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Original Research Article

Dietary high lipid and high plant-protein affected growth performance, liver health, bile acid metabolism and gut microbiota in groupers

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ABSTRACT

High lipid diets (HLD) and high plant-protein diets (HPD) exhibit potential fishmeal-saving effects but negatively impact liver health and growth performance in fish. We hypothesized that HLD and HPD impair liver health in pearl gentian groupers (*Epinephelus fuscoguttatus*♂ × *Epinephelus lanceolatus*♀) through the modulation of intestinal microbiota and bile acid (BA) metabolism. Four diet groups were tested: control diet (CD, 46.21% crude protein, 9.48% crude lipid), HLD (46.37% crude protein, 16.70% crude lipid), HPD (46.50% crude protein, 9.38% crude lipid), and high lipid-high plant-protein diet (HLPD, 46.54% crude protein, 16.67% crude lipid). A total of 300 fish (average body weight = 15.22 ± 0.03 g) were randomly divided into 4 diet treatments (ensuring 3 tanks replicates of each diet treatment, each tank containing 25 fish). After an eight-week feeding period, the HLD and HPD significantly decreased the final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR) and feed intake (FI) in comparison to CD group, with HLPD exacerbating these indicators ($P < 0.05$). Compared to CD group, the content of total cholesterol (T-CHO) and triglyceride (TG) in liver and serum were significantly increased in HLD group ($P < 0.05$). Compared to HPD group, the content of T-CHO in liver was significantly decreased, the content of TG in liver and serum were significantly increased in HLPD group ($P < 0.05$). HLD, HPD, and HLPD impaired liver health by inducing histological damage, inflammation, and oxidative stress. Compared to CD group, the mRNA relative expression of bile salt export pump (*bsep*) and multidrug resistance protein 3 (*mdr3*) were significantly increased in HLD group, whereas the mRNA relative expression of sterol-27-hydroxylase (*cyp27a1*), microsomal epoxide hydrolase (*meh*), apical sodium-dependent bile acid transporter (*asbt*), multidrug resistance-associated protein 3 (*mrp3*), farnesoid X receptor (*fxr*) and G protein-coupled bile acid receptor 5 (*tgr5*) were significantly decreased ($P < 0.05$). Compared to CD group, the mRNA relative expression of *mdr3*, *asbt*, *mrp3*, organic anion transporters 1 (*oatp1*), *meh*, *fxr* and *tgr5* were significantly decreased in HPD group ($P < 0.05$). In summary, HLD affects intestinal microbiota, BA metabolism, and lipid metabolism, leading to lipid deposition and liver damage. HPD regulates gut microbiota, BA metabolism, inflammatory responses, and BA

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receptor expression, impairing grouper liver health. HLPD synergistically combines the adverse effects of HLD and HPD on grouper liver health.

1. Introduction

The grouper, a prominent marine fish, recorded a production of 205,816 t in China in 2022, marking a 0.83% increase on the previous year (Bureau of Fisheries of MARA, 2023). Notably, the pearl gentian grouper (*Epinephelus fuscoguttatus*♂ × *Epinephelus lanceolatus*♀), highly prized by farmers and consumers alike, is extensively cultivated along the coasts of Southeast Asia and China (Xu et al., 2022a). As a carnivorous species, grouper feed typically contains 50% protein, with fishmeal serving as the predominant ingredient due to its palatability, balanced amino acid profile, and essential nutrients (Shapawi et al., 2018). However, the escalating demand coupled with limited fishmeal production has resulted in increased prices, significantly affecting feeding costs (Liang et al., 2013). Consequently, there has been a growing adoption of high plant-protein diets (HPD) and high lipid diets (HLD) to diminish fishmeal usage, fostering sustainable grouper aquaculture (Pan et al., 2016; Zhang et al., 2022a).

Previous research demonstrated that partial substitution of fishmeal with cottonseed protein concentrate (CPC) did not impact the growth performance of largemouth bass (*Micropterus salmoides*), large yellow croaker (*Larimichthys crocea*), rainbow trout (*Oncorhynchus mykiss*), and pearl gentian groupers (He et al., 2021, 2022; Liu et al., 2022; Tian et al., 2022). However, it is imperative to consider that excessive levels of substitution could detrimentally affect the survival, growth, and enterohepatic health of aquatic animals (He et al., 2022; Xie et al., 2023). Additionally, HPD have been linked to adverse effects on liver health in common carp (*Cyprinus carpio* L.) and Amur sturgeon (*Acipenser schrenckii*), manifesting as disrupted bile acid (BA) circulation and diminished BA levels (Wei et al., 2020; Yao et al., 2021). Notably, liver damage in common carp and shrimp (*Litopenaeus vannamei*) caused by HPD was mitigated by BA supplementation (Li et al., 2022b; Yao et al., 2021). This suggests that the liver injury induced by HPD may be attributed to an imbalance in BA homeostasis.

Additionally, some researchers have observed that HLD may disrupt lipid metabolism, potentially leading to tissue damage in organs such as the intestines and liver over time, and in severe cases, even growth and survival, as observed in common carp (Yang et al., 2023), yellow catfish (Zheng et al., 2023) and largemouth bass (Yin et al., 2021). In our previous study, we noted that a HLD resulted in reduced BA levels in the pearl gentian grouper, leading to impaired liver health (Xu et al., 2022b). Similarly, Zheng et al. (2017) demonstrated that the impact of a HLD on hepatic fat deposition in mice may be correlated with its effect on the BA pool size. Moreover, following BA supplementation, pearl gentian groupers and Chinese perch (*Siniperca chuatsi*) exhibited enhanced lipid metabolism and antioxidant capacity, indicative of healthy livers (Xu et al., 2022b; Zhang et al., 2022b). These findings suggest that disruption of BA homeostasis may significantly contribute to HLD-induced liver damage in fish.

BA, a class of steroidal carboxylic acids derived from cholesterol, serve dual roles as emulsifiers that enhance nutrient absorption and transport, and as intricate metabolic integrators and signaling agents that modulate diverse metabolic pathways within the body (He et al., 2023; Zheng et al., 2023). The enterohepatic circulation of BA involves the liver producing primary BA, which are then transformed by the intestinal microbiota into secondary BA. These are subsequently reabsorbed and returned to the liver (Xiong et al., 2022). Notably, the intestinal microbiota generates essential enzymes that are critical for BA metabolism in the enterohepatic circulation, thereby significantly affecting host metabolism (Luo et al., 2023). Therefore, changes in BA profiles mediated by the microbiota may be crucial in regulating host health. Nonetheless, the exact mechanisms by which gut microbiota influence BA homeostasis and the effects of gut microbe-BA interactions on fish health require further clarification.

While both HLD and HPD are trending in the aquafeed industry, and they have been found to induce liver damage and disruption of BA homeostasis in fish, few studies have focused on how these two feed environments impair liver health by modulating BA homeostasis, and the similarities and differences in the mechanisms of action behind them. On the other hand, liver damage is an important factor limiting the promotion and application of low fishmeal feeds (Nankervis et al., 2022; Zhou et al., 2023). Although both HLD and HPD have the ability to save fishmeal, little attention has been paid to the potential to save fishmeal when the two are applied in combination (high lipid-high plant-protein diet, HLPD), and the limiting factors behind this. Consequently, this study aimed to explore the effects of HLD and HPD on the intestinal microbiota, BA metabolism, and liver health in pearl gentian grouper. Furthermore, we assessed the impacts of HLD, HPD, and their combination on grouper liver health to evaluate the potential of HLPD in reducing fishmeal in feeds. The primary objective of this research is to elucidate the mechanisms by which gut microbes and BA metabolism interact to regulate liver health in fish. As the challenge of balancing fishmeal production with aquaculture expansion persists, discovering more efficient strategies to decrease fishmeal content in diets and understanding the limitations of low-fishmeal diets will become increasingly vital for the aquaculture sector.

2. Materials and methods

2.1. Animal ethics statement

Present study followed the recommendations of Care and Use of Laboratory Animals in China, Animal Ethical and Welfare Committee of China Experimental Animal Society. The protocol was approved by the Ethical Committee of the Guangxi Academy of Sciences, Nanning, China, and processing ID: GXAS-EC-202303157215.

2.2. Chemicals

The study employed primary antibodies against sterol-regulator element-binding protein 1 (srebp1, ab28481), nuclear factor (erythroid-derived 2)-like 2 (nrf2, ab137550) and Kelch-like erythroid cell-derived protein 1 (keap1, ab227828) from Abcam Co (Cambridge, UK). The antibody against glyceraldehyde-phosphate dehydrogenase (GAPDH, 2118S) was purchased from Cell Signaling Technology (MA, USA). The secondary antibody (AB0101) was purchased from Abways (Shanghai, China). The kits of total protein (TP, A045-4-2), triglyceride (TG, A110-1-1), total cholesterol (T-CHO, A111-1-1), aspartate aminotransferase (AST, C010-2-1), alanine aminotransferase (ALT, C009-2-1), malondialdehyde (MDA, A003-1-2), total bile acids (TBA, E003-2-1),

superoxide dismutase (SOD, A001-3-2) and catalase (CAT, A007-1-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.3. Diet preparation, animals and sample collection

Previous studies have demonstrated that an optimal dietary lipid of the pearl gentian grouper level falls within the range of 7% to 13%, while a lipid content of ≥15% leads to fat accumulation in the liver (Zou et al., 2019). Thus, in this study, a HLD was prepared by adding soybean oil to a control diet (CD), resulting in a crude lipid content of 16.70% for the HLD and 9.48% for the CD (Table 1). To formulate a HPD, we replaced 50% of the fishmeal in the CD diet (base level of 500 g/kg) with CPC, as previous laboratory studies have shown that the maximum level that CPC could replace fishmeal is 50% (Ye et al., 2020). In addition, the HLPD (16.67% crude lipid) with 50% fishmeal replaced by CPC was prepared.

Juvenile healthy pearl gentian groupers (*E. fuscoguttatus*♀ × *E. lanceolatus*♂) were carefully selected from a local fish farm in Beihai, Guangxi Province. The fish were fed high-quality commercial diets with 50% crude protein and 10% crude fat, and were expertly domesticated for 7 d in salinity of 28‰. Then, 300 fish were randomly divided into 4 diet treatments (ensuring 3 tanks replicates of each diet treatment, each tank containing 25 fish, for a total of 12 experimental tanks). During the feeding period, two-thirds of the tank water daily was replaced. The water temperature, dissolved oxygen concentration, ammonia nitrogen, and nitrate concentration throughout the experimental process were maintained at optimal levels of 28 to 30 °C, 7 mg/L, less than 0.03 mg/L, and less than 0.03 mg/L, respectively (Xu et al., 2022b). Our previous study provided a detailed description of the methods for diet preparation and rearing conditions (Xu et al., 2022b).

