Transcriptome analysis of grass carp provides insights into disease-related genes and novel regulation pattern of bile acid feedback in response to lithocholic acid

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ABSTRACT

Gut microbiota and its metabolites affect lipid metabolism and liver health. Grass carp (\textit{Ctenopharyngodon idellus}) often suffers from functional disorders of liver and gallbladder, which is usually accompanied by accumulation of lipids in the liver. Here we studied liver transcriptome and microbial fluctuation in the gut in response to dietary lithocholic acid (LCA; a metabolite of gut microbiota) supplementation in grass carp. A total of 1802 differentially expressed genes (DEGs) were identified between LCA and Control groups according to the liver transcriptome analysis. Of these DEGs, 8 nonalcoholic fatty liver disease-related genes were all upregulated, and 11 of 12 cancer related genes were upregulated in the LCA group. We also found significantly upregulated bile acid receptor, hepatocyte nuclear factor 4-beta (HNF4B), in the LCA group. Oil O staining of livers showed a higher abundance of lipid droplets in the LCA group. LCA also changed the composition of gut microbiota, with increasing proportion of Proteobacteria and Firmicutes, whereas Fusobacteria decreased. Co-occurrence between DEGs and microbial taxa was mainly identified between signal transduction and genetic information processing-related genes and Proteobacteria and Firmicutes taxa. Overall, our results elucidate the effects of LCA on liver and gut microbiota in fish, and suggest a novel pattern of bile acid feedback in grass carp. These results contribute to our understanding of the interplay between gut microbiota and liver diseases in grass carp.

1. Introduction

The gut microbiota is involved in the regulation of host metabolism, giving rise to interactive host-microbiota metabolic signaling and immune-inflammatory axes that physiologically connect the gut, liver, muscles, and brain (Nicholson et al., 2012). Within these metabolic axes, gut microbiota can modulate metabolic reactions, so the metabolism of some substrates is often combined of microbial and host's metabolic steps. An example of this is the production of bile acids (BAs), choline, and short-chain fatty acids, all of which are essential for host health (Nicholson and Wilson, 2003). Production of these metabolites by microbes contributes to the host’s metabolic phenotype and hence to disease risk. Secondary BAs, including lithocholic acid (LCA) and deoxycholic acid (DCA), are metabolites of specific gut bacteria with the capability of 7a-dehydroxylation activity (Ridlon et al., 2006). In turn, secondary BAs also appear to regulate the structure of gut microbiome and host physiology (Ridlon et al., 2016). It is well known that they can serve endocrine functions in the body, largely through binding and activation of nuclear receptors, e.g. the Farnesoid X Receptor (FXR) (Makishima et al., 1999a; Parks et al., 1999) and the plasma membrane BA receptor, also known as G protein-coupled BA receptor 1 (GPBAR1 or TGR5) (Kawamata et al., 2003). Activation of these two receptors can regulate the primary BA synthesis, conjugation, transport and detoxification, as well as lipid, glucose, and energy homeostasis (Li and Chiang, 2015).

As it receives most of its blood supply from the intestine, the liver represents the first line of defence against gut-derived xenobiotics, including metabolites of gut microbiota and bacterial endotoxins. The gut
microbiota can play a major role in liver health through this gut–liver axis (Compare et al., 2012). For example, several lines of evidence indicate that the gut microbiota plays a role in the etiology of nonalcoholic fatty liver disease (NAFLD), including the progression to its more advanced stage, and nonalcoholic steatohepatitis (NASH) in mammals (Abu Shanab and Quigley, 2010). Gut microbiota-derived metabolites, i.e. secondary BAs (LCA and DCA), have been associated with liver cancers in mammals (Yoshimoto et al., 2013). Supplementation of LCA to diet resulted in cirrhotic and necrotic damage in the liver of rabbits, which appeared to correlate with bile composition (Fischer et al., 1974).

