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Original Research Article

Enhancing growth, liver health, and bile acid metabolism of tilapia (*Oreochromis niloticus*) through combined cholesterol and bile acid supplementation in plant-based diets

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ABSTRACT

The present study aimed to compare the nutritional effects of cholesterol, bile acids, and combination of cholesterol with bile acids in plant-based diets on juvenile genetically improved farmed tilapia (GIFT; *Oreochromis niloticus*). The isonitrogenous (321 g/kg crude protein) and isolipidic (76 g/kg crude fat) diets (Con diet) were based on plant protein sources, which included corn gluten meal, soybean meal, cottonseed meal and rapeseed meal. The Con diet was supplemented with 12 g/kg cholesterol (CHO diet), 0.2 g/kg bile acids (BAs diet), a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids (CHO–BAs diet), respectively. Each diet was fed to three tanks in an indoor recirculating aquaculture system for 9 weeks. Results showed that compared to the Con group, fish had a higher weight gain rate, hepatosomatic index, and a lower feed conversion ratio in the CHO–BAs group. The highest levels of whole-fish fat and ash were found in the Con group. Serum parameters, including activities of alanine aminotransferase (ALT) and aspartate transaminase (AST), along with levels of glucose (GLU) and triglyceride (TG) except for total cholesterol (TCHO), were lower in the CHO, BAs, and CHO–BAs groups than those in the Con group ($P < 0.001$). Histological examination revealed that fish in the Con group exhibited severe hepatocyte vacuolization and diminished hepatocyte proliferation. Gene expression analysis indicated that the transcriptional levels of bile acid metabolism-related genes (including *fxr*, *fgf19*, *bsep*) were up-regulated in the CHO–BAs group ($P < 0.05$), whereas cholesterol metabolism-related genes (*acly* and *hmgcr*) were down-regulated in both CHO and CHO–BAs groups ($P < 0.001$). Moreover, UPLC-MS/MS analysis revealed that the higher taurine-conjugated bile acids (T-BAs), followed by free bile acids (Free-BAs) and glycine (G-BAs) were determined in tilapia bile. Among these, taurochenodeoxycholic bile acid was the predominant bile acid. Dietary bile acids supplementation also increased the proportion of T-BAs (tauro β-muricholic acid and taurodehydrocholic acid) while decreasing Free-BAs in the fish bile. In conclusion, the incorporation of cholesterol with bile acids into plant-based diets can effectively reduce cholesterol uptake, suppress bile acids synthesis, enhance bile acids efflux, and promote hepatocyte proliferation, which is helpful for maintaining the normal liver morphology in tilapia, and thus improving its growth performance.

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1. Introduction

The rapid development of aquaculture has accelerated the increased demand for fish meal and fish oil, which conversely poses a serious threat to marine fish resources (Lei et al., 2023). Considering the importance of sustainable development in aquaculture,

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the issue of fish meal and fish oil replacement should be at the forefront in fish nutrition research and industrial application (Kamalam et al., 2016). Plant ingredients have been increasingly used in aquatic feed because of their easy availability and relatively low cost. However, an extremely low cholesterol levels may lead to a cholesterol deficiency in plant-based diets (Zhang et al., 2019). The sterols contained in plant ingredients mainly include β-sitosterol, campesterol, stigmasterol and brassicasterol. Previous studies have shown that some phytosterols could suppress intestinal cholesterol absorption, reduce blood cholesterol content, and thus have adverse impacts on the growth and body composition of aquatic animals (Kumar et al., 2020; Zhang et al., 2019).

Cholesterol is an essential substance for the cellular structure and function due to its role as an important component within the cell membrane (Li et al., 2023). Bile acids, the end products of cholesterol metabolism, are the main components of bile and mainly stored in the gallbladder in the form of bile salts (Chiang and Ferrell, 2020). Bile acids have multiple physiological properties, such as their function in the absorption and transport of fat and fat-soluble vitamins, the prevention of gallstone formation, and the metabolic regulation of bile acids and cholesterol (Cao et al., 2021; González-Pena et al., 2017; Romano et al., 2020). Recently, many studies have shown that dietary supplementation of cholesterol or bile acids has positive effects on fish growth (Adhami et al., 2017; Deng et al., 2013b; Jiang et al., 2018). For example, an addition of 0.9% to 1.2% cholesterol to a plant-based diet significantly improved the feeding and growth of rainbow trout (*Oncorhynchus mykiss*) (Deng et al., 2013a, 2014). Similarly, the addition of 0.8% to 3.2% cholesterol to a high-fat diet increased the growth of tilapia (*Oreochromis niloticus*) (Li et al., 2023). Dietary supplementation of appropriate amounts of bile acids also could promote the growth and lipid metabolism of grass carp (*Ctenopharyngodon idllus*) and common carp (*Cyprinus Carpio*) (Peng et al., 2019; Ting et al., 2021; Zhou et al., 2018). Although these studies have documented the advantages of exogenous cholesterol or bile acids on fish growth and health, the combined impacts of cholesterol and bile acids, especially in plant-based diets, are still unclear.

Tilapia is the second most farmed fish worldwide, and its production has increased approximately four-fold over the past few decades owing to its easiness of aquaculture, high nutritional value, and marketability (Prabu et al., 2019). Commonly, in tilapia aquaculture, the plant-based feed ingredients are a good alternative for fishmeal in consideration of fish digestive capability (Olsen and Hasan, 2012). It is noted that a lower content of cholesterol was found in these practical diets, which may impair the normal bile acid metabolism in fish. Studies on child nutrition reported that feeding on vegetarian diets significantly reduced serum cholesterol levels and altered metabolism of bile acids (Hovinen et al., 2021). Therefore, we hypothesized that cholesterol deficiency in a plant-based diet may lead to abnormal bile acid metabolism in tilapia. Consequently, the incorporation of dietary cholesterol with bile acid in plant-based diets may be better for maintaining cholesterol-bile acid metabolic balance, improving fish growth. The present study aims to investigate the nutritional effects of cholesterol-bile acid on tilapia, based on several parameters including growth performance, body composition, liver tissue morphology, and bile acid composition in the gallbladder. These findings would deepen our understanding of cholesterol-bile acid metabolism in aquatic animals, and provide theoretical basis for better utilization of bile acids in aquatic feed.

2. Materials and methods

2.1. Animal ethics

Fish management and sampling protocols were authorized by the Animal Experimental Ethical Inspection of Laboratory Animal Centre, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (Permit Number: YFI2022JM02).