At the end of the 8-week feeding period, the fish in each tank were anaesthetized using MS-222 (Sigma Aldrich, MO, USA) at a concentration of 100 mg/L following 24 h of fasting. The final body weight of all fish in each tank, along with the weight and length of three randomly selected fish, were recorded. Following euthanasia and dissection, 15 fish from each tank were randomly chosen for sampling. Whole-body, blood, muscle, and liver samples were collected. Muscle and whole-body samples were frozen at −20 °C for composition analysis. Blood was drawn from the caudal vein with sterile syringes, centrifuged at 3000 × *g* and 4 °C for 10 min to isolate serum, which was then rapidly frozen in liquid nitrogen and stored at −80 °C for enzyme activity and biochemical indicator measurements. The distal intestinal contents were carefully collected, frozen in liquid nitrogen, and stored at −80 °C till the bacterial composition analysis. Each liver was quartered: one part was fixed in 4% paraformaldehyde for histology, another was stored at −20 °C for composition analysis, and the remaining portions were quick-frozen in liquid nitrogen and stored at −80 °C for biochemical testing, gene, and protein expression analysis.

2.4. Growth performance analyses

The growth parameters were calculated:

Weight gain rate (WGR, %) = 100 × (final body weight - initial body weight)/initial body weight;

Specific growth rate (SGR, %/d) = 100 × (Ln final body weight - Ln initial body weight)/experiment duration;

Feed intake (FI, %/fish) = 100 × diet fed/(final body weight + initial body weight)/(2 × experimental duration).

Table 1
Composition and nutrient levels of diets (air-dry basis, %).

Item	Diet[1]			
	CD	HLD	HPD	HLPD
Ingredients				
Fishmeal	50.00	50.00	25.00	25.00
Vital wheat gluten	8.00	8.00	8.00	8.00
Wheat flour	12.50	12.50	12.50	12.50
Cottonseed protein concentrate			25.20	25.20
Corn gluten meal	5.00	5.00	5.00	5.00
Casein	5.00	5.00	5.00	5.00
Gelatine	1.00	1.00	1.00	1.00
Fish oil	1.50	1.50	3.45	3.45
Soybean oil	1.50	9.00	1.50	9.00
Soybean lecithin	2.00	2.00	2.00	2.00
Calcium monophosphate	1.00	1.00	1.00	1.00
Vitamin C	0.03	0.03	0.03	0.03
Choline chloride	0.50	0.50	0.50	0.50
Vitamin premix[2]	0.50	0.50	0.50	0.50
Mineral premix[3]	0.50	0.50	0.50	0.50
Antioxidant[4]	0.05	0.05	0.05	0.05
Attractant[5]	0.10	0.10	0.10	0.10
Cellulose microcrystalline	10.82	3.32	7.81	0.31
Methionine			0.31	0.31
Lysine			0.55	0.55
Total	100.00	100.00	100.00	100.00
Nutrient levels				
Crude protein	46.21	46.37	46.50	46.54
Crude lipid	9.48	16.70	9.38	16.67
Moisture	9.15	8.31	9.09	8.74

[1] CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid.

[2] Vitamin premix (g/kg vitamin premix): vitamin B_1 17.00 g, vitamin B_2 16.67 g, vitamin B_6 33.33 g, vitamin B_{12} 0.07 g, vitamin K 3.33 g, vitamin E 66.00 g, retinyl acetate 6.67 g, vitamin D 33.33 g, nicotinic acid 67.33 g, D-calcium pantothenate 40.67 g, biotin 16.67 g, folic acid 4.17 g, inositol 102.04 g, cellulose 592.72 g. All ingredients were diluted with corn starch to 1 kg. The mixture was provided by Beijing Enhalor International Tech Co., Ltd., Beijing, China.

[3] Mineral premix (g/kg mineral premix): $CaCO_3$ 350 g, $NaH_2PO_4 \cdot H_2O$ 200 g, KH_2PO_4 200 g, NaCl 12 g, $MgSO_4 \cdot 7H_2O$ 10 g, $FeSO_4 \cdot 7H_2O$ 2 g, $MnSO_4 \cdot 7H_2O$ 2 g, $AlCl_3 \cdot 6H_2O$ 1 g, $CuCl_2 \cdot 2H_2O$ 1 g, KF, 1 g, $NaMoO_4 \cdot 2H_2O$ 0.5 g, $NaSeO_3$ 0.4 g, $CoCl_2 \cdot 6H_2O$ 0.1 g, KI, 0.1 g, zeolite powder 219.9 g. The mixture was provided by Beijing Enhalor International Tech Co., Ltd., Beijing, China.

[4] Antioxidant: ethoxyquin, provided by Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China.

[5] Attractant: betaine, provided by Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China.

2.5. Nutrient composition of diets and body tissues

Following the previously described methods (AOAC, 2002), the proximate composition of crude protein (method 968.06), crude lipid (method 922.06) and moisture contents (method 926.08) in diets, whole-body, muscle and liver were analyzed. In brief, the level of crude protein (N × 6.25) was measured by the Kjeldahl nitrogen method via Auto Kjeldahl System usage (8400-Autoanalyzer, FOSS, Hoganas, Sweden); the level of crude lipid was measured by the Soxhlet method via ether extraction system (Tecator, Sweden); the level of moisture was measured by oven drying the sample at 105 °C until constant weight.

2.6. Liver staining

Liver tissue samples were fixed, dehydrated, and paraffin-embedded before being stained with hematoxylin and eosin (H&E). The stained sections were observed under 200× magnification (Olympus, Tokyo, Japan) to examine the morphology of the hepatocyte and the unstained fatty vacuole area.

2.7. Enzyme activity, biochemical assays and real-time fluorescence quantitative polymerasechain reaction (RT-qPCR) analyses

Commercial kits were used to determine the levels of TP, TG, T-CHO, AST, ALT, MDA, TBA, SOD and CAT (Chiu et al., 2018). The reagent configuration, sample pre-treatment and determination steps were carried out according to the instructions of the kit manufacturers. The concentration of total RNA in all samples was diluted to 1000 ng/μL. Reverse transcription was performed using the Evo M-MLV kit with gDNA Eraser (Accurate Biotechnology (Hunan) Co., Ltd, Hunan, China). The RT-qPCR assay was carried out using SYBR Green Pro Taq HS (Accurate Biotechnology) on the qTower3G IVD system (Jena, Germany). Primers were designed based on the full-length sequence of the pearl gentian grouper transcriptome. The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method with 18s rRNA (*18s*) and β-actin as reference genes (Xu et al., 2022c). The following genes were tested in the present study (Table S2): lipogenesis (acetyl-CoA carboxylase [*acc*], fatty acid synthase [*fas*]); lipolysis (adipose triglyceride lipase [*atgl*], carnitine palmitoyltransferase 1 [*cpt1*], hormone-sensitive lipase [*hsl*]); transcriptional factors (liver X receptor alpha [*lxr*], peroxisome proliferator-activated receptor alpha [*pparα*], *srebp1*); keap1/nrf2 pathway (*keap1*, *nrf2*, heme oxygenase-1 [*ho-1*]); pro-inflammatory cytokines (interleukin 1β [*il1β*] and tumor necrosis factor-alpha [*tnfα*]); BA synthesis (cholesterol 7α-hydroxylase [*cpy7a1*], sterol-27-hydroxylase [*cyp27a1*]); BA transport (bile salt export pump [*bsep*], multidrug resistance protein 3 [*mdr3*]); BA reabsorption (apical sodium-dependent BA transporter [*asbt*], multidrug resistance-associated protein 3 [*mrp3*]; BA recycling (organic anion transporters 1 [*oatp1*], microsomal epoxide hydrolase [*meh*]); BA receptors (farnesoid X receptor [*fxr*] and G protein-coupled bile acid receptor 1 [*tgr5*]).

2.8. Western blot analyses

The Western blot analysis was conducted following the protocol outlined in our recent study (Xu et al., 2022c). Three replicate wells were made for each protein sample. In detail, liver samples were lysed in radio immunoprecipitation assay lysis buffer (RIPA) buffer to extract protein. The protein was denatured in a boiling water bath and electrophoretically separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel containing. The separated proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with primary antibodies: srebp1 (1:1000), nrf2 (1:500), keap1 (1:1500), and GAPDH (1:1000). Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies were used to detect the probes. The target protein bands were visualized using enhanced chemiluminescence (ECL) reagent (Billerica, MA, USA) and quantified using Image J software (version 1.42, National Institutes of Health, USA).

2.9. Untargeted metabolomics analyses

The untargeted metabolomics analyses were performed by Suzhou BioNovo Gene Company. The distal intestine was taken and weighed accurately. Tissue extracts were added, thoroughly ground, centrifuged, and dried. The samples were then re-dissolved by adding 2-chloro-l-phenylalanine (dissolved in an acetonitrile solution). The supernatant was filtered and transferred to a detection vial for LC-MS analysis. The Vanquish UHPLC system (Thermo Fisher Scientific, USA) equipped with an ACQUITY UPLC HSS T3 (2.1 mm × 100 mm, 1.8 μm) (Waters, Milford, MA, USA) was used for chromatographic separations. The eluents employed were 0.1% formic acid acetonitrile (vol/vol) (eluent B2) and acetonitrile solution (eluent B3). The elution gradients were carried out as follows: 0 to 1 min, 8% B; 1 to 8 min, 8% to 98% B; 8 to 10 min, 98% B; 10 to 10.1 min, 98% to 8% B; 10.1 to 12 min, 8% B. The LC-ESI (+)-MS analysis elution solution was B2, while the LC-ESI (−)-MS analysis was B3. The parameters for the ESI Ion Source mass spectrometry of the Orbitrap Exploris 120 (Thermo Fisher Scientific, USA) were set as follows: sheath gas pressure = 40 arb; auxiliary gas flow = 10 arb; spray voltage: ESI (+) was set to 3.50 kV, while ESI (−) was set to −2.50 kV. The capillary temperature was maintained at 325 °C. The MS1 range was set to *m*/*z* 100 to 1000 with a resolution of 60,000 FWHM. The number of correlation scans per cycle of data was 4. The MS/MS resolution was set to 15,000 FWHM, with a normalised collision energy of 30%. The dynamic exclusion time was set to automatic. The metabolomics raw data (accession number: MTBLS9403) have been deposited in the MetaboLights database.

For metabolomics analysis, raw Triple-TOF/MS data were processed using Progenesis QI 2.0 (Waters, MA, USA) for nonlinear alignment and normalization. The software automatically handled deconvolution of the total ion chromatogram, data normalization, peak picking, ion peak alignment, and extraction of ion chromatograms using default settings. It assessed all runs for automatic alignment and peak picking with a default threshold of 3. Metabolite annotation involved matching MS and MS/MS data against the METLIN and HMDB databases with a mass error tolerance of 5 mg/L. Chemical similarity enrichment analysis (ChemRICH) was employed for statistical enrichment analysis based on chemical similarity. Subsequently, we normalized the peak areas of the BA metabolites and expressed them as the relative contents of BA. These values were then subjected to further analysis and comparison in subsequent steps.

