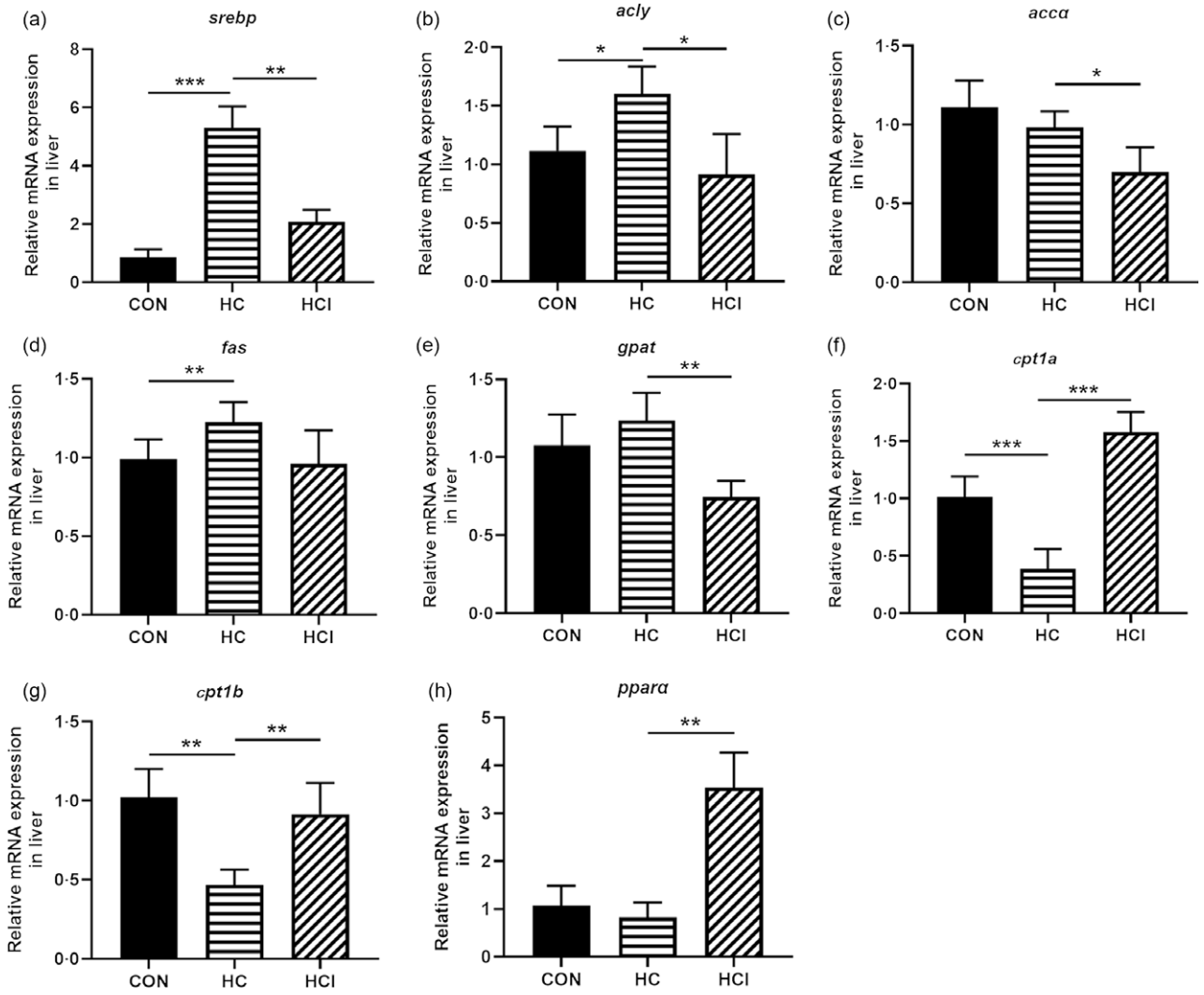

Fig. 3. Inulin influenced the gene expression of lipogenesis and energy expenditure. The gene expression of sterol-regulatory element binding proteins (*srebp*) (a), ATP citrate lyase (*acly*) (b), acetyl-CoA carboxylase α (*accα*) (c), fatty acid synthase (*fas*) (d), glycerol-3-phosphateacyl transferase (*gpat*) (e), carnitine palmitoyltransferase 1a (*cpt1a*) (f), carnitine palmitoyltransferase 1b (*cpt1b*) (g) and *pparα* (h) in liver of Nile tilapia. Data are mean values with their standard errors. Mean values were significantly different: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$ (one-way ANOVA). CON, fish fed with 35 % starch; HC, fish fed with 45 % starch; HCl, fish fed with 45 % starch supplemented with 5 g/kg inulin.

hepatic lipogenesis ($P < 0{\cdot}05$, Fig. 3(b)). Excessive carbohydrate had no influence on gene expression of acetyl-CoA carboxylase α, but inulin distinctly down-regulated the expression level of this gene in liver ($P < 0{\cdot}05$, Fig. 3(c)). High carbohydrate significantly up-regulated the expression level of fatty acid synthase ($P < 0{\cdot}01$), and no difference was found between HC and HCl treatment (Fig. 3(d)). Consistent with the reduced TAG level in liver, expression level of glycerol-3-phosphateacyl transferase, which encodes key enzyme in TAG synthesis, was not influenced by high-carbohydrate diet, but it was distinctly suppressed in the HCl group ($P < 0{\cdot}01$, Fig. 3(e)).