2.2. Experimental diet

According to a similar study (Jiang et al., 2018), dietary bile acid supplementation (approximately 0.19 g/kg) can promote the growth performance and lipid metabolism of tilapia. Data from a previous trial showed that dietary supplementation with 12 g/kg cholesterol had beneficial effects on the growth and health of tilapia (Li et al., 2023). Therefore, a basal diet (Con) without the addition of cholesterol and bile acids was formulated, which contained 321 g/kg crude protein and 76 g/kg crude lipid. Protein sources in diet were provided by a combination of different plant ingredients including corn gluten, soybean meal, cottonseed meal and rapeseed meal. Since tilapia lack specific requirements for n-3 (omega-3) polyunsaturated fatty acids (Corrêa et al., 2023), soybean and corn oil were used as dietary lipid sources to prevent the introduction of exogenous cholesterol. Four practical test diets were prepared by supplementing 12 g/kg cholesterol (CHO), 0.2 g/kg bile acid (BAs) or 12 g/kg cholesterol and 0.2 g/kg bile acids (CHO–BAs) to the basal diet (Table 1). No animal protein and fat sources were used in the test diets. The formulations of these four experimental diets were prepared according to the routine procedure as described in our previous study (Jiang et al., 2018). In brief, to prepare the experimental diets, dry ingredients were finely ground to particles smaller than 300 μm and weighed accurate to 0.1 g. They were then mixed with oil, and distilled water (400 mL per kilogram of the diet) was added to form a uniform moist dough, which was extruded through a meat grinder (TY-432; Shanghai Tai Yi Machinery, China). The resultant noodle-like product was dried at room temperature (18–25 °C) using electrical fans to expedite the process and broken into small pellets (approximately 2.0 mm). The bile acid used in this study was provided by Shandong Longchang Animal Health Product Co., Ltd, Jinan, China, and it mainly contained 699.2 g/kg of hyodesoxycholic acid, 189.2 g/kg of chenodeoxycholic acid and 77.5 g/kg hyocholic acid, which was estimated by high-performance liquid chromatography (HPLC), respectively.

2.3. Experimental fish and feeding

Tilapia were purchased from the Guangxi Tilapia National Breeding Station (Nanning) and transported to an indoor recirculating aquarium culture system (RAS), then were cultivated for 2 weeks with the Con diet at the Yangtze River Fisheries Research Institute (Wuhan, China). At the beginning of the feeding trial, the fish were fasted for 24 h and anesthetized with 40 mg/L MS-222 (Sigma, USA). A total of 360 uniform-sized fish at average weight (6.08 ± 0.12 g, $n = 30$) were selected, and 30 fish were weighed and randomly assigned to one polycarbonate tank (diameter 82 cm × height 80 cm × water depth 75 cm). Each diet was fed randomly to three tanks, with a total of twelve tanks were in this study. Fish were hand-fed slowly to apparent satiation three times daily (08:30, 12:30 and 16:30), and the feeding lasted for 9 weeks. During the culture period, all tanks were supplied with continuous flow of circulating aerated water (about 400 L/h). The water was sourced from municipal tap water, and approximately 15% of the water per tank was exchanged twice a day. Several aquaculture parameters were monitored as below: water temperature was 28 to 30 °C, dissolved oxygen level was 6 to 7 mg/L, and pH was 6.8 to 7.3. The study was carried out with natural photoperiod, and fish mortality was monitored daily.

Table 1
Formulation and proximate composition of the test diets (as air-dried basis, g/kg).

Item	Con	CHO	BAs	CHO–BAs
Ingredients				
Corn gluten meal[1]	100	100	100	100
Soybean meal[2]	200	200	200	200
Cottonseed meal[3]	80	80	80	80
Rapeseed meal[3]	300	300	300	300
Whole wheat flour[4]	197	197	196.8	196.8
Corn oil[5]	30	24	30	24
Soybean oil[5]	30	24	30	24
Choline chloride	1	1	1	1
Vitamin premix[6]	10	10	10	10
Mineral premix[7]	10	10	10	10
DL-Methionine	2	2	2	2
Bentonite	20	20	20	20
$Ca(H_2PO_4)_2$	20	20	20	20
Bile acids[8]	0	0	0.2	0.2
Cholesterol[9]	0	12	0	12
Total	1000	1000	1000	1000
Proximate composition, g/kg diet as fed				
Crude protein	321	321	325	324
Crude lipid	76	74	73	70
Ash	85	83	84	83
Moisture	58	56	59	54

Con = basal diet; CHO = basal diet supplemented with 12 g/kg cholesterol; BAs = basal diet supplemented with 0.2 g/kg bile acids; CHO–BAs = basal diet supplemented with a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids.

[1] Henan Julong Bioengineering Co., Ltd.
[2] Cofco (Dongguan) Grain and Oil Industry Co., Ltd.
[3] Xinjiang Jianlan Plant protein Co., Ltd.
[4] Yihaijiali (Wuhan) Grain and oil industry Co., Ltd.
[5] Yihaijiali Arowana Food Co., Ltd.
[6] The premix provided the following per kilogram of diets: retinol acetate 5000 IU; cholecalciferol 2000 IU; α-tocopheryl acetate 60 mg; L-ascorbyl-2-monophosphate-Mg 120 mg; menadione 5 mg; thiamin hydrochloride 5 mg; riboflavin 20 mg; pyridoxine hydrochloride 10 mg; nicotinic acid 120 mg; calcium pantothenate 10 mg; folic acid 1 mg; biotinn 0.1 mg; inositol 400 mg.
[7] The premix provided the following per kilogram of diet: $Ca(CH_3CHOHCOO)_2$ 6540 mg, $FeSO_4$ 42.5 mg, $MgSO_4$ 1340 mg, NaH_2PO_4 1744 mg, NaCl 870 mg, $AlCl_3$ 3 mg, KIO_3 2.5 mg, KCl 1500 mg, $CuCl_2$ 2 mg, $MnSO_4$ 16 mg, $CoCl_2$ 20 mg, $ZnSO_4$ 60 mg.
[8] Shang dong Longchang Animal Health Product Co., Ltd.
[9] Shanghai yuanye Bio-Technology Co., Ltd.

2.4. Sample collection

At the end of 9-week feeding trial, fish were fasted for 24 h before sampling. All fish were anesthetized with 40 mg/L MS-222 (Sigma, USA) before recording the total survival and total weight of fish from each aquarium. Four fish from each replicate were randomly collected, killed by an overdose of MS-222, and stored at −20 °C for final whole-body analysis. Another twelve fish per tank were randomly selected to measure body weight and body length to calculate the condition factor (CF). Blood from each fish was obtained by puncturing the caudal vein using a 1-mL syringe. Blood samples were allowed to clot at 4 °C for 4 h then centrifuged (900 × *g*, 15 min) to collect serum samples. Following blood collection, each of the six fish from each tank were dissected to obtain the liver, placed in 4% paraformaldehyde in phosphate buffer saline (PBS) for 24 h, then preserved in 70% ethanol for H&E staining. Another six fish were collected to obtain liver and bile samples, quickly frozen with liquid nitrogen, and then stored in a freezer at −80 °C until analyzed.

2.5. Sample processing and analysis

The whole fish samples were freeze-dried by a vacuum freeze drier (Christ Beta 2–4 LD plus LT; Marin Christ Corporation) and then smashed. Gross composition analysis for fish and diets were conducted using the standard methods (AOAC, 1995) as follows: crude protein content was calculated by Kjeldahl method (N × 6.25) after the determination of total nitrogen by LECO auto-analyser (KjelFlex K-360; BUCHI Labortechnik AG); crude fat was determined by Soxhlet extraction through petroleum ether extract in a liquid phase extraction apparatus; ash was obtained by calcinating samples in a muffle furnace (SX-4-10) at 550 °C for 8 h.