As opposed to mammals, the effects (and associated mechanisms) of secondary BAs on the regulation of metabolic pathways and physiology in lower vertebrates, e.g. fish, remain poorly understood. Grass carp (Ctenopharyngodon idellus), the major farmed fish globally in terms of the total production output (Faoro, 2015), often suffers from functional disorders of liver and gallbladder, which is accompanied by the accumulation of lipids in liver, and can result in high mortality (Ni and Wang, 1999; Cai and Curtis, 1989; Li et al., 2016). Elucidating the interactions between secondary BA and liver in grass carp may help us find a treatment for these diseases and better understand the metabolism and physiology of secondary BA in lower vertebrates. Herein, we aimed to assess the effects of commercially produced LCA on fish liver health and composition of gut microbiota as well as studying the interactions between changes of gut microbiota composition and liver transcriptome.

2. Materials and methods

2.1. Fish and feeding trial

Juvenile grass carp (n ≈ 200) were obtained from a fish hatchery in Wuthan, China. Prior to the experiment, fish were reared in 1000-L plastic tanks for two weeks to acclimatize them to the experimental conditions. Then, 90 similarly-sized fish (40 ± 5 g) were randomly distributed into six aquariums (125 L of water each) at the stocking density of 15 fish per tank. Three aquariums were randomly chosen as control groups and fed the control (normal) diet, while the other three aquariums were set as the LCA group and fed the experiment diet with an addition of sodium taurocholate (TLCAS) at the concentration of 0.2 mmol per kg of the control diet. The dose of TLCAS used here was selected on the basis of the fact that a similar dose of a mixture of BAs can promote the growth of grass carp (Chen, 2016). TLCAS was purchased from Sigma-Aldrich (St. Louis, MO). The normal (control) diet was composed of 32.7% protein, 5.6% fat and 5.0% fiber. Fish were fed to apparent satiation three times daily (08:30, 12:30 and 17:30) for six weeks. During the feeding trial, fish were maintained under the natural photoperiod conditions, water temperature ranged from 22 to 23 °C, pH value fluctuated between 7.2 and 7.4, and water replacement rate was adjusted to keep the total ammonia nitrogen and nitrites below 0.2 and 0.005 mg/L, respectively. At the end of the feeding trial, three fish were sampled randomly from each tank (adding up to nine specimens per treatment), they were anesthetized in diluted tricaine methanesulfonate (MS-222, Sigma, USA) at the concentration of 100 mg/L. Blood samples were collected from all three anesthetized fish from each tank, whereas liver and hindgut contents were sampled only from two specimens (euthanized and dissected) per tank. Blood samples were collected from the caudal vein by a non-heparinized syringe and then centrifuged at 3500 × g for 10 min. The resulting serum samples, liver and hindgut contents were stored at −80 °C for subsequent analyses. All animal handling procedures and experiments were reviewed and approved by the ethics committee of the Institute of Hydrobiology, Chinese Academy of Sciences.

2.2. Biochemical analysis

Serum alanine aminotransferase (ALT) activities and aspartate transaminase (AST) were quantified by the enzymatic colorimetric assay using a commercial assay kit from Fosun Long March Medical Science Co., Ltd., Shanghai, P.R. China. t-test was performed for the activities of each parameter between two groups.