Genes related to fatty acid oxidation, including carnitine palmitoyltransferase 1a and carnitine palmitoyltransferase 1b, were markedly decreased in HC diet compared with CON ($P < 0{\cdot}01$), while these two genes were significantly up-regulated in HCl diet ($P < 0{\cdot}01$) (Fig. 3(f) and (g)). The gene expression level of *pparα*, a transcription factor to active β-oxidation, was not affected by high-carbohydrate diet, but notably up-regulated in inulin-added diet ($P < 0{\cdot}01$, Fig. 3(h)). Collectively, addition of inulin in high-carbohydrate diet could alleviate the hepatic lipid accumulation by down-regulating genes related to lipogenesis and up-regulating genes related to β-oxidation.

Addition of inulin in high-carbohydrate diet increased glycogen synthesis in liver

In order to investigate whether the beneficial impacts of inulin also acted on glucose homoeostasis in Nile tilapia, an oral glucose tolerance test was performed. Compared with CON treatment, plasma glucose was significantly higher at 1·5 and 3 h despite the excessive content of plasma insulin in HC treatment ($P < 0{\cdot}05$, Fig. 4(a) and (b)). Although the HCl group had lower plasma insulin than the HC group ($P < 0{\cdot}05$), the plasma glucose was lower at 1·5 and 3 h post-glucose injection compared with the HC group ($P < 0{\cdot}05$, Fig. 4(a)