Serum chemistry parameters, including the activities of alkaline phosphatase (ALP), aminotransferase (AST) and alanine aminotransferase (ALT), along with the contents of albumin (ALB), triglyceride (TG), glucose (GLU), total protein (TP) and total cholesterol (TCHO) were detected with the automatic biochemistry analyzer (CHEMIX-800, Mikan hisen) (Zhu et al., 2021).

2.6. Quantitative real-time PCR analysis

Six genes related to cholesterol and bile acids metabolism were selected to assess their expression levels in response to different dietary treatments. These genes included ATP citrate lyase a (*acly*, lipogenesis-related gene), 3-hydroxy-3-methylglutaryl-CoA reductase a (*hmgcr*, cholesterol synthesis-related gene), low density lipoprotein receptor a (*ldlr*, cholesterol uptake-related gene), nuclear receptor subfamily 1, group H, member 4 (*fxr*, cholesterol uptake-related gene), fibroblast growth factor 19 (*fgf19*, cholesterol uptake-related gene), ATP-binding cassette (*bsep*, cholesterol uptake-related gene), and their primer information is listed in Table 2. The method of quantitative real-time PCR (qRT-PCR) was performed as described previously (Lu et al., 2021). Briefly, high-quality RNA was extracted from liver tissue and then used for cDNA synthesis. The reaction system consisted of 2 μL cDNA sample, 0.4 μL PCR forward/reverse primers (10 μM), 10 μL SYBR Premix Ex Taq and 7.2 μL enzyme-free water. The cycle process contained 95 °C 30 s, 40 cycles of 95 °C 10 s, 60 °C 15 s, 72 °C 20 s. All kits used for qRT-PCR experiment were purchased from Takara Biotech. Beta-actin was selected as the reference gene. The mRNA relative expression was quantified by $2^{-\Delta\Delta CT}$.

2.7. 5-ethynyl-2′-deoxyuridine (EdU) labeling of the liver

To assess the effects of dietary cholesterol and bile acids on hepatocyte proliferation, three fish were selected from each tank and intraperitoneally injected with EdU (Beyotime, Shanghai, China). Subsequently, these fish were transferred to new tanks with clean water. The injection dosage used was in accordance with the manufacturer's guidelines (50 μg/g body weight). The fish were euthanized 12 h after EdU injection. Their liver tissues were then extracted for further processing, sectioning and staining to identify EdU-labeled cells. To accomplish this, the BeyoClick EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, Shanghai, China) was utilized following the manufacturer's instructions. The tissue sections were subjected to a 30-min incubation with Click Additive Solution, and subsequently stained with Hoechst 33342 for 10 min to visualize the cell nuclei (Wu et al., 2024).

2.8. Bile acid quantitative analysis

To investigate the effects of dietary cholesterol and bile acids on bile acid profile, the bile samples were analyzed using ultra-performance liquid chromatography (UPLC, Shim-pack UFLC SHIMADZU CBM30A) and tandem mass spectrometry (MS/MS, Applied Biosystems 6500 QTRAP). Waters ACQUITY UPLC HSS T3 C18 (1.8 μm,

Table 2
Primers used in experiment.

Gene	Gene ID	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
bsep	XM_019352153.2	GGAAGAGCACCGCCATTCAACT	GAGAACGCAGCCACTGGATGTT
ldlr	XM_003443172.5	GGACCACACCGAGGACCATACT	GGATTCCCTGGCTGGAGCAAAG
hmgcr	XM_003437610.5	GAACCAACCTGCCTCCACAACA	AGCCAGAGCAGCCATCAGTGA
fxr	XM_005470943.4	GAACAGTACAGCGCAGACGA	TGAACCCTTCAGCAGAGCAA
fgf19	XM_003456559.5	TCGCACGCCTACAGCAGAGA	CCCAGACTGAGCAGGACTCCAT
acly	XM_003442027.5	CCAGAGGAGGTTGCCGAGGTAT	AGGTGAGGCTGGAGATGAGGTC
β-actin	XM_003443127.5	TCGTGCGTGACATCAAGGAGAAG	CAAGGAAGGAAGGCTGGAAGAGG

bsep = ATP-binding cassette; *ldlr* = low density lipoprotein receptor a; *hmgcr* = 3-hydroxy-3-methylglutaryl-CoA reductase a; *fxr* = nuclear receptor subfamily 1, group H, member 4; *fgf19* = fibroblast growth factor 19; *acly* = ATP citrate lyase a; β-actin = housekeeping gene.

2.1 mm × 100 mm) was used for separating the components. The separation conditions included a flow rate of 0.35 mL/min, a column temperature of 40 °C, and a sample size of 2 μL. The solvent system consisted of water-0.04% acetic acid (A) and acetonitrile-0.04% acetic acid (B), with the elution gradient conditions set as following: starting with water to acetonitrile ratio of 95:5 (vol:vol) for 0 min, transitioning to a ratio of 5:95 (vol:vol) at 11 min, and maintaining this ratio until minute 12 when it transitioned back to a ratio of 95:5 (vol:vol) until minute 14. The eluted metabolites were detected using high-resolution MS/MS analysis. The employed mass spectrometry conditions included an electrospray ionization temperature of 500 °C, a mass spectrum voltage of 5500 V, and a curtain gas pressure of 25 psi. The collision-activated dissociation parameter was set to the high level for enhanced fragmentation.

To compare the differences in the metabolites, the mass spectrum peaks of each metabolite over different samples were corrected to guarantee the accuracy of qualitative and quantitative analyses. Fig. S1 shows the integral correction results of quantitative analysis of metabolites in randomly selected samples. The horizontal coordinate is the residence time (min) of the metabolite, and the vertical coordinate is the ion current intensity of the metabolite ion detection. Metabolites were quantitatively analyzed using triple quadrupole mass spectrometry Multiple Reaction monitoring (MRM) mode (Fraga et al., 2010). Quality control samples were prepared by mixing sample extracts, and the reproducibility of the samples was analyzed by the same treatment method. During instrumental analysis, one quality control sample was analyzed for every ten samples to monitor the repeatability of the UPLC-MS/MS system throughout the inspection process.

2.9. Histological analysis

H&E staining sections were pictured by Leica DM2500 (Leica, Solms, Germany) and scored (1 = normal, 2 = mild, 3 = moderate, 4 = severe) according to their vacuolation area. EdU and DAPI staining sections were pictured by Eclipse Ti-SR (Nikon, Japan), and the color merge area was calculated to determine the cell proliferation rate (%) as 100 × red area (EdU)/blue area (DAPI). Image 6.0 software (Media Cybernetics, USA) was used to quantify the above parameters (Jiang et al., 2020).