2.3. Sequencing and analysis of liver transcriptome data

RNA of livers collected as described above was isolated using RNasy Mini Kit (Qiagen, Germany) according the manufacturer's protocol. RNA concentration was measured using the Qubit RNA assay kit (Life Technologies, USA), and integrity was assessed with the RNA Nano 6000 assay kit (Agilent Technologies, USA). RNA of sufficiently high quality was used for the library construction, conducted using the NEBNext Ultra RNA library prep kit for Illumina (New England Biolabs, USA) following the manufacturer's protocol. Briefly, mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads, and fragmented by the NEBNext first strand synthesis reaction buffer (New England Biolabs). First strand cDNA was synthesized using a random hexamer primer and M-MuLV reverse transcriptase. Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. After adenylation of the 3’ end of DNA fragments, NEBNext adaptors with a hairpin loop structure were ligated in preparation for hybridization. Subsequently, 3 μL USER enzyme (New England Biolabs, USA) was incubated with size-selected, adaptor-ligated cDNA at 37 °C for 15 min, followed by 5 min at 95 °C, to be immediately followed by PCR, using Phusion High-fidelity DNA polymerase, universal PCR primers and index (X) primer. Finally, PCR products were purified using an AMPure XP system and library quality assessed was using an Agilent Bioanalyzer 2100 system. Libraries were sequenced on an Illumina Hiseq4000 platform and 150 bp pair-end reads were generated. Raw data reads in fastq format were initially processed using in-house perl scripts. In this step, clean data (clean reads) were obtained by removing adapter, poly-N and poor quality reads. The Q30 and GC content of the clean data were calculated, and all downstream analysis performed, using the clean high quality data. Clean data were mapped to the grass carp reference genome (Wang et al., 2015) using Hisat2 software (Kim et al., 2015). StringTie v1.2.3 software was used for the transcript assembling and to count the number of reads mapped to each transcript (Pertea et al., 2015). The transcripts were subjected to the BLASTX similarity search against UniProt database with an E-value threshold of 10e5, and UniProt accession IDs were used for the functional annotation of transcripts. Differential expression analysis of two groups was performed using the Ballgown package, and the fragments per kilobase of exon model per million mapped reads (FPKM) were calculated for each transcript on the basis of the length of the transcript and the number of reads mapped to the transcript (Pertea et al., 2016). The resulting P-values were adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate. Transcripts with an adjusted P-value < .05 (q value < 0.05) were identified as differentially expressed genes (DEGs). The Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation analyses were performed with the DAVID v6.8 platform (Huang et al., 2008). In the GO enrichment analysis, only categories with a P-value < .05 were considered as enriched in the network as determined by two-sided hypergeometric statistical tests employing the Benjamini and Hochberg approach to false discovery rate correction. The KEGG database is used to provide high-level functional information on biological systems of molecules, cells, organisms and ecosystems, and is particularly powerful for the evaluation of large-scale molecular datasets generated by genome sequencing and other high-throughput experimental approaches (Kanehisa et al., 2007). In this study, KEGG terms with corrected P < 0.05 were considered to indicate statistical significance.
2.4. Validation of DEGs by RT-qPCR

To confirm the reliability of data obtained by RNA-Seq, 10 DEGs were selected for validation by RT-qPCR. The primers used in RT-qPCR are listed in Additional file 1. First strand cDNAs were obtained using a random hexamer primer and the ReverTra Ace kit (Toyobo, Japan). RT-qPCR was carried out using a fluorescence quantitative PCR instrument (Bio-Rad, USA). Each RT-qPCR mixture contained 0.8 μL forward and reverse primers (for each primer), 1 μL template, 10 μL 2× SYBRgreen master mix (TOYOBO, Japan), and 7.4 μL ddH2O. Three replicates were included for each sample and β-actin gene was used as an internal control to normalize gene expression. The program for RT-qPCR was as follows: 95°C for 10 s, 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. Relative expression levels were calculated using the 2^ΔΔCt method. Data represent the mean ± standard deviation of three replicates.

2.5. Oil red O staining of frozen liver

To confirm the results of transcriptome analysis, we stained liver sections with Oil Red O to detect lipids in liver. Frozen liver sections were stained with Oil Red O to detect lipid droplets and counterstained with Mayer’s hematoxylin (Luna, 1968). The percentage of oil red O-positive area to the internal surface was measured with a computer-assisted morphometry system (Image Pro Plus 6.0, USA). Data represent the mean ± standard deviation of each group. t-test was performed to compare the abundance of lipid droplets between two groups.

2.6. Preparation and analysis of 16S rRNA amplicon sequencing

DNA was extracted from hindgut contents of 12 samples using QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA) was used to check the concentration and quality of the extracted DNA. Extracted DNA was diluted to 10 ng/μL and stored at −80°C for downstream use. Universal primers 515F (5’-GTGYCAGCMGCCGCGGTAA-3’) and 806R (5’-GGACTACNVGGGTWTCTAAT-3’), with a 12 nt unique barcode at 5′-end of 515F, were used to amplify the hypervariable V4 region of the bacterial 16S rRNA gene (Caporaso et al., 2011). Each sample was amplified in triplicate, in a 25 μL reaction mix containing 2×GoTaq Green Master Mix (Promega, USA), 1 μM of each primer and 10 ng genomic DNA. The program included 5 min at 94°C, followed by 23 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and finally 10 min at 72°C. PCR products were purified using the MinElute 96 UF PCR Purification Kit (Qiagen, Valencia, CA, USA). All samples were sequenced using an Illumina Hiseq platform (HiSeq Reagent Kit V2, 500 cycles) by the Novogene Biotechnology Co., Ltd. (Beijing).