2.10. 16S rRNA of microbial samples

The total genome DNA from bacteria in the intestinal content of groupers was extracted using a TIANamp Marine Animals DNA Kit (TIANGEN, Beijing, China) as per the manufacturer's protocol. The sample size was 3 for the CD, HLD and HLPD groups, while 4 for the HPD group. The V3–V4 regions of the 16S rRNA genes were amplified using primers 338-forward (5′-ACTCCTACGGGAGGCAGCA-3′) and 806-reverse (5′-GGCTACHVGGGTWTCTAAT-3′). Sequencing libraries were prepared with the TruSeq DNA PCR-Free Kit (Illumina, California, USA), incorporating index barcodes, and sequenced on an Illumina NovaSeq platform to produce 250 bp paired-end reads. These were merged with FLASH (v1.2.7) to form raw tags. Qualified reads were clustered into amplicon sequence variants (ASV) at 97% similarity using UPARSE (v 7.1), and taxonomic information was annotated against the Silva Database via the Mothur algorithm. To study the phylogenetic relationship of different ASV, and the differences in terms of dominant species in different groups, multiple sequence alignments were conducted using the MUSCLE software (v 3.8.31). ASV abundance information was normalized using a standard of the sequence number corresponding to the sample with the fewest sequences. The α diversity indices were computed using QIIME2 (2019.4) and displayed using R software (v 2.15.3). Alpha diversity was estimated using various metrics, such as Shannon index, Simpson index, Chao1 index, Observed-species, Faith-pd, Pielou-e, and Goods-coverage. Unweighted Pair Group Method with Arithmetic mean (UPGMA) trees (Jaccard distance) were created. Beta diversity was calculated using weighted and unweighted UniFrac and principal coordinate analysis (PCoA). In addition, linear discriminant analysis (LDA) effect size (LEfSe) algorithm was performed combining Kruskal–Wallis test or Wilcoxon rank-sum test with LDA scores to estimate the effect size of differentially abundant features with biological

Table 2
The growth performance and lipid deposition in groupers[1].

Item	Diet[2]				*P*-value[3]		
	CD	HLD	HPD	HLPD	L	HP	L × HP
IBW, g	15.21 ± 0.027	15.21 ± 0.027	15.24 ± 0.023	15.20 ± 0.023	–	–	–
FBW, g	84.70 ± 2.633^{c}	80.39 ± 2.054^{b}	76.78 ± 1.656^{b}	71.28 ± 2.418^{a}	0.005	< 0.001	0.065
WGR, %	456.70 ± 15.857^{c}	428.41 ± 11.976^{b}	403.79 ± 9.745^{b}	368.96 ± 17.047^{a}	0.004	< 0.001	0.696
SGR, %/d	3.07 ± 0.051^{c}	2.97 ± 0.041^{b}	2.89 ± 0.034^{b}	2.76 ± 0.066^{a}	0.005	< 0.001	0.550
FI, %/fish	2.06 ± 0.004^{c}	1.97 ± 0.027^{b}	2.01 ± 0.019^{b}	1.91 ± 0.022^{a}	< 0.001	0.001	0.792
TG in liver, mmol/g prot	0.37 ± 0.012^{a}	0.54 ± 0.064^{b}	0.30 ± 0.047^{a}	0.52 ± 0.064^{b}	< 0.001	0.145	0.435
T-CHO in liver, mmol/g prot	0.06 ± 0.000^{b}	0.07 ± 0.002^{c}	0.05 ± 0.003^{b}	0.04 ± 0.005^{a}	0.029	< 0.001	0.024
TG in serum, mmol/L	1.88 ± 0.113^{ab}	3.28 ± 0.338^{c}	1.65 ± 0.029^{a}	2.25 ± 0.126^{b}	< 0.001	< 0.001	0.006
T-CHO in serum, mmol/L	1.37 ± 0.010^{b}	1.67 ± 0.090^{c}	1.07 ± 0.025^{a}	1.07 ± 0.053^{a}	0.005	< 0.001	0.065

IBW = initial body weight; FBW = final body weight; WGR = weight gain rate; SGR = specific growth rate; FI = feed intake; TG = triglyceride; T-CHO = total cholesterol.
$^{a-c}$ Different letters indicate significant differences between groups ($P < 0.05$).
[1] Values are presented as means ± standard deviation (SD).
[2] CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid.
[3] L means lipid level; HP means plant-protein level; L × HP means lipids and plant-protein interacted level.

consistency and statistical significance. Differentially expressed genes (DEG) were identified between libraries at a false discovery rate (FDR) threshold of less than 0.05. Subsequently, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed on these DEG. The microbial sequences obtained in this study are available in NCBI's SRA database under accession number PRJNA1065578.

2.11. Statistical analyses

The results were presented as mean ± standard deviation (SD). Normality and homogeneity of variance were tested using the Shapiro–Wilk and Levene tests, respectively. Data were evaluated using one-way ANOVA and two-way ANOVA. Multiple comparisons of means were performed using Duncan's multiple range tests when significant differences were found between groups. A significance level of 0.05 was used. The statistical analysis was performed using SPSS 23.0 (IBM, Armonk, NY, USA).

The mathematical model for one-way ANOVA can be expressed as:

$$Y_{ij} = \mu + \alpha_i + \epsilon_i,$$

where Y_{ij} is the j-th observation under the i-th level; μ is the overall mean, representing the grand mean of all observations; α_i is the main effect of the i-th level, indicating the influence of that level on the dependent variable; ϵ_{ij} is the random error term, representing the influence of other random factors beyond the independent variable on the dependent variable.

The mathematical model for two-way ANOVA can be expressed as:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk},$$

where two factors of lipid level and plant-protein level, labeled as L and HP, each with different denoted as L1, L2, …, L_j for factor L and HP1, HP2, …, HP_k for factor HP; Y_{ijk} is the value of the dependent variable for the k-th observation at the j-th level of factor HP within the i-th level of factor L; α_i is the main effect of the i-th level of factor L, indicating the influence of factor L on the dependent variable; β_j is the main effect of the j-th level of factor HP, indicating the influence of factor HP on the dependent variable; $(\alpha\beta)_{ij}$ is the interaction effect between factor L and factor HP, representing the combined influence of both factors on the dependent variable; ϵ_{ijk} is the random error term, representing the influence of other random factors beyond factors L and HP.

3. Results

3.1. Dietary high lipid/plant-protein impaired the growth performance and affected the lipid deposition of groupers

The HLD and HPD significantly decreased the FBW, WGR, SGR and FI in comparison to the CD group ($P < 0.05$) (Table 2). In addition, compared to the HLD or HPD groups, the HLPD significantly decreased the FBW, WGR, SGR and FI ($P < 0.05$). Thus, the HLD and HPD impaired growth performance, and their combined effects (HLPD) further undermined it.

Furthermore, compared to the CD group, the content of T-CHO and TG in liver and serum were significantly increased in the HLD group, whereas the content of T-CHO in serum was significantly decreased in the HPD group ($P < 0.05$) (Table 2). In addition, compared to the HPD group, the content of T-CHO in liver was significantly decreased, and the TG in liver and serum were significantly increased in the HLPD group ($P < 0.05$).

The HLD significantly increased the levels of crude lipid in whole-body, muscle and liver, compared with the CD group ($P < 0.05$) (Table 3). The HPD did not significantly alter the levels of crude lipid in the whole-body, muscle and liver compared with the CD group ($P > 0.05$) (Table 3). When compared to the HLD/HPD group, the crude lipid in whole-body, muscle and liver were significantly decreased/increased in the HLPD group ($P < 0.05$) .

3.2. Dietary high lipid/plant-protein impaired the liver health of grouper

The fish fed CD exhibited normal morphology, with most hepatocytes having regular round nuclei (Fig. 1A). However, hepatocyte swelling with large numbers of diffuse lipid vacuoles was more common, accompanied by lysis of some hepatocyte nuclei in the HLD group. In the HPD and HLPD group, hepatocyte nuclear abnormalities, including deviation and cleavage, were more common. Moreover, the HLD significantly increased the activities of ALT and AST in serum compared to the CD group ($P < 0.05$). Likewise, the HPD resulted in a significant increase in the activity of ALT in serum ($P = 0.001$) (Table 4). The HLPD significantly decreased the activity of AST in serum compared to the HLD group ($P = 0.031$).

Table 3
Average proximate composition of the whole-body, muscle, and liver in groupers (% of wet matter)[1].

Item	Diet[2]				*P*-value[3]		
	CD	HLD	HPD	HLPD	L	HP	L × HP
Whole-body							
Moisture	77.16 ± 0.061[d]	71.73 ± 0.068[a]	75.85 ± 0.095[c]	73.93 ± 0.085[b]	< 0.001	< 0.001	< 0.001
Crude protein	11.97 ± 0.286[a]	14.70 ± 0.205[c]	13.86 ± 0.142[b]	14.40 ± 0.190[c]	< 0.001	< 0.001	< 0.001
Crude lipid	6.21 ± 0.325[a]	9.52 ± 0.177[c]	6.01 ± 0.306[a]	7.47 ± 0.292[b]	< 0.001	< 0.001	< 0.001
Muscle							
Moisture	80.59 ± 0.332[b]	79.42 ± 0.184[a]	80.51 ± 0.277[b]	80.75 ± 0.110[b]	0.010	0.002	0.001
Crude protein	16.12 ± 0.618	16.73 ± 0.154	16.38 ± 0.544	16.29 ± 0.833	0.472	0.786	0.336
Crude lipid	1.84 ± 0.070[ab]	2.49 ± 0.046[c]	1.76 ± 0.115[a]	2.03 ± 0.162[b]	< 0.001	0.002	0.013
Liver							
Moisture	68.08 ± 0.173[b]	67.46 ± 0.136[a]	68.25 ± 0.125[b]	68.31 ± 0.156[b]	0.010	< 0.001	0.004
Crude lipid	4.37 ± 0.151[a]	5.69 ± 0.287[c]	4.17 ± 0.146[a]	4.93 ± 0.120[b]	< 0.001	0.002	0.032

[a-d] Different letters indicate significant differences between groups ($P < 0.05$).
[1] Values are presented as means ± standard deviation (SD).
[2] CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid.
[3] L means lipid level; HP means plant-protein level; L × HP means lipids and plant-protein interacted level.

In liver, the mRNA relative expression of *il1β* and *tnfα* was significantly increased in the HLD and HPD groups, compared to the CD group ($P < 0.05$) (Fig. 1B). Compared to the HLD or HPD groups, the mRNA relative expression of *il1β* was significantly decreased, and the mRNA relative expression of *tnfα* was significantly increased in the HLPD group ($P < 0.05$). Furthermore, compared to the CD group, the activities of CAT and SOD in liver were significantly decreased in the HLD and HPD groups ($P < 0.05$) (Table 4). Compared to the HPD group, the activity of SOD was significantly increased in the HLPD group ($P < 0.001$). Meanwhile, compared to the CD group, the expression of NRF2 was significantly increased in the HLD group, while the expression of KEAP1 and NRF2 was significantly increased in liver of the HPD group ($P < 0.05$) (Fig. 1D–E). Compared to the HLD/HPD groups, the HLPD treatment significantly increased/decreased the expression of KEAP1/NRF2 in liver ($P < 0.05$).