2.10. Statistical analysis

All data analysis was performed by using SPSS 22.0 (IBM, New York, USA). The data were analyzed with one-way ANOVA and Tukey's multiple range tests. All data are shown as mean and standard deviation (SD) in tables and figures ($n = 3$). Figures were created in GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA). Compared with the Con group, statistically significant differences are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3. Results

3.1. Growth performance

The growth performance of tilapia is shown in Table 3. Fish in the CHO−BAs group exhibited the highest weight gain rate (WGR) and specific growth rate (SGR), with statistical significance ($P < 0.001$). Additionally, this group demonstrated the lowest feed conversion ratio (FCR) and feed intake (FI), also with statistical significance ($P < 0.001$). The highest value of viscera somatic index (VSI) of tilapia was detected in the Con group. Hepatosomatic index (HSI) in the CHO, BAs, CHO−BAs groups was significantly higher than that in the Con group ($P < 0.001$). No significant difference was observed among the four groups in condition factor (CF) and survival rate (SR) ($P > 0.05$).

3.2. Proximate composition of whole fish

The proximate composition of whole fish and liver is presented in Table 4. Crude fat and ash contents of whole fish in the CHO group were significantly lower than those in the Con group ($P < 0.001$). Dietary cholesterol and bile acid supplementation significantly increased crude fat and protein contents, but decreased moisture level in liver ($P < 0.001$).

3.3. Serum biochemistry parameters

The serum biochemistry indices are presented in Table 5. The highest activities of serum ALT and AST were recorded in the Con group ($P < 0.001$). The levels of TG and GLU in the CHO group were significantly lower than other treatment groups ($P < 0.001$). In addition, no significant difference was observed among four groups in serum TP and ALP values ($P > 0.05$).

3.4. Liver histology

The histological structure and scores of hepatocytes stained with H&E in different treatments are illustrated in Fig. 1A and B. Fish fed the Con diet exhibited cell enlargement, nuclear migration, partial cell membrane rupture and boundary blurring in liver tissues. Conversely, fish fed the CHO, BAs, CHO−BAs diets showed normal hepatocytes. Furthermore, liver EdU staining (Fig. 2A) revealed enhanced positive signals in all three treatment groups, with particularly noticeable signal detection in the CHO−BAs group. The CHO−BAs group showed the highest cell proliferating rate in the liver compared to the other groups (Fig. 2B).

3.5. Gene expression in liver metabolism pathway

The hepatic expression of CHO and BA metabolism-related genes in tilapia is illustrated in Fig. 3. The mRNA levels of *fxr*,

Table 3
Growth performance of juvenile GIFT fed the test diets for 9 weeks.

Item	Con	CHO	BAs	CHO–BAs	*P*-value
IBW, g	6.16 ± 0.30	6.07 ± 0.02	6.04 ± 0.10	6.08 ± 0.03	0.193
FBW, g	44.56 ± 2.40^{a}	46.85 ± 0.46^{a}	49.04 ± 1.26^{b}	53.97 ± 1.32^{c}	<0.001
WGR[1], %	628.42 ± 37.20^{a}	658.43 ± 59.31^{ab}	707.77 ± 14.15^{bc}	786.15 ± 19.31^{c}	<0.001
SGR[2], %/d	3.16 ± 0.02^{a}	3.21 ± 0.07^{a}	3.31 ± 0.04^{a}	3.53 ± 0.04^{b}	<0.001
FCR[3]	1.41 ± 0.04^{b}	1.37 ± 0.06^{b}	1.24 ± 0.06^{a}	1.16 ± 0.04^{a}	<0.001
FI[4], g/fish	57.73 ± 0.66^{c}	54.18 ± 0.23^{b}	54.26 ± 0.43^{b}	51.23 ± 0.77^{a}	<0.001
HSI[5], %	1.57 ± 0.15^{a}	1.84 ± 0.10^{b}	1.95 ± 0.20^{b}	1.86 ± 0.16^{b}	<0.001
VSI[6], %	10.41 ± 0.78^{b}	9.25 ± 0.68^{a}	9.91 ± 0.41^{ab}	9.82 ± 0.70^{ab}	<0.001
CF[7], g/cm^3	3.76 ± 0.28	3.95 ± 0.16	3.94 ± 0.17	3.90 ± 0.11	0.063
SR[8]	93.67 ± 5.77	94.89 ± 6.94	93.33 ± 6.67	93.33 ± 3.33	0.484

IBW = initial mean weight; FBW = final mean weight; GIFT = genetically improved farmed tilapia.
Con, basal diet; CHO, basal diet supplemented with 12 g/kg cholesterol; BAs, basal diet supplemented with 0.2 g/kg bile acids; CHO–BAs, basal diet supplemented with a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids.
Data was presented as mean ± SD, $n = 3$. Values in the same row sharing different superscript letters are significantly different, as determined by Turkey's test ($P < 0.05$).
[1] WGR (weight gain rate, %) = (FBW - IBW)/IBW × 100.
[2] SGR (specific growth rate, %/d) = 100 × ln (FBW/IBW)/feeding days.
[3] FCR (feed conversion ratio) = dry feed intake (g)/wet weight gain (g).
[4] FI (feed intake, g/fish) = (dry diet given − dry remaining diet recovered)/number of fish.
[5] HSI (hepatosomatic index, %) = (liver weight, g)/(body weight, g) × 100.
[6] VSI (viscerosomatic index, %) = (viscera weight, g)/(body weight, g) × 100.
[7] CF (condition factor, g/cm^3) = (body weight, g)/(body length, cm)^3 × 100.
[8] SR (survival rate, %) = (number of final fish/number of initial fish) × 100.

Table 4
Proximate composition of whole fish and liver in juvenile genetically improved farmed tilapia (GIFT) fed test diets for 9 weeks.

Item	Con	CHO	BAs	CHO–BAs	*P*-value
Whole fish					
Moisture, %	69.33 ± 2.40	71.03 ± 1.79	71.80 ± 1.22	70.06 ± 1.10	0.089
Crude protein, %	14.42 ± 1.47	14.87 ± 1.89	14.62 ± 1.43	15.10 ± 0.97	0.282
Crude fat, %	9.99 ± 0.45^{c}	7.68 ± 0.37^{a}	8.14 ± 0.56 ^{ab}	8.76 ± 0.37^{b}	<0.001
Ash, %	4.19 ± 0.11^{c}	3.99 ± 0.26^{a}	4.00 ± 0.10^{a}	4.08 ± 0.10^{b}	<0.001
Liver					
Moisture, %	72.46 ± 0.23^{c}	71.80 ± 0.17^{c}	70.51 ± 0.15^{b}	68.77 ± 0.38^{a}	<0.001
Crude protein, %	10.44 ± 0.10^{b}	9.69 ± 0.03^{a}	11.45 ± 0.02^{b}	12.14 ± 0.04^{c}	<0.001
Crude fat, %	9.76 ± 0.03^{b}	8.81 ± 0.07^{a}	8.92 ± 0.02^{a}	10.19 ± 0.06^{c}	<0.001

Con, basal diet; CHO, basal diet supplemented with 12 g/kg cholesterol; BAs, basal diet supplemented with 0.2 g/kg bile acids; CHO–BAs, basal diet supplemented with a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids.
Data was presented as mean ± SD, $n = 3$. Values in the same row sharing different superscript letters are significantly different, as determined by Turkey's test ($P < 0.05$).

bsep, *fgf19*, and *ldlr* were significantly upregulated in the CHO–BAs group compared to those in the Con group ($P < 0.05$). Conversely, the expression levels of *acly* and *hmgcr* in CHO and CHO–BAs groups were lower than those in the Con group ($P < 0.05$).