The raw sequence data were processed using QIIME Pipeline-Version 1.8.0 (http://qiime.org/) (Caporaso et al., 2010). Overlapping paired end reads were merged using the FLASH-1.2.8 software (Magoc and Salzberg, 2011). Only the merged sequences with high-quality reads (length > 250 bp, without ambiguous bases BN, and average base quality score > 30) were used for further analysis. All sequences were trimmed and assigned to each sample based on their barcodes (barcode mismatches = 0). Chimeras were removed using the Uchime algorithm (Edgar et al., 2011). Non-chimera sequences were subsampled to the same sequence depth (11,784 reads per sample) using daisychopper.pl (Gilbert et al., 2009). This subset of sequences was clustered into operational taxonomic units (OTUs) at 97% identity threshold using CD-HIT algorithm (Li and Godzik, 2006). Singleton sequences were filtered out. OTU identities were assigned in Greengenes database (release 13.8) (De Santis et al., 2006) using UCLUST algorithm (Edgar, 2010). Sequences classified as unassigned and C_Chloroplast were removed. Alpha diversity (Chao1, PD_whole_tree, Shannon and Simpson index) and beta diversity (weighted UniFrac metric and Bray-Curtis distance) indices of bacterial communities were calculated. Principal coordinate analysis (PCoA) was used to visualize similarities between groupings with weighted unifrac distance (Navas Molina et al., 2013). Permutational multivariate analysis of variance (PERMANOVA) analysis was performed to test for significant differences between groups in overall microbial composition with weighted_unifrac distance (Kelly et al., 2015). t-test was performed to compare the taxonomic abundance of OTUs between the two groups.

2.7. Co-expression network

The relative errors of gut OTU abundance and FPKM of DEGs in liver were used to conduct the co-expression network analysis with the WGCNA packages in R (Langfelder and Horvath, 2008). The modules were obtained using the automatic network construction adjacency with default settings; with the exception of using ‘soft threshold’ (power = 40). The networks were visualized using Cytoscape v.3.6.1 (Shannon et al., 2003). Significance of correlation between liver genes and bacterial taxa was evaluated using the Pearson correlation coefficient (r, P) with R software.

2.8. Data accessibility

Raw sequencing reads data of liver transcriptomes has been deposited to Sequence Read Archive in NCBI in BioProject PRJNA477492. The obtained raw 16S rRNA sequences were deposited in the NCBI/EBI/DDBJ Sequence Read Archive (Bioproject: PRJNA416986) with the accession numbers SRR6251438 and SRR6251440.

3. Results

3.1. ALT and AST activities

In the LCA group, the average activities of serum ALT and AST were both higher than that in the Control group (Table 1), but neither was significantly different between two groups (P = 0.06; P = 0.07, respectively).

3.2. Transcriptome sequence and differential expression analysis

RNA sequencing resulted in about 193.6 million (Control) and 169.2 million (LCA) raw reads. After filtration, 154.0 (Control group) and 123.4 million (LCA group) of clean reads were obtained, and these sequences were used for subsequent analyses. On average, 70% of the clean reads were mapped to the reference genome (Table 2). The differential expression analysis results showed that 1802 genes were significantly differently expressed, including 407 up-regulated genes (fold change (fc) ≥ 2, P < 0.05) and 389 down-regulated genes (fc ≤ 0.5, P < 0.05) (Fig. 1A). Among these 1802 DEGs, known BA receptors, FXR, TGR5, HNF4B, and VDR, were not included, but we found another nuclear receptor, a member of the hepatocyte nuclear factor-4 family (HF4B), upregulated in the liver of the LCA group (fc = 2.34, P = 0.001).

3.3. GO and KEGG enrichment analysis of differentially expressed genes

The results of the GO enrichment analysis of DEGs were classified into three categories: biological process (23 subclasses), cellular component (11 subclasses) and molecular function (20 subclasses).