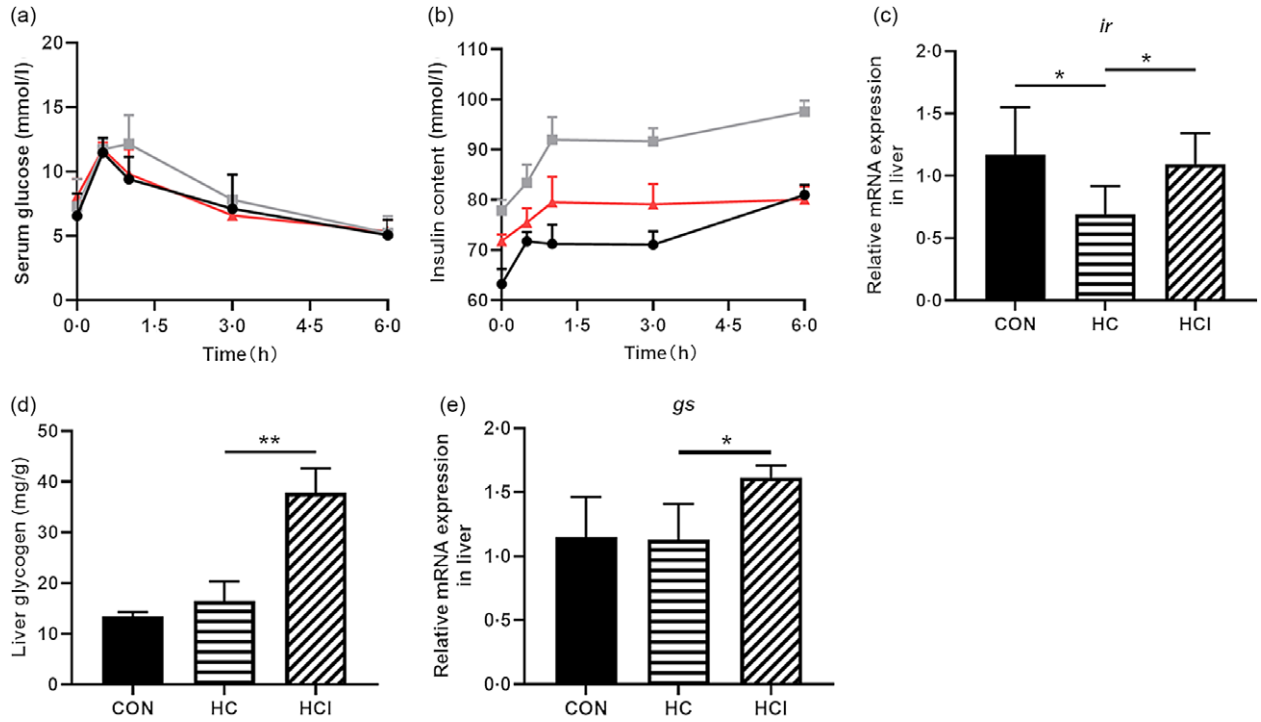

Fig. 4. Addition of inulin influenced glycogen synthesis and gluconeogenesis of Nile tilapia. (a) Glucose tolerance test. —●—, Fish fed with 35 % starch (CON); —■—, fish fed with 45 % starch (HC); —▲—, fish fed with 45 % starch supplemented with 5 g/kg inulin (HCI); (b) insulin concentrations after glucose load (500 mg/kg body weight); (c) gene expression level of insulin receptor (*ir*); (d) glycogen content in liver; (e) gene expression level of glycogen synthase (*gs*) of Nile tilapia. Data are mean values with their standard errors. Mean values were significantly different: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ (one-way ANOVA).

and (b)). Because liver is an important metabolic organ for blood glucose regulation, the expression level of genes related to glucose metabolism was also detected. The result showed that HC diet obviously reduced the gene expression of insulin receptor compared with CON ($P < 0{\cdot}05$), but the supplement of inulin distinctly up-regulated the expression level of this gene ($P < 0{\cdot}05$, Fig. 4(c)). We also found that the glycogen content in HC was similar with CON, but it was higher in HCI ($P < 0{\cdot}05$, Fig. 4(d)). In line with the tendency, gene expression of glycogen synthase (*gs*), coding a key enzyme for glycogen synthesis, was not influenced by high level of carbohydrate, but the expression level of this gene was significantly higher in HCI compared with HC ($P < 0{\cdot}05$, Fig. 4(e)). The above data suggested that inulin could improve high-carbohydrate-induced glucose metabolism disorder by increasing insulin sensitivity and liver glycogen content.