3.6. Bile acids profile in tilapia bile

UPLC-MS/MS analysis revealed the presence of 51, 51, 54, and 55 bile acid compounds in the Con, CHO, CHO–BAs and BAs groups, respectively (Fig. 4). Taurine-conjugated bile acids (T-BAs) were the predominant form of bile acids present. The top 20 bile acid spectra in tilapia fed with different diets have been summarized in Table S1. Among these, taurochenodeoxycholic acid (TCDCA) was found to be the major compound followed by taurocholic acid (TCA), accounting for approximately 57% to 72% and 8% to 13%, respectively. Cholic acid (CA) and chenodeoxycholic acid (CDCA) were identified as the main free bile acids (Free-BAs). Notably, there were similar types and percentage changes of bile acids observed between the CHO–BAs group and BAs group in tilapia bile samples.

Table 5
Serum biochemical indices of juvenile genetically improved farmed tilapia (GIFT) fed test diets for 9 weeks.

Item	Con	CHO	BAs	CHO–BAs	*P*-value
ALB, g/L	8.91 ± 1.08^{b}	7.80 ± 0.72^{a}	9.53 ± 0.27^{c}	9.01 ± 0.41^{b}	0.017
AST, U/L	333.00 ± 45.08^{b}	248.33 ± 30.11^{ab}	274.00 ± 37.51^{ab}	228.67 ± 23.86^{a}	<0.001
ALT, U/L	48.67 ± 5.51^{c}	35.00 ± 4.58^{a}	43.00 ± 5.00^{ab}	39.67 ± 4.04^{ab}	<0.001
ALP, U/L	25.67 ± 3.21	27.67 ± 0.58	28.67 ± 0.58	28.67 ± 4.62	0.063
Glu, mmol/L	9.01 ± 0.52^{d}	6.72 ± 0.25^{a}	8.44 ± 0.38^{c}	7.41 ± 0.23^{b}	<0.001
TP, g/L	33.44 ± 2.79	30.01 ± 1.71	31.91 ± 1.25	31.63 ± 2.30	0.408
TG, mmol/L	7.80 ± 0.82^{d}	1.36 ± 0.12^{a}	4.30 ± 0.25^{c}	3.54 ± 0.24^{b}	<0.001
TCHO, mmol/L	2.33 ± 0.11^{a}	3.94 ± 0.45^{c}	3.52 ± 0.10^{b}	3.47 ± 0.40^{b}	<0.001

ALB = albumin; AST = aspartate transaminase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; GLU = glucose; TP = total protein; TG = triglyceride; TCHO = total cholesterol.
Con, basal diet; CHO, basal diet supplemented with 12 g/kg cholesterol; BAs, basal diet supplemented with 0.2 g/kg bile acids; CHO–BAs, basal diet supplemented with a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids.
Data was presented as mean ± SD, $n = 3$. Values in the same row sharing different superscript letters are significantly different, as determined by Turkey's test ($P < 0.05$).

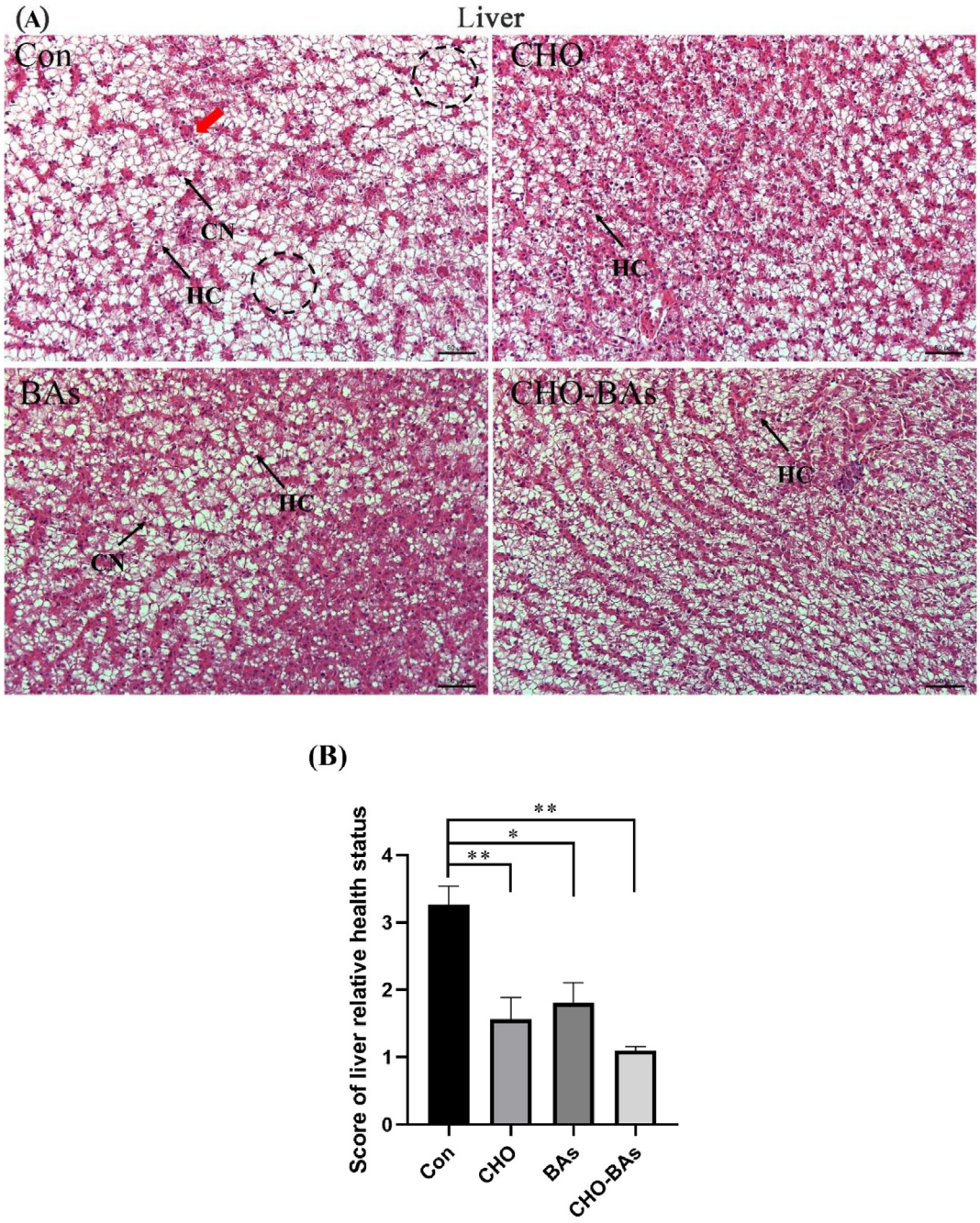

Fig. 1. Dietary cholesterol bile acid supplementation can improve the liver health of GIFT. (A) The histologically observed livers of juvenile GIFT fed test diets (20×, scale bar = 50 μm). CN, cell nucleus; HC, hepatocyte cell. Black circles indicate cellular vacuolization; red arrows indicate abnormal cell nucleus. (B) Liver relative health status of tilapia was scored according to the degree of its vacuolization (1 = normal, 2 = mild, 3 = moderate, 4 = severe). The values are expressed as mean ± SD (*, $P < 0.05$; **, $P < 0.01$, $n = 10$). GIFT = genetically improved farmed tilapia; Con = basal diet; CHO = basal diet supplemented with 12 g/kg cholesterol; BAs = basal diet supplemented with 0.2 g/kg bile acids; CHO–BAs = basal diet supplemented with a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids.