Table 1

<table>
<thead>
<tr>
<th>Serum biochemical parameters</th>
<th>Control</th>
<th>LCA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT(μL-1)</td>
<td>25.62 ± 6.36</td>
<td>30.54 ± 10.23</td>
<td>0.06</td>
</tr>
<tr>
<td>AST(μL-1)</td>
<td>121.25 ± 30.12</td>
<td>158.67 ± 35.44</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Significant GO terms in these three categories and associated DEGs are shown in Fig. S1. The KEGG pathway enrichment analysis, conducted to further elucidate biological functions of DEGs, revealed that 447 of 1802 DEGswere annotated to 193 signaling pathways (Additional file 1). Among these, 9 DEGs were assigned to cytochrome P450 family, 5 of which were upregulated and 4 downregulated in the LCA group; 4 of 11 DEGs related to metabolism of xenobiotics by cytochrome P450 were upregulated and 7 were downregulated in the LCA group; all 8 DEGs involved in the NAFLD pathway, including Cytochrome C1 (CYC1), Ubiquinol-Cytochrome C Reductase Core Protein 2 (UQCRC2), NADH: Ubiquinone Oxidoreductase Core Subunit S1 (NDUFS1), NADH: Ubiquinone Oxidoreductase Subunit A13 (NDUFA13), NADH: Ubiquinone Oxidoreductase Core Subunit S8 (NDUFS8), one insulin receptor (INSR), AMP-activated protein kinase subunit beta-1 (PRKAB1) and Mitogen-activated protein kinase protein 5 (MAP2K5), were upregulated in the LCA group; all 3 DEGs involved in p53 signaling pathway were upregulated in the LCA group; and finally, 11 of 12 DEGs related to pathways in cancer were upregulated (1 downregulated) in the LCA group, including Cadherin-1 (CDH1), FMS-like tyrosine kinase 3 (FLT3), Cyclin dependent kinase inhibitor 1A (CDKN1A), Peroxisome proliferator-activated receptors (PPARD), Guanine nucleotide-binding proteins (GNAI1), β-catenin (CTNNB1), Signal transducer and activator of transcription 1 (STAT1), Guanine nucleotide-binding proteins (GNB2), Phospholipase C beta 2 (PLCB2), Protein tyrosine kinase 2 (PTK2) and NF-kappa-B inhibitor alpha (NFKBIA), whereas the only downregulated DEG was EGL-9 family hypoxia inducible factor 2 (EGLN2) (Fig. 1B).

Table 2
Summary of transcriptome sequencing and mapping.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Raw Reads</th>
<th>Raw Base(G)</th>
<th>GC Content (%)</th>
<th>Filter reads</th>
<th>Mapped reads ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont_1</td>
<td>33,336,412</td>
<td>5.00</td>
<td>55.62</td>
<td>26,437,327</td>
<td>47.13</td>
</tr>
<tr>
<td>Cont_2</td>
<td>30,767,896</td>
<td>4.62</td>
<td>49.58</td>
<td>23,846,486</td>
<td>60.08</td>
</tr>
<tr>
<td>Cont_4</td>
<td>32,169,494</td>
<td>4.83</td>
<td>48.92</td>
<td>25,476,690</td>
<td>71.98</td>
</tr>
<tr>
<td>Cont_5</td>
<td>26,457,466</td>
<td>3.97</td>
<td>48.17</td>
<td>21,793,835</td>
<td>46.27</td>
</tr>
<tr>
<td>Cont_7</td>
<td>43,421,092</td>
<td>6.51</td>
<td>49.17</td>
<td>34,745,901</td>
<td>75.14</td>
</tr>
<tr>
<td>Cont_8</td>
<td>27,547,120</td>
<td>4.13</td>
<td>47.83</td>
<td>21,655,248</td>
<td>79.83</td>
</tr>
<tr>
<td>LCA_1</td>
<td>37,715,756</td>
<td>5.66</td>
<td>48.77</td>
<td>28,533,311</td>
<td>74.32</td>
</tr>
<tr>
<td>LCA_2</td>
<td>35,682,910</td>
<td>5.35</td>
<td>50.02</td>
<td>25,864,879</td>
<td>77.85</td>
</tr>
<tr>
<td>LCA_4</td>
<td>24,534,026</td>
<td>3.68</td>
<td>49.55</td>
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<td>71.98</td>
</tr>
<tr>
<td>LCA_5</td>
<td>28,550,342</td>
<td>4.28</td>
<td>50.69</td>
<td>20,552,725</td>
<td>80.56</td>
</tr>
<tr>
<td>LCA_7</td>
<td>18,901,408</td>
<td>2.83</td>
<td>51.16</td>
<td>13,782,393</td>
<td>76.01</td>
</tr>
<tr>
<td>LCA_8</td>
<td>23,791,420</td>
<td>3.57</td>
<td>50.44</td>
<td>16,927,923</td>
<td>81.71</td>
</tr>
</tbody>
</table>