Addition of inulin in high-carbohydrate diet altered intestinal microbiota composition and their metabolites

A total of 1 798 795 high-quality sequences were obtained from twenty-one samples (seven individuals in each group) with an average of 85 656 sequences per fish. The α-diversity indices displayed that Chao1, Simpson and Shannon indexes were not influenced by high carbohydrate and inulin (Fig. 5(a)–(c)). The microbial community composition analysis showed that the most dominant phylum in all samples was Proteobacteria, representing 70, 55 and 44 % of the total sequences in CON, HC and HCI, respectively. Actinobacteria was accounted for 27 % in HC which was higher than that in CON (3 %), and the proportion of this phylum was 11 % in HCI. The relative abundance of Fusobacteria (9 %) was lower in HC treatment than in CON (19 %), but it increased to 30 % in HCI treatment (Fig. 5(d)). The microbiota composition at the phylum level of each sample was similar within the same treatment, but was different among three diets (Fig. 5(e)). Principal component analysis was conducted according to the proportion of OTU in different groups, and the results showed that intestinal microbiota in these three groups were obviously distinguished, suggesting that either high-carbohydrate diet or addition of inulin could influence gut microbiota (Fig. 5(f)). Compared with CON, six OTU including OTU1142 (Microbacteriaceae), OTU971 (Microbacteriaceae), OTU624 (Rhodobacteraceae), OTU449 (Rhodobacteraceae), OTU228 (Rhodobacteraceae) and OTU1135 (Xanthobacteraceae) were significantly increased in HC, while they were decreased in HCI ($P < 0{\cdot}05$). High-carbohydrate diet increased the abundance of OTU327 (Microbacteriaceae) which was further increased in inulin supplementation diet ($P < 0{\cdot}05$). In contrast with this tendency, the abundance of OTU1249 (Rhodobacteraceae) was lower in the HC group than in the CON group and was lowest in the HCI group ($P < 0{\cdot}05$). No significant difference was found in the abundance of six OTU, including OTU1329 (Mycobacteriaceae), OTU2450 (Fusobacteriaceae), OTU116 (Fusobacteriaceae), OTU571 (Brucellaceae), OTU816 (Comamonadaceae) and OTU364 (Oxalobacteraceae), between CON and HC treatment, but addition of inulin significantly increased the abundance