The differences in bile acids among the Con group and the three treatment groups are shown in Fig. 5. Compared with the Con group, hyodeoxycholic acid (HDCA), murideoxycholic acid (MDCA), ω-muricholic acid (ω-MCA), glycoursodeoxycholic acid (GUDCA), tauro β-muricholic acid (Tβ-MCA), 12-ketolithocholic acid (12-KLCA), 5-β-cholanic acid-3α-ol-6-one (6-ketoLCA), taurodehydrocholic acid (TDHCA) and hyocholic acid (HCA) levels were significantly increased in the CHO–BAs group and BAs group ($P < 0.01$), whereas levels of norcholic acid (NCA) and taurodeoxycholic acid (TDCA) were significantly decreased in the CHO group and BAs group ($P < 0.05$). Chenodeoxycholic acid-3-β-D-glucuronide (CDCA-3Gln) was significantly decreased in the BAs group ($P < 0.05$). In addition, compared with the Con group, chenodeoxycholic acid-3-β-D-glucuronide (GLCA-3S) in the CHO group increased significantly ($P < 0.05$), while tauroursodeoxycholic acid (TUDCA), NCA and taurohyodeoxycholic acid (sodium salt) (THDCA) decreased significantly ($P < 0.01$ for TUDCA, $P < 0.001$ for NCA and $P < 0.05$ for THDCA, respectively).

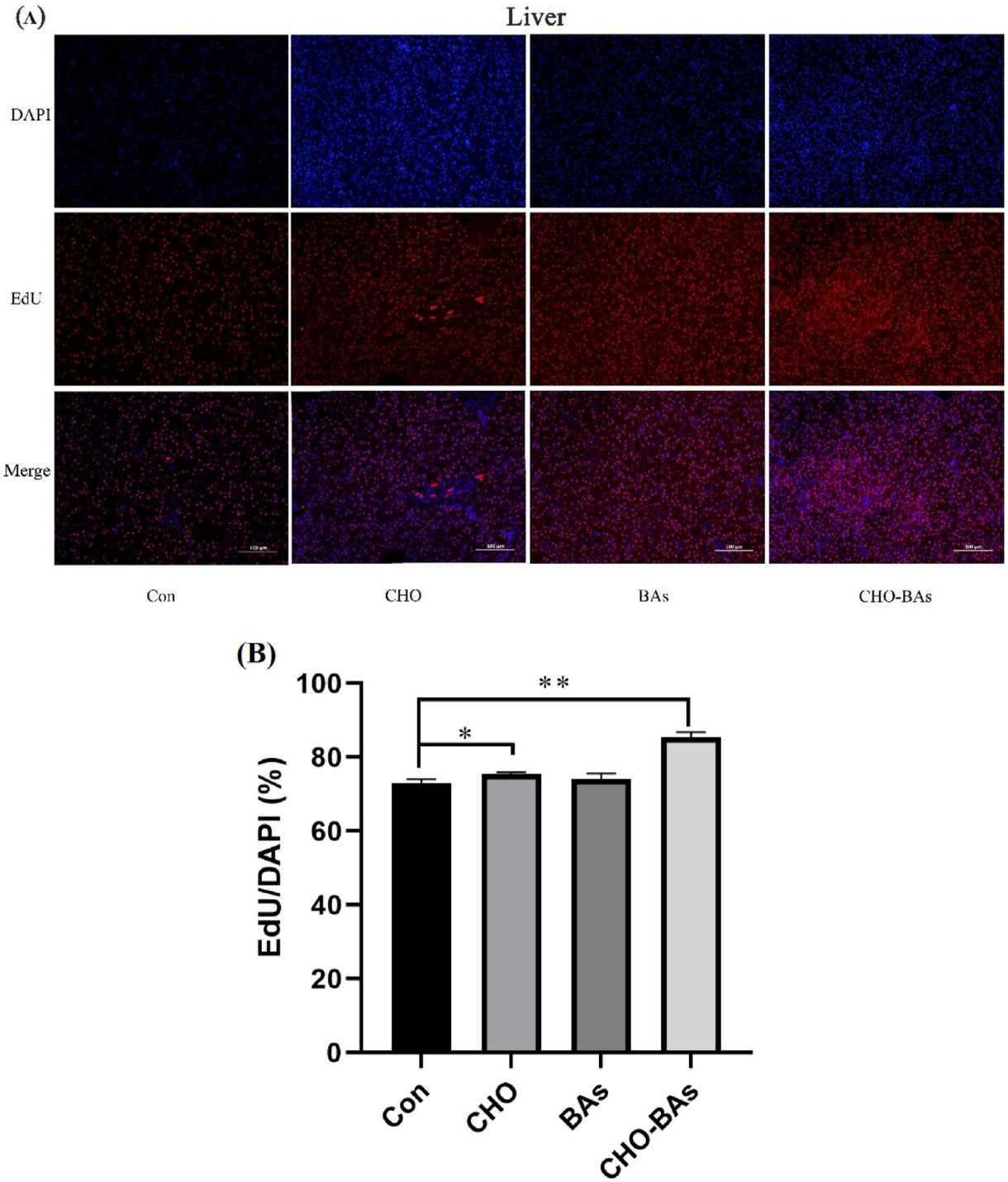

Fig. 2. Dietary cholesterol and bile acid supplementation enhanced the proliferation and renewal of liver cells. (A) Detection of cell proliferation of liver after 12 h of intraperitoneal injection using 5-ethynyl-2′-deoxyuridine (EdU). 4′,6-diamidino-2-phenylindole (DPAI) (blue, Ex/Em λ: 340–380/435–485), EdU (red, Ex/Em λ: 550/590). (B) Percentage of proliferating cells in liver. The values are expressed as mean ± SD (*, $P < 0.05$; **, $P < 0.01$, $n = 3$). GIFT = genetically improved farmed tilapia; Con = basal diet; CHO = basal diet supplemented with 12 g/kg cholesterol; BAs = basal diet supplemented with 0.2 g/kg bile acids; CHO–BAs = basal diet supplemented with a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids.