Fig. 1. Distribution trends of differentially expressed genes between LCA and Control groups. (A) Volcano Plot. The log2 fold (LCA/Control) indicates the mean expression level for each gene. Each dot represents one gene. Red dots represent up-regulated genes and blue dots represent down-regulated genes. Green dots represent genes that were not differentially expressed between the two groups. (B) Heatmap of candidate DEGs. The colors of row bars indicate KEGG families with which candidate DEGs were associated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Validation of differential expression by quantitative real-time PCR. The x-axis indicates the names of candidate genes, the y-axis indicates the relative expression (LCA/Control) of each gene.

KEGG pathways
- Biosynthesis of primary bile acids
- Metabolism of xenobiotics by cytochrome P450
- Steroid biosynthesis
- Mitochondrion inner membrane
- p53 signaling pathway
- Pathways in cancer
- Glycolysis/gluconeogenesis
- Arginine and proline metabolism
- MAPK signaling pathway
- Metabolism of xenobiotics by cytochrome P450
- Peroxisome proliferator-activated receptor signaling pathway
- Claudin 5
- Toll-like receptor signaling pathway
- Phosphatidylinositol 3-kinase signaling pathway
- Schwann cell differentiation
- Peroxisome proliferator-activated receptor signaling pathway
- Regulation of actin cytoskeleton
- Notch signaling pathway
- Mitochondrion inner membrane
- Notch signaling pathway
3.4. The validation of candidate genes

To corroborate the transcriptomics results, we selected relevant 10 DEGs, among which 5 were upregulated and 5 downregulated, and tested their expression in liver tissue by qRT-PCR. The results (Fig. 2) were fully congruent between the two methods, thereby indirectly confirming the validity of the RNA-seq analysis.

3.5. Oil red staining of liver sections

The lipid droplet area percentage (%) was 4.6 ± 0.9 in the Control group and 11.2 ± 2.5 in the LCA group (P = 0.03) (Fig. 3B). Some vacuolations were observed, but as we used frozen samples for the staining, they could be caused by freeze-thaw of the tissue.

3.6. Gut microbiota

After the initial quality filtering, chimera checking, and OTU picking, 360 non-singleton OTUs were identified at the 97% similarity level, 33 of which exhibited significant differences between two groups (P < 0.05). Among these different OTUs, 20 belonged to Proteobacteria, 6 belonged to Fusobacteria, 4 belonged to Tenericutes, 1 belonged to Actinobacteria and 1 belonged to Thermi. The abundance of almost all Proteobacteria OTUs (19 of 20) was upregulated, and all 6 OTUs of Fusobacteria were downregulated, in the LCA group (Fig. 4A). At the phylum level, the composition of gut microbiota showed that LCA supplementation increased the proportion of Proteobacteria and Firmicutes, while decreasing that of Fusobacteria (Fig. 4B, Fig. S2). The Firmicutes/Bacteroidetes (F/B ratio) was significantly (P = 0.01) higher in the LCA group (7.4 ± 6.4) in comparison to the Control group (0.13 ± 0.13). Both heatmap (Fig. 4A) and PCoA with weighted_unifrac distance (Fig. 4C) showed that two samples from the LCA group (LCAS and LCB) clustered together with the Control group, which indicates individual differences in the LCA group. However, PERMANOVA analyses showed a significant difference between the LCA and Control groups, with P = 0.006 and F = 9.716. Therefore, the results indicate that populations of gut microbiota were significantly different between the two groups.
In summary, LCA supplementation significantly changed the composition of gut microbiota, increasing the proportion of Proteobacteria and Firmicutes, while decreasing that of Fusobacteria.