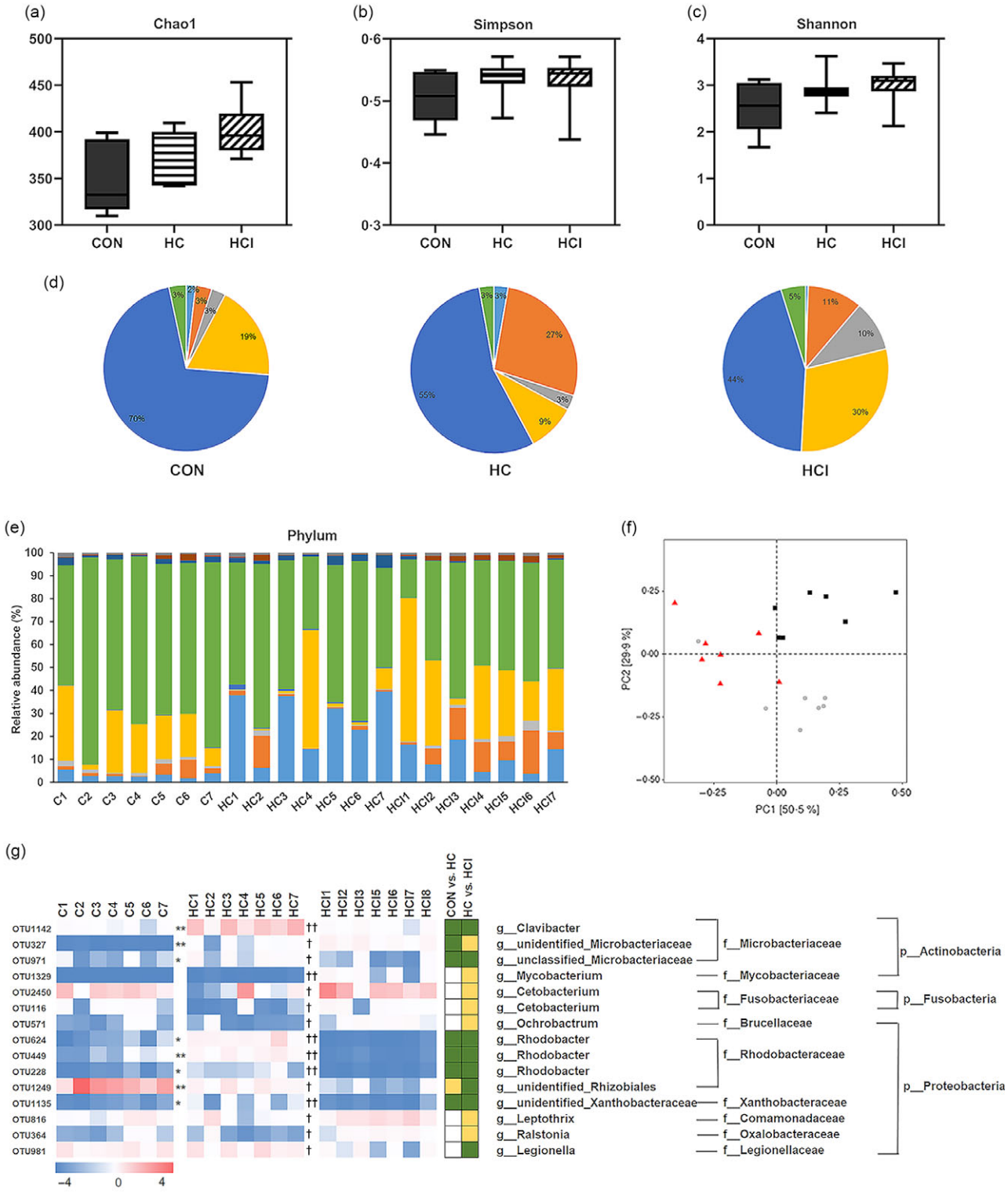

Fig. 5. Intestinal microbiota composition was altered by inulin. (a) Chao1; (b) Simpson; (c) Shannon indexes; (d) proportion of dominant phyla (■, Verrucomicrobia; ■, Actinobacteria; ■, Thermomicrobia; ■, Fusobacteria; ■, Proteobacteria; ■, others); (e) microbiota composition at phylum level of each sample (■, others; Bacteroidetes; ■, Verrucomicrobia; ■, Proteobacteria; ■, Planctomycetes; ■, Fusobacteria; ■, Firmicutes; ■, Thermomicrobia; ■, Actinobacteria); (f) principal component analysis (PCA). ■, Fish fed with 35 % starch (CON); ■, fish fed with 45 % starch (HC); ○, fish fed with 45 % starch supplemented with 5 g/kg inulin (HCI). Heat map analyses of operational taxonomic units (OTU) showing significantly different among groups. Green indicated that the abundance of OTU was higher in the HC group, and yellow indicated that abundance of OTU was lower in the HC group (g). Data are mean values with their standard errors. Mean values of CON and HC groups were significantly different: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$. Mean values of HC and HCI groups were significantly different: † $P < 0{\cdot}05$, †† $P < 0{\cdot}01$.

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of these OTU ($P < 0{\cdot}05$) (Fig. 5(g)), suggesting that inulin did not influence microbial complexity of Nile tilapia, but altered the gut microbiota composition.

The production of SCFA was also detected. Compared with the control diet, high-carbohydrate diet did not affect production of acetic acid and propionic acid (Fig. 6(a) and (b)). But the supplement of inulin significantly increased the content of acetic acid and propionic acid in gut ($P < 0{\cdot}05$) (Fig. 6(a) and (b)). In addition, the production of butyric acid was markedly decreased in HC treatment ($P < 0{\cdot}01$), while there was no difference between HC and HCI treatments (Fig. 6(c)).