4. Discussion

This study has shown that dietary cholesterol and bile acid supplementation can significantly improve the growth performance and feed utilization of tilapia. Cholesterol is an important precursor of steroid hormones, vitamin D_3 and bile acids (Kortner et al., 2014). Generally, cholesterol is considered as a non-essential nutrient for fish because they are capable of synthesizing cholesterol in vivo (An-Yuan et al., 2015; Sealey et al., 2001). In this experiment, the weight gain rate of tilapia in the CHO group was slightly but insignificantly higher than that in the Con group. Similar studies have also shown that cholesterol supplementation in a plant-based diet does not promote the growth of Atlantic salmon (*Salmo salar L.*) (Kortner et al., 2014) and *Epinephelus*

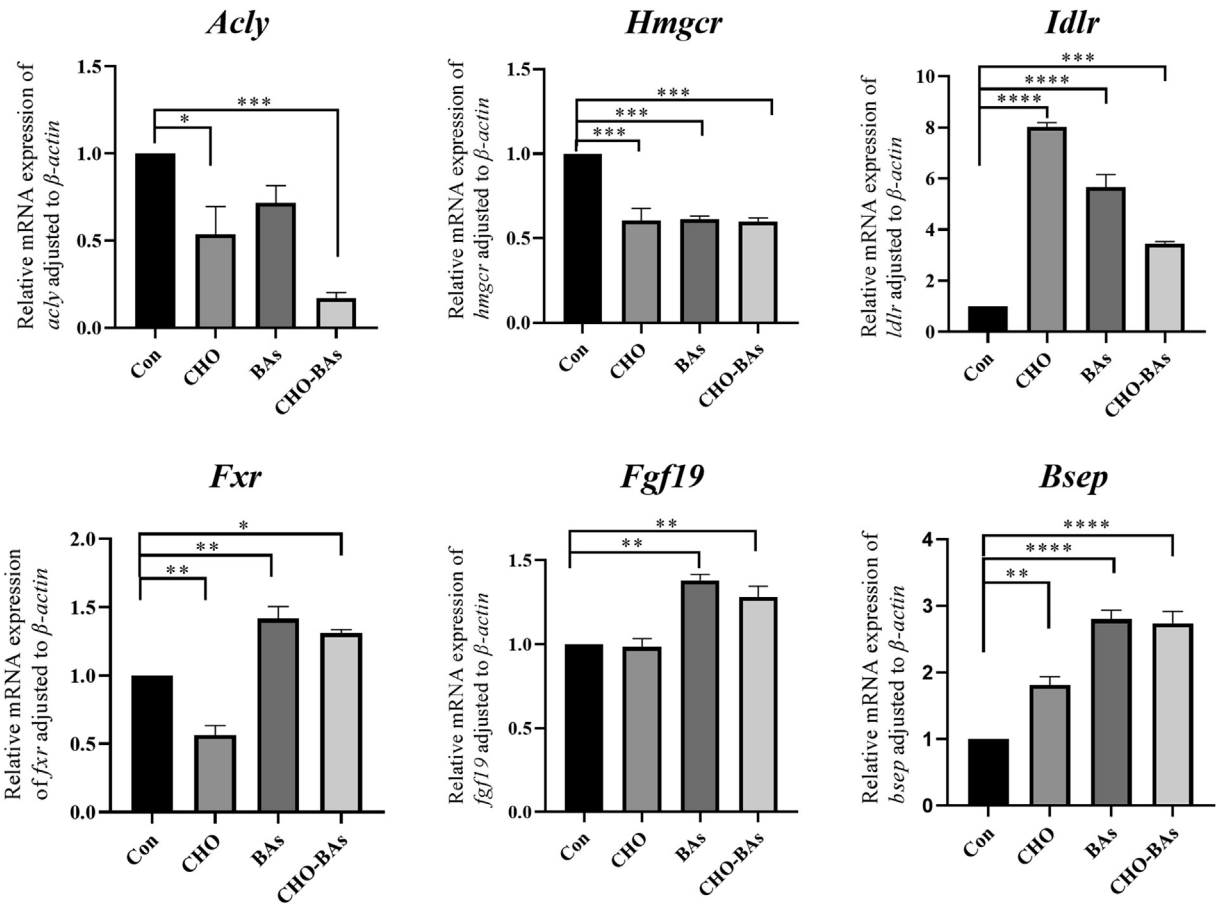

Fig. 3. Relative mRNA expression of genes acting as cholesterol and bile acid metabolism regulators. *acly* = ATP citrate lyase a; *hmgcr* = 3-hydroxy-3-methylglutaryl-CoA reductase a; *ldlr* = low density lipoprotein receptor a; *fxr* = nuclear receptor subfamily 1, group H, member 4; *fgf19* = fibroblast growth factor 19; *bsep* = ATP-binding cassette. The values are expressed as mean ± SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, $n = 3$). GIFT = genetically improved farmed tilapia; Con = basal diet; CHO = basal diet supplemented with 12 g/kg cholesterol; BAs = basal diet supplemented with 0.2 g/kg bile acids; CHO–BAs = basal diet supplemented with a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids.

lanceolatus (Sealey et al., 2001). However, some studies have found that adding an appropriate amount of cholesterol to the diet can significantly improve the growth performance and feed utilization rate of *Ictalurus punctatus* (Zhao et al., 2011) and turbot (*Scophthalmus maximus L.*) (Yun et al., 2012). The variation across above trials may be attributed to culture species and diet formulations.

Bile acids, the main components of bile, are widely used in commercial aquatic feed as a highly active emulsifier (Geng et al., 2022). In this experiment, the weight gain rate of fish in the BAs group was significantly higher than that in the Con group, which was consistent with our previous results (Jiang et al., 2018). Moreover, compared with the control group, the crude fat content of whole fish and serum TG level in the CHO, BAs and CHO–BAs groups were significantly reduced. Among four experimental groups, the best growth performance was found in the CHO–BAs group, which may be due to the synergistic effects between bile acids and cholesterol. Bile acids can combine with cholesterol to form a mixture, which may promote the dissolution and emulsification of cholesterol and makes it more easily absorbed by the intestine (Di Ciaula et al., 2022), thereby improving its nutritional effect on fish.

In the experiment, the addition of cholesterol and bile acids to the diet stimulated fish liver metabolism, resulting in an increased HSI value and an enhanced hepatocyte regeneration. The HSI was significantly higher in the CHO, BAs and CHO–BAs groups than that in the Con group, which is similar to the results in the study of Atlantic salmon (*S. salar L.*) (Bjerkeng, 1999). Although an increase in HSI may be considered a side effect, it also reflects liver metabolism (Huang et al., 2023). The addition of cholesterol and bile acids in the diet will stimulate the fish liver and promote the cholesterol metabolism process. In addition, bile acids can stimulate the gallbladder to discharge bile, increase the amount of bile secreted in the intestine, and promote the digestion and absorption of lipids (Di Ciaula et al., 2022). These physiological processes require many enzymes and metabolites, and inevitably aggravate the metabolic burden in fish liver. Moreover, EdU staining showed that the proliferation and renewal ability of tilapia hepatocytes in the CHO, BAs and CHO–BAs groups were better than that in the Con group, which was consistent with the increase of HSI in tilapia.