3.3. Dietary high lipid/plant-protein altered the intestinal microbiota of grouper

For alpha diversity (Fig. 2A–G), compared to the CD group, the HLD treatment significantly increased the Pielou_e and Goods_coverage indexse ($P < 0.05$); the HPD treatment significantly increased the Simpson, Shannon and Pielou_e indexse ($P < 0.05$); the HLPD treatment significantly increased the Simpson, Shannon, Pielou_e, and Goods_coverage indexse ($P < 0.05$). Furthermore, there was a statistically significant effect on the clustering of these communities ($P = 0.001$, Jaccard-based Adonis), and 29.15% of this variance was accounted for by the difference among groups (PERMANOVA $R^2 = 0.890918$) (Table S2).

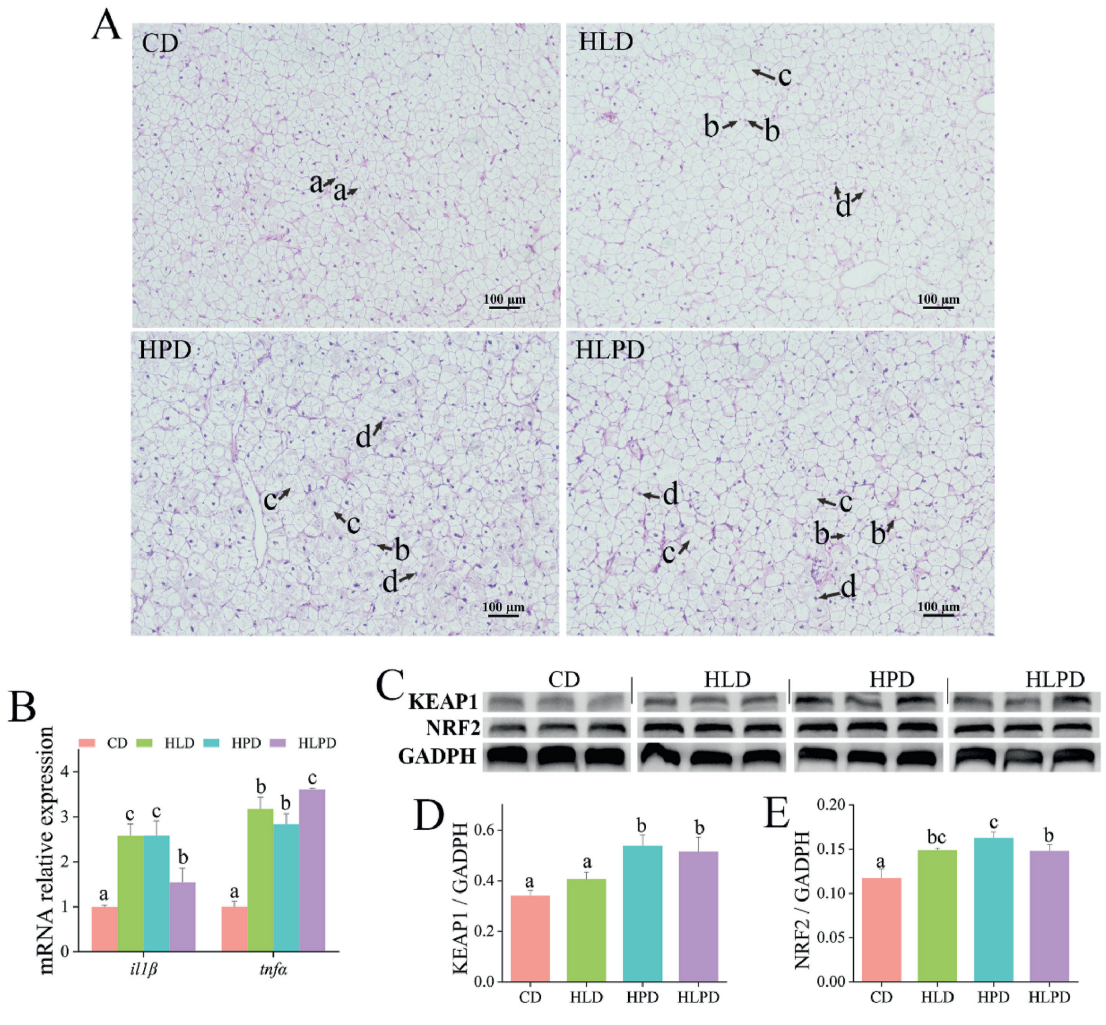
Un-weighted UniFrac based PCoA plots revealed that the samples from the CD and HLD groups were distributed together and were not well differentiated (Fig. 3A). Whereas the samples of the HPD group were distributed independently from those of the CD group along the direction of Axis1, there was an independent distribution of the samples of the HPLD group from the CD group along both Axis1 and Axis2. UPGMA analysis based on the Jaccard similarity revealed that the samples in the CD, HLD and HPD groups were broadly distinguishable (Fig. 2H). Meanwhile, the samples in the HLPD group had some distributional randomness among the HLD and HPD groups, which further confirmed the synergistic effects (HLD and HPD) on microbiota structure in the HLPD group. The LEfSe algorithm revealed that the main biomarkers (LDA score > 4.0) of microbiota in the CD group were: phylum Firmicutes, class Bacilli, species *Lactobacillus_delbrueckii*, genera *Lactobacillus*, order Lactobacillales, families Lactobacillaceae; whereas in the HPD group it was the genera *Bosea*, species *Bosea_genosp*, families Bradyrhizobiaceae (Fig. 3B).

Compared to the CD group, the HLPD significantly increased the relative abundance of Proteobacteria and decreased that of Firmicutes ($P < 0.05$) (Fig. 2I-L). By two-way ANOVA, the dietary lipid significantly affected the relative abundance of *Bosea* and *Chryseobacterium* ($P < 0.05$), which might be the sensitive microorganisms to lipid levels (Fig. 4). The dietary plant-protein significantly affected the relative abundance of *unclassified_Peptostreptococcaceae*, *Lactobacillus*, *Lactococcus*, *Staphylococcus*, *Clostridium*, and *Bosea* ($P < 0.05$), which might be the sensitive microorganisms to plant-protein levels. Meanwhile, the relative abundance of *Streptococcus*, *Lactobacillus* and *Bosea*, were affected by the interaction of dietary lipid and plant-protein ($P < 0.05$) (Fig. 4).

3.4. Dietary high lipid/plant-protein impacted the metabolomics of the intestine of groupers

The hierarchical clustering dendrograms roughly separated samples from the CD and HLD groups, while samples from the HPD and HLPD groups were mixed together in both positive and negative ion patterns (Fig. S1A-B). Meanwhile, the results of principal component analysis (PCA) indicated that the metabolite structures of the samples from the CD, HLD, and HPD groups were mostly separable along the axial direction with a slight overlap. However, the metabolite structures of the samples from the HLPD and HPD groups showed a large overlap (Fig. S2 and S3). In addition, compared to the CD group, there were 87 differential expressions of metabolites (DEM) in the HLD group, while 76 DEM in the HPD group (Fig. S3C). Compared to the HLD group, there were 67 DEM in HLPD group. Compared to the HPD group, there were 22 DEM in the HLPD group (Fig. S1C).

In the identified 14 kinds of BA (Table S3), dietary lipid significantly affected the levels of allocholic acid (ACA), glychenodeoxycholic acid (GCDCA) and taurohyocholate (THCA), while the plant-protein significantly affected the levels of ACA, GCDCA and 3b-Hydroxy-5-Cholenoic acid ($P < 0.05$). In detail, compared to the CD group, the levels of ACA and GCDCA were significantly decreased in the HLD group ($P < 0.05$). Compared to the CD group, the levels of ACA, GCDCA and 3b-Hydroxy-5-Cholenoic acid were significantly decreased in the HPD group,



P-value	*il1β*	*tnfα*	KEAP1/GADPH	NRF2/GADPH
L	0.110	< 0.001	0.376	0.076
HP	0.108	< 0.001	< 0.001	0.001
L × HP	< 0.001	< 0.001	0.094	0.001

Fig. 1. The liver health in response to dietary high lipid and high plant-protein in groupers. (A) Photomicrographs of representative hematoxylin and eosin-stained histological liver sections in 200× magnification. (B) The mRNA relative expression of inflammatory cytokines. (C) The Western blot analysis, three replicate wells were made for each protein sample. (D-E) The relative quantification of protein levels. CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid; L means lipid level; HP means plant-protein level; L × HP means lipids and plant-protein interacted level. Arrows indicate examples of a: normal hepatocytes with a regular, round nucleus; b: abnormal nucleus; c: swollen hepatocytes with large diffused lipid vacuoles; d: absent nucleus. Values are presented as means ± error bars (SD). *tnfα* = tumor necrosis factor-alpha; *il1β* = interleukin 1β; KEAP1 = Kelch-like erythroid cell-derived protein 1; NRF2 = nuclear factor (erythroid-derived 2)-like 2; GADPH = glyceraldehyde-phosphate dehydrogenase. [a-c] Different letters indicate significant differences between groups ($P < 0.05$).

while the levels of taurocholic acid (TCA) were significantly increased ($P < 0.05$). In addition, compared to the HLD group, the HLPD treatment significantly increased the level of deoxycholic acid (DCA). Conclusively, these results demonstrated that HLD and HPD mainly affected the levels of ACA, GCDCA, THCA, TCA and DCA.

To further determine the association between changes in the profiles of intestinal BA and the abundance of microorganisms sensitive to high lipid or plant-protein levels, spearman correlation analysis was conducted (Fig. 5). The results showed that the level of ACA was positively correlated with the relative abundances of *unclassified_Peptostreptococcaceae* and *Clostridium* ($P < 0.01$), and negatively correlated with the relative abundances of *Lactobacillus* and *Lactococcus* ($P < 0.05$); the level of TCA was positively correlated with the relative abundances of *Streptococcus* and *Lactococcus* ($P < 0.05$); the level of GCDCA was positively correlated with the relative abundances of *unclassified_Peptostreptococcaceae* and *Clostridium* ($P < 0.01$); the level of THCA was positively correlated with the relative abundance of *Streptococcus* ($P < 0.05$), and negatively correlated with the relative abundances of *unclassified_Peptostreptococcaceae* and *Clostridium* ($P < 0.01$). These results showed that HLD and HPD could induce the variations in BA profiles by altering the *unclassified_Peptostreptococcaceae*, *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Staphylococcus* and *Clostridium*.