Discussion

Inulin is widely used in aquaculture, but the influence of inulin on growth condition of fish remains controversial. Recent studies have found that grass carp (*Ctenopharyngodon idella*) and rainbow trout (*O. mykiss*) fed with inulin resulted in higher weight gain, specific growth rate and feed condition rate[26,27]. Ibrahem *et al.* and Tiengtam *et al.* also found that supplementation of 5 g/kg inulin in diet enhanced the final weight of Nile tilapia[16,28]. While some studies showed that addition of inulin did no influence on the growth condition of juvenile beluga (*Huso huso*), Indian white shrimp (*Fenneropenaeus indicus*) and common carp (*Cyprinus carpio*)[29–32]. Host phylogeny, the concentration of inulin, the duration of the feeding experiment and the diet composition may account for the inconsistency. In order to identify the influence of inulin on fish fed with high-carbohydrate diet and to reveal the possible mechanism, we detected the growth condition and intestinal microbial composition in Nile tilapia. We found no difference on growth performance or body composition among three treatments, but addition of inulin changed the metabolic characteristics of fish and altered intestinal microbiota composition and their metabolites.

It has been reported that high-carbohydrate diet could suppress innate immunity and thus affect the health status and disease resistance[1,5]. Dietary inulin always acts as immunostimulant to improve immune status in aquaculture. For example, inulin supplementation improved the resistance of Pacific white shrimp (*Litopenaeus vannamei*) and Nile tilapia to *Vibrio alginolyticus* or *A. hydrophila* and the enhancement of immunity response including higher nitroblue tetrazolium activity and higher gene expression of TLR were observed[28,33]. Sheikholeslami *et al.* also found that rainbow trout fed with 5 and 20 g/kg inulin could increase lysozyme activity and leucocytes to resist against *Streptococcus* sp.[34]. However, the connection between inulin and immunostimulant effect in aquatic animals remains unknown. In mammals, the beneficial effects of inulin treatment on immune dysfunction were attributed to the increase in SCFA formation[35]. In the present study, the survival rate was higher in the HCI group than that in the HC group post a pathogen challenge. We also found the addition of inulin altered the composition of gut microbiota and increased the content of acetate and propionate. It has been shown that addition of acetate in high-carbohydrate diet could alleviate intestinal inflammation by inhibiting MAPK and NF-κB signalling pathways in Nile tilapia[5]. Hoseinifar *et al.* found that dietary sodium propionate improved mucosal and non-specific immune responses of Caspian white fish (*Rutilus frisii kutum*) including total Ig, lysozyme activity and alternative haemolytic complement activity[36]. These results suggested that in spare of the different composition of gut microbiota in fish and mammals, inulin could improve the immune response and pathogen resistance in fish by modulating the intestinal composition and their metabolites.

Excessive carbohydrate in the diet induced lipid accumulation and glucose intolerance in fish[37,38]. In the present study, we found that supplement of inulin in high-carbohydrate diet alleviated the lipid accumulation by down-regulating genes related to lipogenesis and up-regulating genes related to *β*-oxidation. And inulin also regulated glucose homoeostasis by improving insulin sensitivity. Supplement of inulin could alleviate lipid accumulation by reducing lipogenesis and increasing energy expenditure which was closely related to the alteration of intestinal microbiota composition and the production of SCFA in mammals[39,40]. However, how inulin regulates the metabolic characteristics in fish remains elusive. In order to identify the mechanism by which inulin regulates the metabolic characteristics in fish, we detected the gut microbiota composition and their metabolites. We found that inulin did not influence gut microbiota diversity, but changed the gut microbiota composition. In addition, we found that addition of inulin obviously increased the abundance of Fusobacteria in Nile tilapia, especially *Cetobacterium*. This genus commonly exists in the intestinal tracts of fishes and whales[41], and it could produce acetic and propionic acids by fermenting