### 3.7. Co-occurrence

The WGCNA results showed that co-occurrence network could be divided into 8 modules. Interactions between gut OTUs and liver genes mainly happened in the second (M2), fifth (M5) and sixth (M6) modules, but rarely in others (Fig. 5). In the second module (M2), 28 Proteobacteria OTUs, 4 Firmicutes OTUs, 3 Verrucomicrobia OTUs, 3 Actinobacteria OTUs, 1 Chlamydiae OTU and 9 DEGs were involved. Among these 9 involved DEGs, 2 DEGs did not have a KEGG Orthology (KO) assigned, 6 were related to signal transduction and genetic information processing, and 1 was related to the immune system (Table 3). In the fifth module (M5), 10 Proteobacteria OTUs, 4 Verrucomicrobia OTUs, 4 Bacteroidetes OTUs and 6 DEGs were involved. Of these involved DEGs, 2 DEGs had no KO assigned, 3 were related to signal transduction and genetic information processing and 1 was related to signaling and cellular processes. In the sixth module (M6), 16 Proteobacteria OTUs, 5 Firmicutes OTUs, 2 Bacteroidetes OTUs, and 1 Thermi OTU were involved, and 7 DEGs showed interactions with these microbial taxa. Of these involved DEGs, 1 DEG did not have a KO assigned, 4 were related to the digestive system and metabolism, 1 was related to signal transduction, and 1 was related to signaling and cellular processes. Among the overall involved DEGs in three modules, 5 DEGs did not have a KO assigned, 5 were related to signal transduction (4 of which were related to the PI3K-Akt signaling pathway), 5 were related to genetic information processing, 4 were related to digestive system and metabolism, 2 were related to signaling and cellular processes, and 1 was related to the immune system (Table 3).

In summary, most OTUs involved in these interactions were assigned to Proteobacteria and Firmicutes, and most of the involved DEGs were related to signal transduction and genetic information processing, especially the PI3K-Akt signaling pathway.
conserved between zebrafish and mammals, which indicates that enzyme activities and physiological functions are also relatively conserved (Goldstone et al., 2010). We found that gene expression levels of two important rate-limiting enzymes, Cyp7a1 and Cyp7b1 (involved in the conversion of cholesterol to bile acid) (Wu et al., 1999), as well as that of Cyp27a1, involved in alternative pathways of bile acid synthesis (Russell, 2009), were all downregulated in livers of the LCA group. This result indicates a decreased bile acid synthesis, which is consistent with the effect of LCA in both murine liver (Song et al., 2011) and primary human hepatocytes (Liu et al., 2014). In humans and mice, these three enzymes are strongly regulated by bile acid receptors named FXR (Makishima et al., 1999b) and TGR5 (Kawamata et al., 2003). Although LCA can upregulate the mRNA expression of these two receptors in primary human hepatocytes (Liu et al., 2014), we did not find any evidence of upregulation of these two receptors. This may be attributed to interspecific differences in BA biosynthesis, BA signaling, BA transport, and BA toxicity (Handschin et al., 2005). For example, it has been reported that CDCA and LCA could not activate the FXR in zebrafish (Reschly et al., 2008). Interestingly, we found that the expression of another nuclear receptor, a member of hepatocyte nuclear factor-4 family, HNF4B, was upregulated in livers of the LCA group. As HNF4 is involved in the feedback regulatory mechanism of bile acid synthesis (Makishima et al., 1999b; De Fabiani et al., 2001; Watt et al., 2003), our results suggest that HNF4B receptor might also play an important role in the feedback regulation of bile acid synthesis in grass carp.