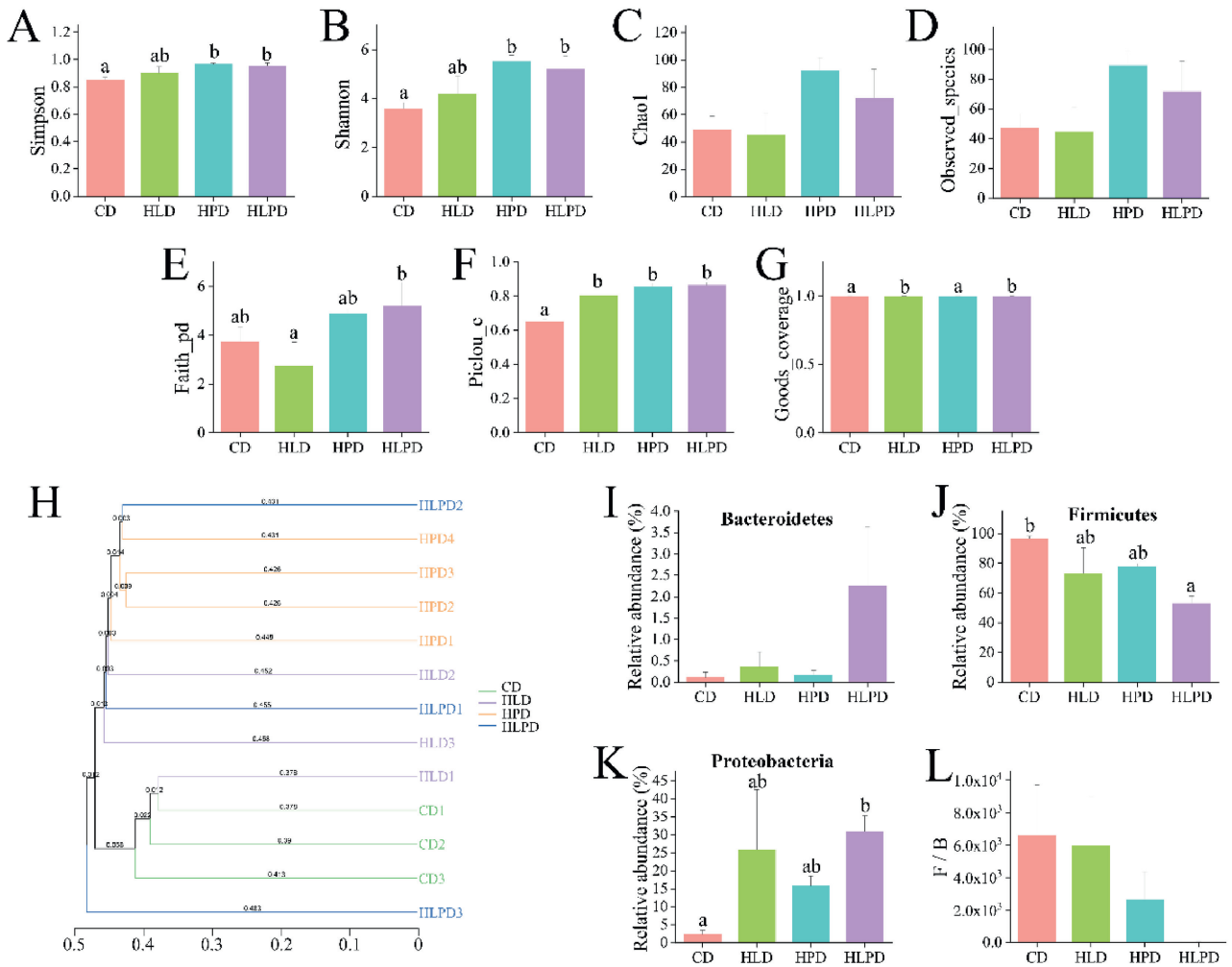
3.5. Dietary high lipid/plant-protein affected the lipid metabolism of groupers

All high lipid or plant-protein treatments effectively suppressed the body's BA level (BA pool), as revealed by the significantly lower levels of TBA in the intestine, liver, serum in HLD, HPD and HLPD

Table 4
The levels of markers of liver impairment in grouper[1].

Item	Diet[2]				*P*-value[3]		
	CD	HLD	HPD	HLPD	L	HP	L × HP
ALT in serum, U/L	55.36 ± 3.008[a]	79.48 ± 5.318[b]	68.92 ± 1.905[b]	77.47 ± 1.133[b]	0.113	0.001	0.043
AST in serum, U/L	21.59 ± 0.448[a]	25.72 ± 0.574[b]	22.66 ± 1.607[a]	22.01 ± 0.453[a]	0.185	0.092	0.031
CAT in liver, U/mg prot	12.10 ± 0.197[c]	6.64 ± 0.629[a]	8.19 ± 0.404[b]	9.07 ± 0.330[b]	0.115	0.001	< 0.001
SOD in liver, U/mg prot	29.41 ± 1.315[b]	23.08 ± 1.625[a]	23.25 ± 0.815[a]	34.66 ± 1.504[c]	0.079	0.097	< 0.001
MDA in liver, nmol/mg prot	1.06 ± 0.025	1.03 ± 0.062	1.12 ± 0.109	1.03 ± 0.107	0.758	0.471	0.737

ALT = alanine aminotransferase; AST = aspartate aminotransferase; CAT = catalase; SOD = superoxide dismutase; MDA = malondialdehyde.
[a–c] Different letters indicate significant differences between groups ($P < 0.05$).
[1] Values are presented as means ± standard deviation (SD).
[2] CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid.
[3] L means lipid level; HP means plant-protein level; L × HP means lipids and plant-protein interacted level.



P-value	Chao1	Simpson	Shannon	Pielou_e	Observed_species	Faith_pd	Goods_coverage	Bacteroidetes	Firmicutes	Proteobacteria	F/B
L	0.421	0.407	0.739	0.002	0.497	0.633	< 0.001	0.107	0.017	0.039	0.504
HP	0.034	0.007	0.009	< 0.001	0.035	0.028	0.103	0.163	0.044	0.28	0.059
L × HP	0.591	0.265	0.313	0.004	0.594	0.374	0.247	0.189	0.965	0.617	0.677

Fig. 2. The structure of gut microbiotas in grouper. (A-G) The alpha diversity of the intestinal microbiota. (H) The Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree with Jaccard distances. (I-L) The proportion of microbiotas at phylum levels. CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid; L means lipid level; HP means plant-protein level; L × HP means lipids and plant-protein interacted level. Values are presented as means ± error bars (SD). F/B = the ratio of Firmicutes/Bacteroidetes. [a,b] Different letters indicate significant differences between groups ($P < 0.05$).

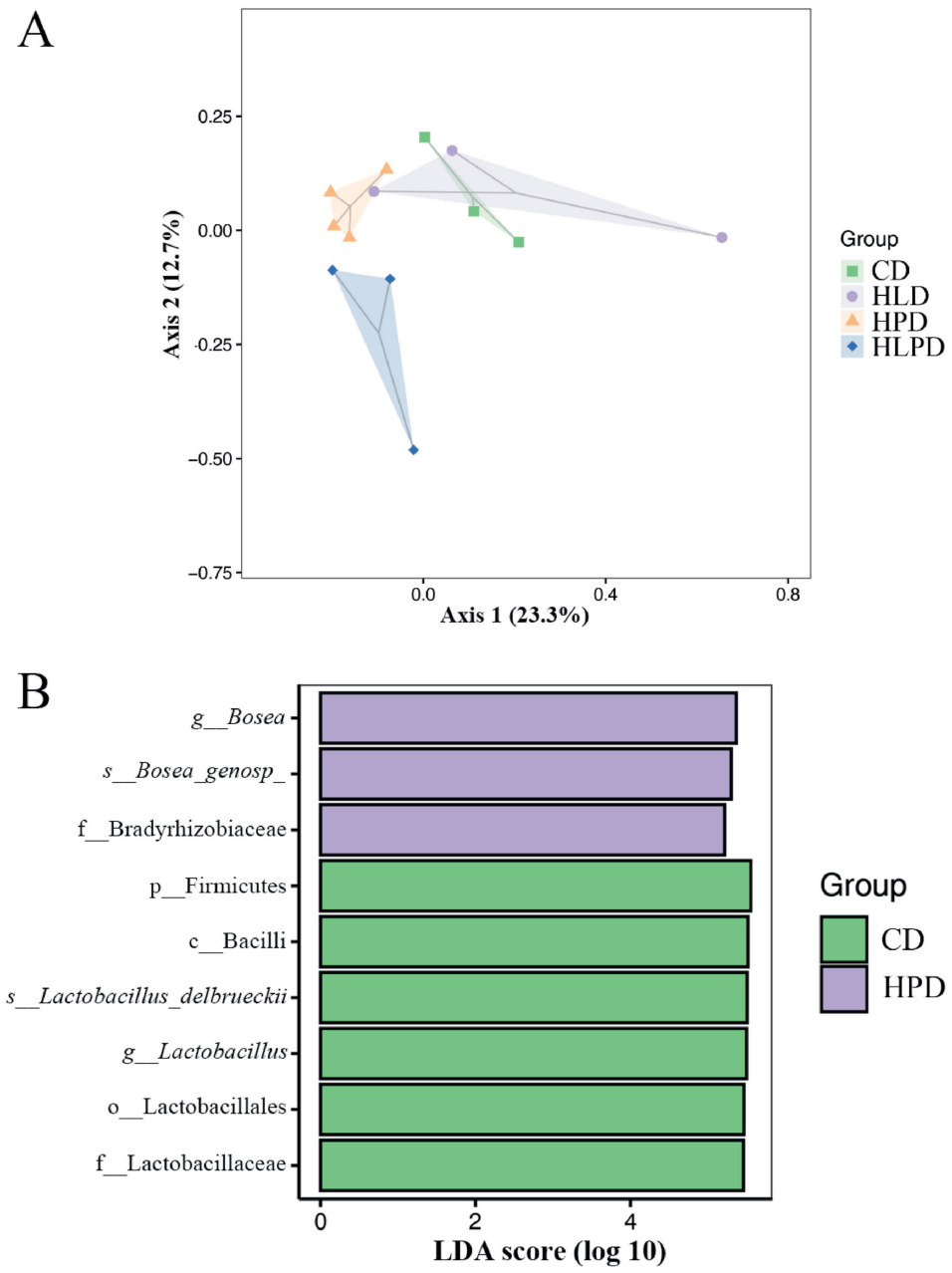
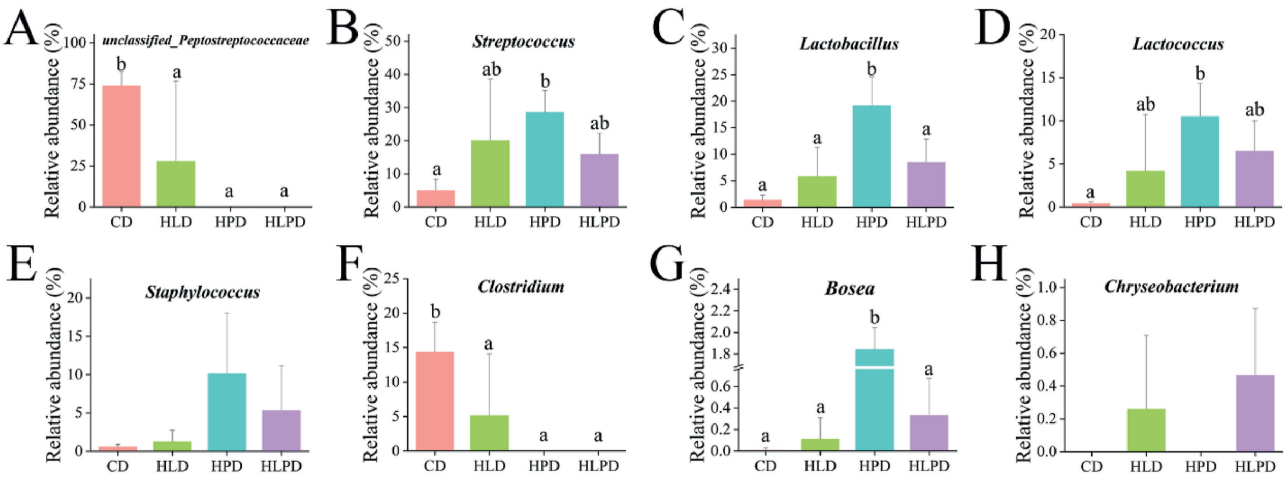