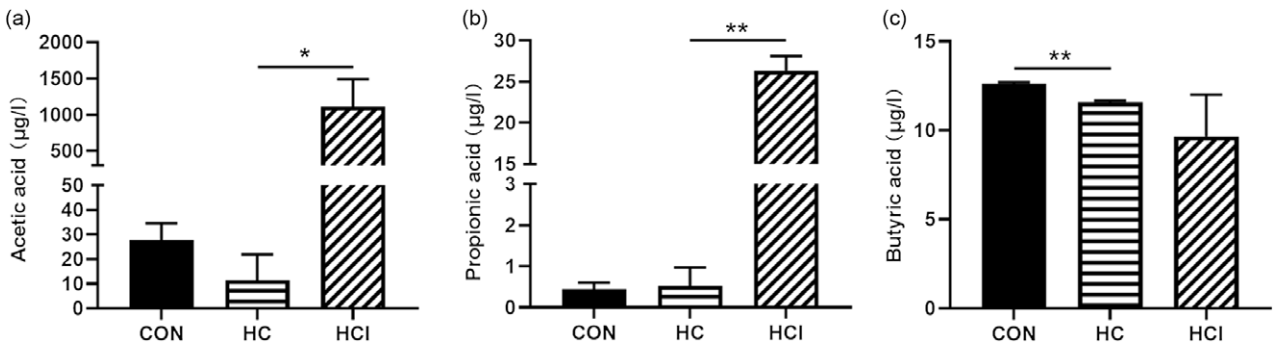

Fig. 6. Concentration of SCFA of three treatments. (a) Acetic acid; (b) propionic acid and (c) butyric acid in the gut of Nile tilapia. Data are mean values with their standard errors. Mean values were significantly different: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ (one-way ANOVA). CON, fish fed with 35 % starch; HC, fish fed with 45 % starch; HCI, fish fed with 45 % starch supplemented with 5 g/kg inulin.

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carbohydrates[42]. 16S rRNA sequencing, which provides valuable information on the comparison of microbiota composition among different groups, has been widely used to identify the intestinal microbiota in aquatic animals[43]. However, 16S rRNA sequencing cannot reveal the bacterial function directly[44]. In order to identify the bacterial function, we analysed the content of SCFA, which are produced by microbial fermentation in the gut. We found that inulin supplementation increased the production of acetic acid and propionic acid but did not influence the concentration of butyrate in fish gut. Geraylou *et al.* found that addition of prebiotics enhanced growth performance and immune responses in Siberian sturgeon (*Acipenser baerii*) and giant freshwater prawn (*Macrobrachium rosenbergii*) and increased production of acetic acid and propionic acid may account for these changes[45,46]. It has been reported that diet supplemented with acetate or propionate enhanced the growth and immune parameters in Nile tilapia and zebrafish (*Danio rerio*)[5,47]. Although the influence of SCFA on fish health is just starting, SCFA showed multiple beneficial effects on mammals. Zou *et al.* found that inulin prevented dysglycaemia and hepatosteatosis induced by high-fat diet in a microbiota-dependent manner and enhanced the production of acetic acid and propionic acid of C57BL/6 mice[48]. Weitkunat *et al.* found that addition of propionic acid in high-fat diet improved insulin resistance and prevented hepatic steatosis by decreasing hepatic TAG in mice[39]. The above results suggested that the main mechanism by which inulin regulates fish metabolism may attribute to the change of intestinal microbiota and their metabolites.

Conclusions

In conclusion, the present study indicated that inulin could ameliorate inflammation status induced by high-carbohydrate diet to increase the pathogen resistance in Nile tilapia. We also found that supplement of inulin altered the gut microbiota composition and their metabolites, which may account for the alleviation of metabolic syndromes induced by high-carbohydrate diet in fish. To our knowledge, this is the first study revealing the mechanism of inulin in regulating metabolic disorders induced by high-carbohydrate diet. In order to improve the efficiency and sustainability of aquaculture production, the potential beneficial effects of prebiotics and their underlying mechanisms should be identified deeply.

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M.-L. Z. and Z.-Y. D. designed the research. T. W., N. Z. and X.-B. Y. conducted all experiments. T. W. analysed the data. F. Q., L.-Q. C. and M.-L Z. provided the assistance in analysing data. T. W. wrote the manuscript. M.-L. Z. revised the manuscript. M.-L. Z. had primary responsibility for final content. All authors read and approved the final manuscript.

The authors declare no conflicts of interest.

Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S000711452000402X

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