LCA supplementation also changed the composition of gut microbiota, increasing the proportion of Proteobacteria and Firmicutes, while decreasing that of Fusobacteria. Fusobacteria is the dominant bacterial taxon among the fish gut microbiota (Larsen et al., 2014). Fish bacterial diseases are usually caused by the Proteobacteria genera, such as Pseudomonas (Wu et al., 2012). In this aspect, LCA-induced gut microbiota changes might increase the probability of bacterial infection in fish. The F/B ratio was significantly higher in the LCA group (P = 0.01). The so-called “obese microbiota”, which appear to extract more energy from a diet (Turnbaugh et al., 2006), are usually associated with higher F/B ratios. This result suggests that LCA-induced gut microbiota changes might also alter the host’s capability to extract energy from a diet. As LCA is a metabolite of specific gut bacteria, possessing the 7α-dehydroxylation capability, treatments aimed at reducing the concentration of LCA in bile and feces by modifying the bile acid 7α-dehydroxylation pathway via genetic knockouts (Ridlon et al., 2006) or supplementation of bacteria with hydroxysteroid dehydrogenases (HSDH) to detoxify bile acid (Devlin and Fischbach, 2015) could probably alleviate the negative impact on liver health in grass carp. Human intestinal bacteria capable of bile acid 7α-dehydroxylation have been isolated (Hirano et al., 1981; Wells et al., 2000), and 16S rDNA phylogenetic analysis has led to their classification to the genus Clostridium in Firmicutes (Kitahara et al., 2000, 2001). In our experiment the average ratio of Clostridium was a little higher in the LCA group (0.01%) than in the Control group (average ratio = 0.00%), but the increase was not significant (P = 0.36). The average ratio of Clostridiales was also higher in the LCA group (0.06%, vs 0.00% in the Control), but the difference was also not significant (P = 0.06).

The interaction between gut microbiota OTUs and liver DEGs was identified in three modules, and the involved DEGs indicated functional differences among all three modules. Most of DEGs in the M2 were signal transduction and genetic information processing related, while most of them in the M6 were digestive system and metabolism related. Most of these DEGs were related to signal transduction and genetic information processing, especially the PI3K-Akt signaling pathway, which is closely related to cell survival and cancer (Shulka et al., 2007; Altomare and Testa, 2005). Most OTUs involved in these interactions were from Proteobacteria and Firmicutes. It was reported that gut microbiota and their metabolites were able to engage different signaling pathways in ileum and colon epithelium (Sommer and Backhed, 2015), and liver (Miura and Ohnishi, 2014). Signal transduction was inhibited in the M2, which suggested that LCA probably caused a disorder of mitochondrial respiratory chain and altered the synthesis of ATP in the liver of grass carp.
previously reported to be related with gut microbiota and liver diseases in mice and humans (Qin et al., 2014; Abu Shanab and Quigley, 2010). Here we also found that co-occurrence of DEGs and microbial taxa mainly happened between signal transduction and genetic information. PI3K-Akt signaling pathway could be the most important functional pathway in this interaction in grass carp. These findings provide a new insight into the research of interactions between gut microbiota and liver diseases.

Overall, our research showed LCA could alter lipid metabolism in the liver of grass carp and influence composition of its gut microbiota. These results contribute to our understanding of the interplay between gut microbiota and liver diseases in fish, and offer insights into putative treatments of liver diseases in grass carp.

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Ethics statement

This study has been reviewed and approved by the ethics committee of the Institute of Hydrobiology, Chinese Academy of Sciences.

Conflict of interest

There is no conflict of interest related to this research.

Data accessibility

Raw sequencing reads data of liver transcriptomes has been deposited to Sequence Read Archive in NCBI in BioProject PRJNA477492. The obtained raw 16S rRNA sequences were deposited in the NCBI/EBI/DDBJ Sequence Read Archive (Bioproject: PRJNA416986) with the accession numbers SRR6251438 and SRR6251440.

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Authors' contributions

SW and GW were the principal investigators and contributed to the study design, acquisition of funding and overseeing the study, interpretation of data and manuscript editing. FX was in charge of the design, data collection, analysis and interpretation of data and manuscript writing. MS and LQ contributed to data collection, analysis and interpretation. HZ was in charge of study coordination and quality control, and manuscript editing. WL and ML contributed to fieldwork data collection and manuscript drafting.

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