Fig. 3. The beta diversity and LEfSe algorithm of gut microbiota. (A) The unweighted UniFrac based principal coordinate analysis (PCoA) plots. (B) The linear discriminatory analysis (LDA) effect size (LEfSe). CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid.

groups ($P < 0.05$) (Table 5), which further confirmed the alterations of BA profiles in metabolomics. Furthermore, compared to the CD group, the mRNA relative expression of *cyp7a1*, *bsep* and *mdr3* was significantly increased in the HLD group, whereas the mRNA relative expression of *cyp27a1*, *meh*, *asbt*, *mrp3*, *fxr* and *tgr5* was significantly decreased ($P < 0.05$) (Fig. 6). Compared to the CD group, the mRNA relative expression of *mdr3*, *asbt*, *mrp3*, *oatp1*, *meh*, *fxr* and *tgr5* was significantly decreased in the HPD group ($P < 0.05$). Compared to the HPD group, the mRNA relative expression of *cyp27a1*, *bsep*, *mdr3* and *tgr5* was significantly increased in the HLPD group, while the mRNA relative expression of *asbt* was significantly decreased ($P < 0.05$).

Compared to the CD group, the mRNA relative expression of *acc*, *fas*, *lxr*, *pparα* and *srebp1* was significantly increased in HLD group ($P < 0.05$), while the mRNA relative expression of *atgl* and *hsl* was significantly decreased ($P < 0.05$) (Fig. 7A). Compared to the CD group, the mRNA relative expression of *atgl*, *hsl* and *pparα* was significantly increased in the HPD group, while the mRNA relative expression of *lxr* and *srebp1* was significantly decreased ($P < 0.05$). Compared to the HPD group, the mRNA relative expression of *acc*, *fas*, *lxr* and *srebp1* was significantly increased in the HLPD group, while the mRNA relative expression of *atgl* and *hsl* was significantly decreased ($P < 0.05$). In addition, compared to the CD group, the expression of SREBP1 was significantly increased in the HLD and



P-value	*unclassified_ Peptostreptococcaceae*	*Streptococcus*	*Lactobacillus*	*Lactococcus*	*Staphylococcus*	*Clostridium*	*Bosea*	*Chryseobacterium*
L	0.111	0.830	0.255	0.949	0.511	0.110	< 0.001	0.049
HP	0.003	0.116	0.003	0.025	0.049	0.005	< 0.001	0.531
L × HP	0.111	0.035	0.015	0.126	0.388	0.110	< 0.001	0.531

Fig. 4. The proportion of genus of gut microbiotas in groupers. (A) Unclassified_*Peptostreptococcaceae*. (B) *Streptococcus*. (C) *Lactobacillus*. (D) *Lactococcus*. (E) *Staphylococcus*. (F) *Clostridium*. (G) *Bosea*. (H) *Chryseobacterium*. CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid; L means lipid level; HP means plant-protein level; L × HP means lipids and plant-protein interacted level. Values are presented as means ± error bars (SD). [a,b] Different letters indicate significant differences between groups ($P < 0.05$).

HPD groups, and compared to the HLD group, the HLPD treatment significantly increased the mRNA relative expression of SREBP1 ($P < 0.05$; Fig. 7B-C).

4. Discussion

4.1. High lipid diet impaired the growth performance and liver damage via regulating intestinal microbiotas, BA metabolism and lipid metabolism

In this study, the HLD negatively impacted the growth performance of pearl gentian groupers. Previous research reported mixed outcomes regarding HLD's effect on fish growth—ranging from beneficial (Xu et al., 2022a) to neutral (Zheng et al., 2023) and detrimental (Yang et al., 2023). It has been observed that as dietary lipid content increases, fish tend to exhibit a reduced tolerance to lipid-rich feeds, transitioning from growth enhancement to suppression (Xun et al., 2021; Yue et al., 2020). Such variability in growth performance outcomes under HLD is likely due to differences in lipid tolerance among fish subjected to varying experimental conditions, encompassing base diet formulation, culture environment, and genetic makeup.

Echoing the findings related to other fish species (He et al., 2022; Yang et al., 2023; Zheng et al., 2023), our study also found that HLD led to hepatocyte abnormalities, including nuclear migration and vacuolization, and liver damage. This was evidenced by increased activities of AST and ALT, elevated levels of pro-inflammatory factors, decreased antioxidant enzyme activities, and the activation of the KEAP1/NRF2 pathway, further corroborating the adverse effects of high lipid intake on fish health.

Dietary composition markedly influences the intestinal microbial community, impacting the population size and metabolism of crucial symbiotic species and consequently eliciting significant biological alterations in the host (Ye et al., 2020). In our study, the HLD treatment augmented certain indicators of α-diversity. However, research on golden pompano (*Trachinotus ovatus*) indicates that an HLD does not significantly affect most α-diversity indices (Fang et al., 2021; Xun et al., 2021). Furthermore, the relative abundance of *Clostridium* diminished in grouper subjected to an HLD, aligning with observations in rice field eel (Peng et al., 2019). Conversely, the use of an HLD in mice was associated with an increase in the proportions of *Clostridium* and *Lactobacillus* (He et al., 2023). Considering that *Clostridium* is a lipase-producing bacterium in aquatic animals (Wu et al., 2023), these findings imply that an HLD can modulate BA metabolism by adjusting specific intestinal microbiota, with BA metabolic pathways possibly interconnected with lipid metabolism through these microbial populations.

The intestine serves as a critical organ for assessing the BA profile and pool in fish (Wang et al., 2023). This study observed a marked decrease in the levels of ACA and GCDCA in the distal gut of the HLD group. Consistent with some findings, the HLD elevated GCDCA levels in the gallbladder of Tiger puffer (*Takifugu rubripes*) but did not affect it in the liver of yellow catfish (Liao et al., 2020; Zheng et al., 2023). The size of the circulating BA pool in the body is governed by hepatic BA synthesis, transport, intestinal reabsorption, and recycling to the liver (Luo et al., 2023). In humans, elevated lipid consumption stimulates hepatic BA synthesis, leading to increased BA that are reabsorbed by the ileum (Ocvirk, O'Keefe, 2021). Partially corroborating a previous report (Xu et al., 2022b), this study revealed that HLD augmented BA synthesis and transport, while diminishing BA reabsorption and recycling. Given that BA are efficiently reabsorbed (>95%) from the gut content into enterocytes, and this process is vital for maintaining BA homeostasis (Jia et al., 2018), inflammation-induced impairment of intestinal function significantly reduces the BA pool size (Romano et al., 2020). Although intestinal health was not directly assessed in this study, based on prior research (He et al., 2023; Krogdahl et al., 2015), it was hypothesized that HLD treatment might have

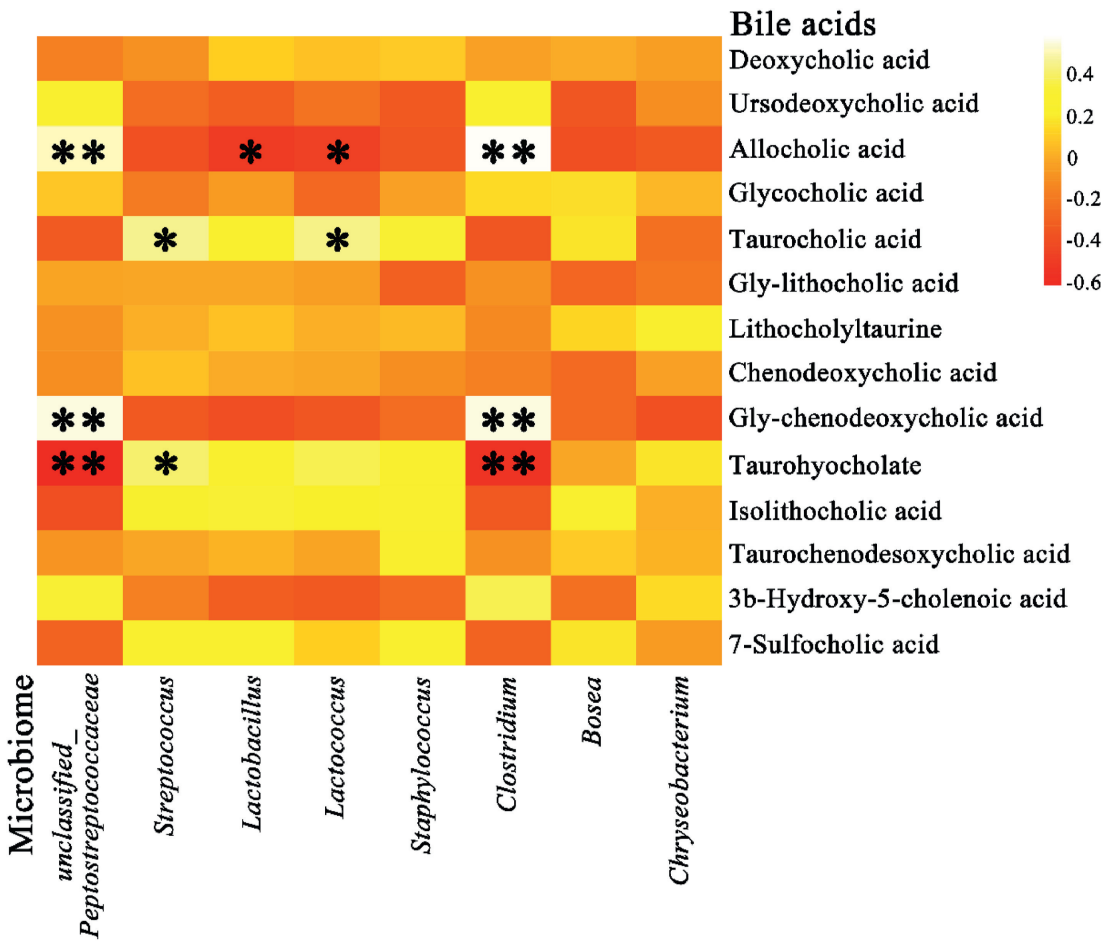


Fig. 5. Spearman's correlation analysis of BA profiles and representative genus in grouper. BA = bile acids. * $P < 0.05$; ** $P < 0.01$.

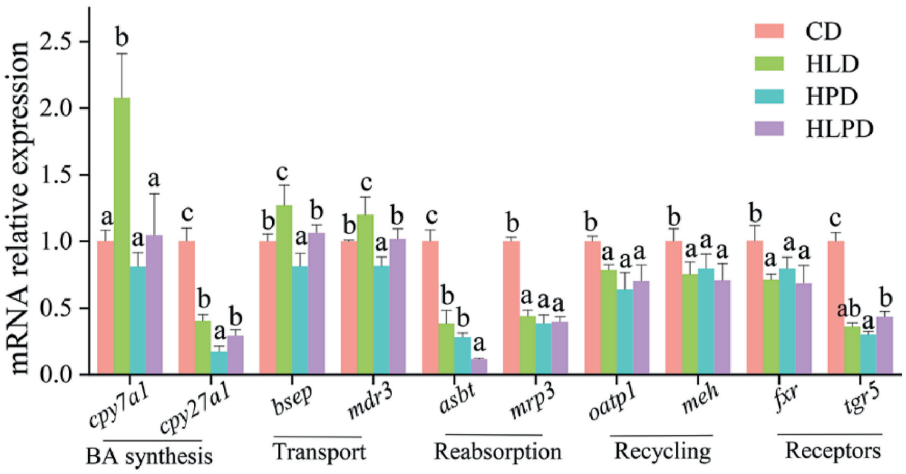
Table 5
The concentrations of TBA in the intestine, liver and serum tissues[1].

Item	Diet[2]				*P*-value[3]		
	CD	HLD	HPD	HLPD	L	HP	L × HP
Intestine, μmol/mg prot	114.70 ± 9.291[b]	72.85 ± 13.921[a]	82.84 ± 9.092[a]	81.16 ± 7.653[a]	0.006	0.082	0.010
Liver, μmol/mg prot	67.62 ± 5.689[b]	45.89 ± 2.924[a]	52.79 ± 5.838[a]	39.36 ± 8.217[a]	0.001	0.015	0.263
Serum, μmol/L	65.34 ± 5.251[b]	46.90 ± 0.782[a]	53.22 ± 4.136[a]	46.17 ± 2.071[a]	0.105	0.007	0.144

TBA= total bile acids.
[a,b] Different letters indicate significant differences between groups ($P < 0.05$).
[1] Values are presented as means ± standard deviation (SD).
[2] CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid.
[3] L means lipid level; HP means plant-protein level; L × HP means lipids and plant-protein interacted level.

diminished BA absorption and pool size due to compromised gut function under these dietary conditions.

Given that BA metabolism is known to regulate lipid and glucose homeostasis through BA receptor activation, alterations in lipid metabolism might be associated with the mRNA relative expression of *fxr* and *tgr5* in this study. Previous findings indicated that feeding a HLD decreased *fxr* mRNA relative expression in Tiger puffer and both *fxr* and *tgr5* mRNA relative expression in pearl gentian groupers (Liao et al., 2020; Xu et al., 2022a). Consistent with these findings, our study showed that HLD, HPD, and HLPD treatments significantly decreased *fxr* and *tgr5* mRNA relative expression. The reduced *fxr* mRNA relative expression might be due to altered BA composition and reduced BA pool size, as GCDCA has been shown to promote FXR expression in rat pancreatic acinar-like AR42J cells and gallbladder cancer cells (Wang et al., 2020; Zhou et al., 2017). Furthermore, the FXR-SHP and FXR-LXR axis inhibited the mRNA relative expression of *srebp1* and its downstream lipogenic genes in the liver (Watanabe et al., 2004; Yang, Wu, 2022). Activation of FXR also upregulated the expression of PPARα and its targets related to fatty acid oxidation (Cyphert et al., 2012). Previous studies have shown that feeding HLD to groupers increases the expression of lipogenic genes such as *srebp1* and *fas*, while decreasing the mRNA relative expression of lipolytic genes such as *cpt1* and *ppar* (Li et al., 2022a; Xu et al., 2022a; Zheng et al., 2023). Consistent with these findings, our study confirmed that the HLD enhanced lipogenesis. This enhancement of lipogenesis is one reason why the HLD



P-value	*cpy7a1*	*cpy27a1*	*bsep*	*mdr3*	*asbt*	*mrp3*	*oatp1*	*meh*	*fxr*	*tgr5*
L	0.001	< 0.001	0.002	0.003	< 0.001	< 0.001	0.189	0.025	0.009	< 0.001
HP	0.002	< 0.001	0.008	0.005	< 0.001	< 0.001	0.003	0.070	0.077	< 0.001
L × HP	0.015	< 0.001	0.856	0.988	< 0.001	< 0.001	0.029	0.221	0.161	< 0.001

Fig. 6. The mRNA relative expression of BA enterohepatic circulation genes in grouper. *cpy7a1* = cholesterol 7α-hydroxylase; *cyp27a1* = sterol-27-hydroxylase; *bsep* = bile salt export pump; *mdr3* = multidrug resistance protein 3; *asbt* = apical sodium-dependent BAs transporter; *mrp3* = multidrug resistance-associated protein 3; *oatp1* = organic anion transporters 1; *meh* = microsomal epoxide hydrolase; *fxr* = farnesoid X receptor; *tgr* = G protein-coupled bile acid receptor. CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid; L means lipid level; HP means plant-protein level; L × HP means lipids and plant-protein interacted level. Values are presented as means ± error bars (SD). [a-c] Different letters indicate significant differences between groups ($P < 0.05$).

promoted fat deposition, as evidenced by increased crude lipid in tissues and elevated hepatic or systemic lipid levels in pearl gentian grouper. Overall, dietary high lipid intake impaired intestinal microbiota, BA metabolism, and lipid metabolism, which in turn induced lipid deposition and liver damage, ultimately impairing the growth performance of pearl gentian groupers.

4.2. High plant-protein diet impaired the growth performance and liver damage via regulating gut microbiota, BA metabolism and inflammatory response

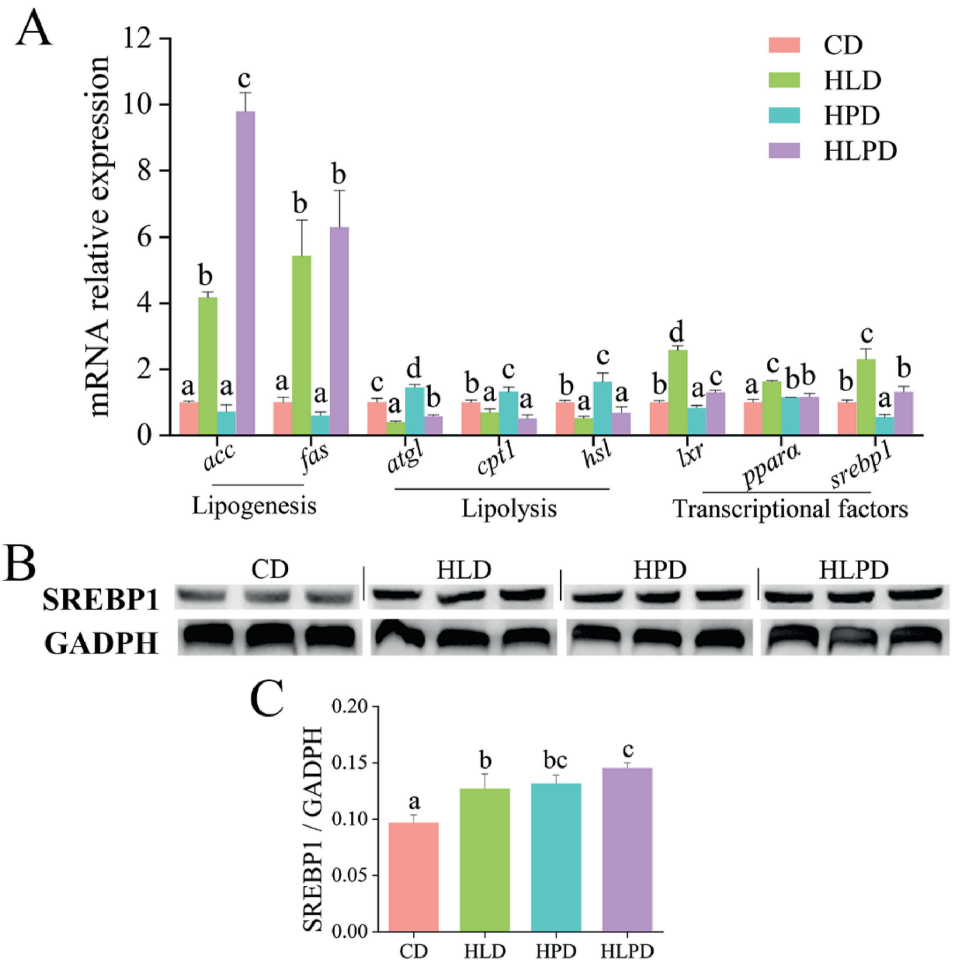
In previous studies, diets enriched with CPC, substituting for fishmeal at approximately 50%, either attenuated or did not impact fish growth performance (Liu et al., 2022; Tian et al., 2022; Xie et al., 2023). Consistent with these observations, the HPD also adversely affected the growth performance of pearl gentian groupers in this study. Reflecting outcomes from research on largemouth bass, where a HPD detrimentally influenced liver functionality (Xie et al., 2023), the current study similarly identified that the HPD induced hepatocyte abnormalities and liver impairment in groupers.

Prior research revealed that a HPD augmented the α-diversity of the gut microbiome in *L. vannamei* (Wang et al., 2022). In alignment with these findings, the present study observed an enhancement in specific α-diversity indicators under the HPD treatment. Given the antimicrobial properties of BA that inhibit the proliferation of pathogenic bacteria (He et al., 2023), it is hypothesized that diminished BA levels could increase intestinal bacterial abundance, consequently enriching microbial diversity within the HPD group. Moreover, variations in protein intake levels have been shown to influence *Lactobacillus* abundance in mice (He et al., 2023). Echoing these insights, our study documented a reduction in *Clostridium* abundance and an elevation in *Lactobacillus* within the HPD group, highlighting the linkage of these genera with BA metabolism. This suggests that feeding an HPD may modulate BA metabolism by altering specific intestinal microbiotas.

Significant reductions in ACA and GCDCA levels were observed in the distal gut of the HPD groups. Partially aligning with our findings, feeding an HPD elevated ACA levels and reduced GCDCA levels in the hemolymph of *L. vannamei* (Li et al., 2023). Correlation analysis indicated that variations in BA profiles (ACA, TCA, GCDCA, and THCA) correlated with changes in the relative abundance of *unclassified_Peptostreptococcaceae*, *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Staphylococcus* and *Clostridium*. Similarly, in mouse liver, TCA levels were negatively correlated with the relative abundance of *Clostridium* and *Lactobacillus* (Chen et al., 2022). Despite *Lactobacillus*'s notable capacity to hydrolyze GCDCA (Liang et al., 2021), no association was found in the current study, signaling a need for further research to elucidate the complex interactions between gut microbiota and BA metabolism.

As fishmeal alternatives often disturb BA homeostasis by either increasing BA excretion/decreasing intestinal reabsorption or decreasing synthesis due to lower cholesterol levels (a BA precursor) and the ability of certain plant-protein compounds to bind bile salts, preventing their reabsorption (Romano et al., 2020), these mechanisms may explain the observed inhibitory effects on BA synthesis, transport, reabsorption, and recycling in grouper attributable to the HPD in our study.

Furthermore, in the present study, the HPD significantly downregulated the mRNA relative expression of *fxr* and *tgr5*, genes essential in regulating fat metabolism. Interestingly, despite HPD treatment, adipogenic genes and regulatory factors did not exhibit upregulation, except for a significant increase in SREBP1 protein expression. Conversely, expressions of lipolysis genes (*atgl*, *cpt1*, and *hsl*) were significantly upregulated. Given that crude lipid levels in liver, serum, and whole-body of fish remained unchanged, it appears that the HPD had minimal impact on lipid metabolism, contrasting with previous findings that indicated the HPD could influence lipid deposition in other fish species (He et al., 2022; Tian et al., 2022; Xie et al., 2023). These discrepancies warrant further investigation.



P-value	*acc*	*fas*	*atgl*	*cpt1*	*hsl*	*lxr*	*pparα*	*srebp1*	SREBP1/GADPH
L	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002
HP	< 0.001	0.627	< 0.001	0.290	0.003	< 0.001	0.005	< 0.001	0.001
L × HP	< 0.001	0.190	0.013	0.005	0.039	< 0.001	< 0.001	0.042	0.129

Fig. 7. The lipid metabolism in response to dietary high lipid and high plant-protein in grouper. (A) The mRNA relative expression of lipid metabolism genes. (B) The Western blot analysis, three replicate wells were made for each protein sample. (C) The relative quantification of protein levels. *acc* = acetyl-CoA carboxylase; *fas* = fatty acid synthase; *atgl* = adipose triglyceride lipase; *cpt1* = carnitine palmitoyltransferase 1; *hsl* = hormone-sensitive lipase; *lxr* = liver X receptor alpha; *pparα* = peroxisome proliferator-activated receptor alpha; *srebp1* = sterol responsive element binding protein 1; GADPH = glyceraldehyde-phosphate dehydrogenase. CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid; HLPD means high lipid-high plant-protein diet, 46.54% crude protein, 16.67% crude lipid; L means lipid level; HP means plant-protein level; L × HP means lipids and plant-protein interacted level. Values are presented as means ± error bars (SD). [a-d] Different letters indicate significant differences between groups ($P < 0.05$).

Additionally, recent studies, including our own, suggest that *tgr5* and *fxr* signaling pathways may mitigate the inflammatory response by inhibiting the nuclear translocation of nuclear factor-κB (NF-κB) in grouper, mice, and humans (Chiang, 2013; Ticho et al., 2019; Xu et al., 2022d, 2023). In accordance with these findings, we observed a significant inverse correlation between the expression of BA receptor genes and pro-inflammatory factors, potentially contributing to liver damage in grouper. Overall, our findings propose a potential mechanism by which feeding an HPD influences gut microbiota, BA metabolism, and inflammatory response, thereby affecting the expression of BA receptors and ultimately compromising liver health and growth performance in pearl gentian grouper.

4.3. High lipid-high plant-protein diet did not further impair liver health, but disrupted growth performance

The current study has elucidated that both the HLD and HPD detrimentally impact the growth performance and liver health of groupers. Notably, the synergistic effects of the HLD and HPD, as observed in the HLPD group, primarily compromised growth performance without exacerbating hepatic damage. Specifically, the serum activities of ALT and AST, and the degree of hepatocyte microstructural damage in the HLPD group were not significantly divergent from those in the HLD and/or HPD groups. Similarly, the HLPD did not augment indicators related to antioxidant capacity, inflammatory response, and lipid metabolism in the liver, nor did it

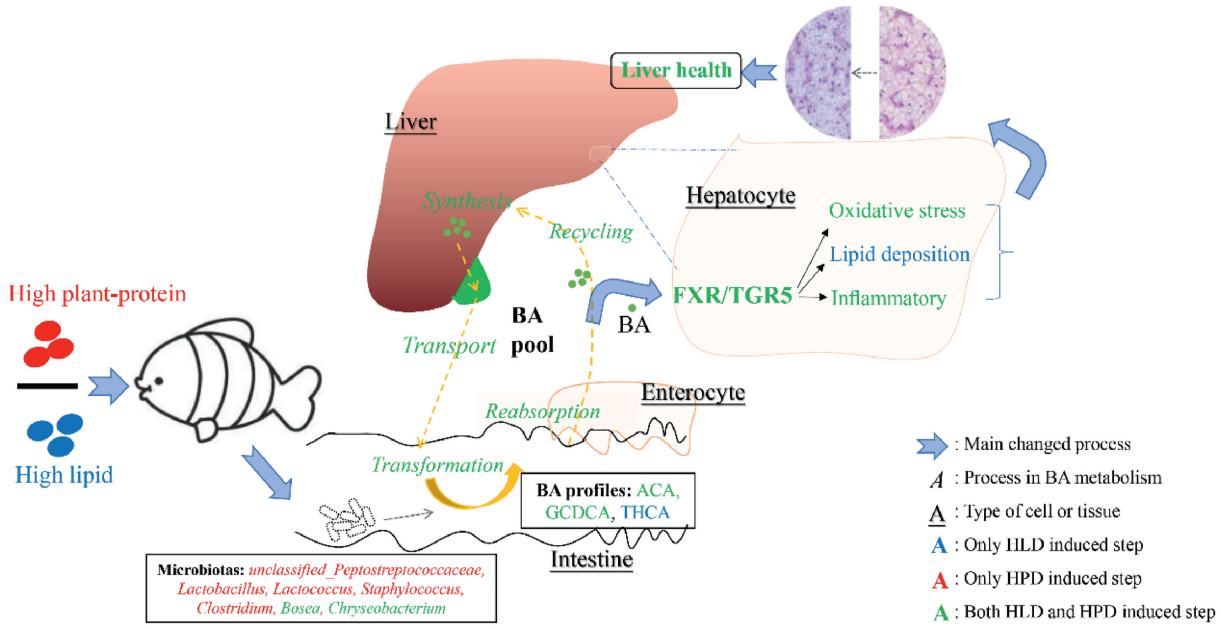


Fig. 8. The potential mechanism by which the high lipid and high plant-protein diets impaired the liver health in grouper. ACA = allocholic acid; GCDCA = gly-chenodeoxycholic acid; THCA = taurohyocholate; BA = bile acids metabolism; FXR = farnesoid X receptor; TGR5 = G protein-coupled bile acid receptor 5. CD means control diet, 46.21% crude protein, 9.48% crude lipid; HLD means high lipid diet, 46.37% crude protein, 16.70% crude lipid; HPD means high plant-protein diet, 46.50% crude protein, 9.38% crude lipid.

modify the principal composition of the intestinal microbiota when juxtaposed with the HLD and HPD treatments. This observation implies that groupers exhibit a robust capacity for self-regulation of hepatic damage, thereby mitigating the potential exacerbation of these indicators by the combined effects of the HLD and HPD. Consequently, the HLPD could be regarded as a viable alternative for preserving feed fishmeal with respect to liver health, albeit with the proviso that specific applications necessitate further scrutiny.

Moreover, given that the HLPD group exhibited the lowest feed intake, it is hypothesized that the compromised growth performance in this cohort is attributable not to hepatic health impairment but to diminished feed intake. This finding underscores the imperative to address issues of palatability and attractiveness in HLPD feeds prior to their implementation. However, this hypothesis remains provisional and requires empirical validation to substantiate the findings of this investigation.

Additionally, examination of primary and secondary metabolite identification maps within the metabolome, incorporating PCA, disclosed a substantial overlap in sample distribution between the HPD and HLPD groups. This observation suggests that the HPD component significantly modulates the effects of the HLPD treatment on the intestinal metabolome. Furthermore, the HLPD treatment mirrored the HLD treatment in terms of lipid deposition, lipolysis, and lipid synthesis in grouper, indicating that the HLD treatment is a primary determinant of the impact of the HLPD treatment on lipid homeostasis. Considering the scarcity of research on HLPD feeds in fish, our findings underscore the necessity for further investigation to elucidate these phenomena.

5. Conclusion

The present study substantiates the hypothesis that dietary high lipid intake detrimentally affects the intestinal microbiota, BA metabolism, and lipid metabolism, culminating in lipid deposition, liver damage, and impaired growth performance of pearl gentian groupers (Fig. 8). Moreover, the investigation has delineated a plausible mechanism by which HPD feeds influence the gut microbiota, BA metabolism, inflammatory responses, and the expression of BA receptors, thereby compromising liver health and growth performance in grouper. Notably, our findings reveal that HLPD feeds do not further impair (compared to the HLD and HPD) liver health in groupers; however, they significantly disrupt growth performance. This research highlights the pivotal role of the interplay between intestinal microbiota and BA metabolism as a critical mechanism by which various diets modulate liver health in fish and possibly other animals. Additionally, this study advances our comprehension of the physiological implications of HLPD feeds and provides fresh feasibility into strategies for minimizing dietary fishmeal levels.

Credit author contribution statement

Jia Xu: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Fan Wang:** Writing – original draft, Investigation, Formal analysis, Data curation. **Chaoqun Hu:** Writing – review & editing, Validation, Resources, Conceptualization. **Junxiang Lai:** Writing – review & editing, Investigation, Conceptualization. **Shiwei Xie:** Writing – review & editing, Conceptualization. **Kefu Yu:** Project administration, Funding acquisition, Conceptualization. **Fajun Jiang:** Writing – review & editing, Project administration, Conceptualization.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.08.005.

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