In this study, dietary supplementation with cholesterol or bile acids significantly inhibited cholesterol synthesis (*hmgcr*) and lipogenesis (*acly*) expression, while induced cholesterol uptake (*ldlr*) gene expression. It may be associated with the reduction in crude fat content of whole fish. A similar study also showed that dietary bile acids treatment could reduce fat accumulation in the liver of large yellow croaker, *Larimichthys crocea*. Bile acids could enter the small intestine through the micro-bile ducts, contribute to the digestion and absorption of lipids, and thereby decrease the triglyceride content (Dana et al., 2012). The lowest value of T-CHO was detected in the Con group, which is illustrated by the fact that

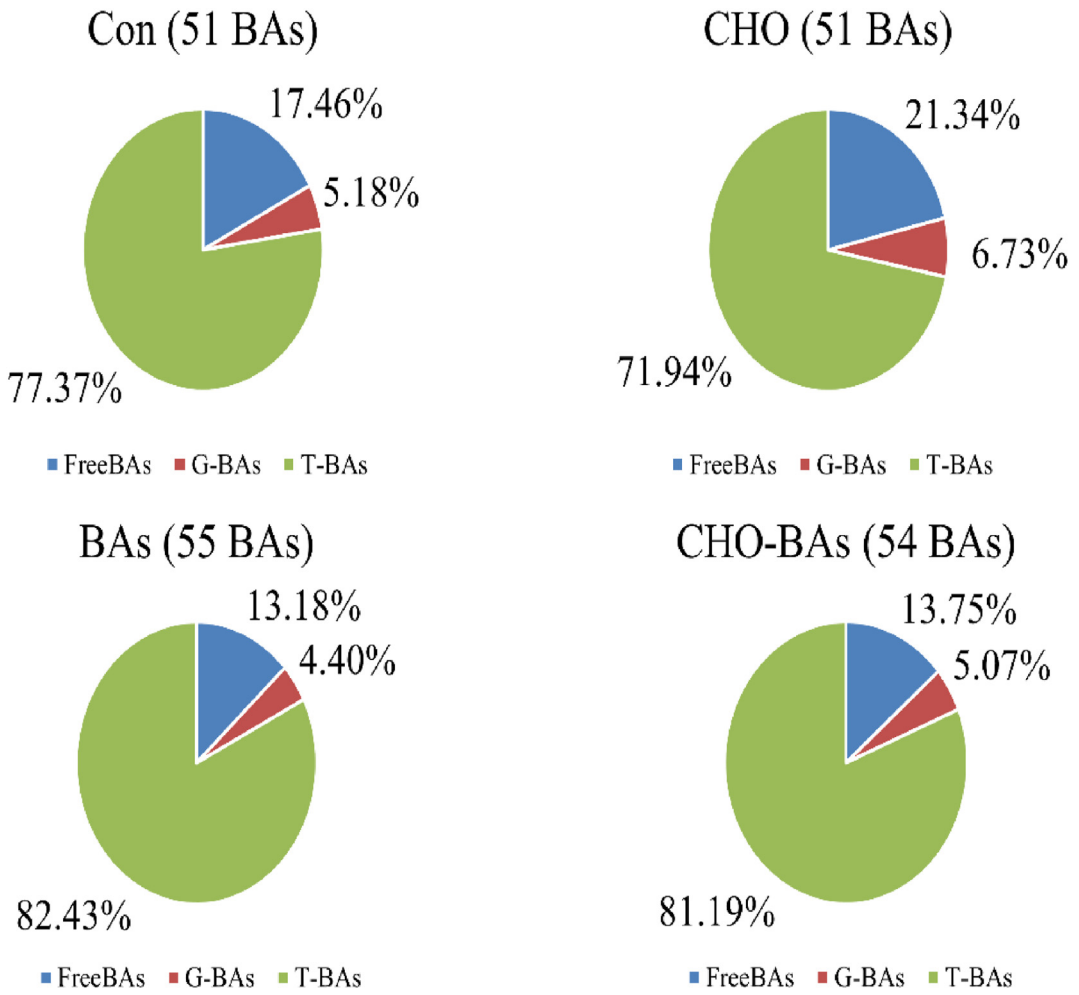

Fig. 4. The profile of bile acids in the bile of tilapia fed test diets. Free-BAs = free bile acids; T-BAs = taurine-conjugation bile acids; G-BAs = glycine-conjugation bile acids. Bile acid supplementation to a whole plant diet increased the proportion of T-BAs while Free-BAs decreased in tilapia bile ($n = 3$). GIFT = genetically improved farmed tilapia; Con = basal diet; CHO = basal diet supplemented with 12 g/kg cholesterol; BAs = basal diet supplemented with 0.2 g/kg bile acids; CHO–BAs = basal diet supplemented with a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids.

these sterols contained in plant inhibited cholesterol absorption in the fish and reduced blood cholesterol content, possibly leading to hypocholesterolemia (Demonty et al., 2006).

AST and ALT are important biochemical indicators that reflect the liver function and health of fish. In the present experiment, the activities of AST and ALT were significantly reduced in the CHO, BAs and CHO–BAs groups, indicating that dietary cholesterol or bile acid supplementation can reduce the liver damage triggered by plant-based diets. The hepatocytes of fish in the experimental group showed alleviation of cell enlargement, nuclear migration, partial cell membrane rupture, and boundary ambiguity. These hepatic histomorphology also confirmed that the liver health was improved by dietary cholesterol or bile acid supplementation.

Different animals have differing types, numbers, and bile acids contents as well as differing active components (Xian et al., 2021). This study revealed that the types of bile acids in tilapia were more diverse than those in other fish. According to our data, the main component of bile acids in tilapia was identified as TCDCA. This is distinct with the report on common carp (*Cyprinus carpio*) which indicated that the TCA is a major component in this fish (Xian et al., 2021). Early observations have shown that free bile acids are mainly conjugated with taurine except for mammals. Therefore, little attention was paid to the presence and function of glycine-type bile acids in fish (Hofmann et al., 2010). Nonetheless, this study revealed that tilapia was capable of conjugating bile acids not only with taurine, but also with glycine. For instance, GCA, GCDCA, GLCA, GLCA-3S, and GDCA were determined in the bile. Generally, the glycine-conjugated bile acids presented in fish may partially originate from fishmeal or other animals-derived ingredients (Xian et al., 2021). However, we hypothesize that the glycine-conjugated bile acids in tilapia may have an endogenous origin due to the plant-based protein diets used in this study. In addition, the increased proportion of bound bile acids with subsequent decreased proportion of free bile acids were observed after exogenous bile acids supplementation. The incorporation of primary bile acids with taurine and glycine may reduce bile acid hydrophobicity and thereby possess the detoxification function. This may also have a certain detoxification effect on the anti-nutritional factors (such as non-starch polysaccharides and plant protease inhibitors) present in plant-based feed, but the specific mechanism needs to be further studied.

Dietary supplementation with bile acids in plant-based diet increased the levels of HDCA, MDCA, ω-MCA, and GUDCA in tilapia bile, which may be attributed to the inclusion of HCA, HDCA and CDCA in bile acids. Previous studies have shown that HCA is partially transformed into HDCA, and MDCA is generated from α/β-

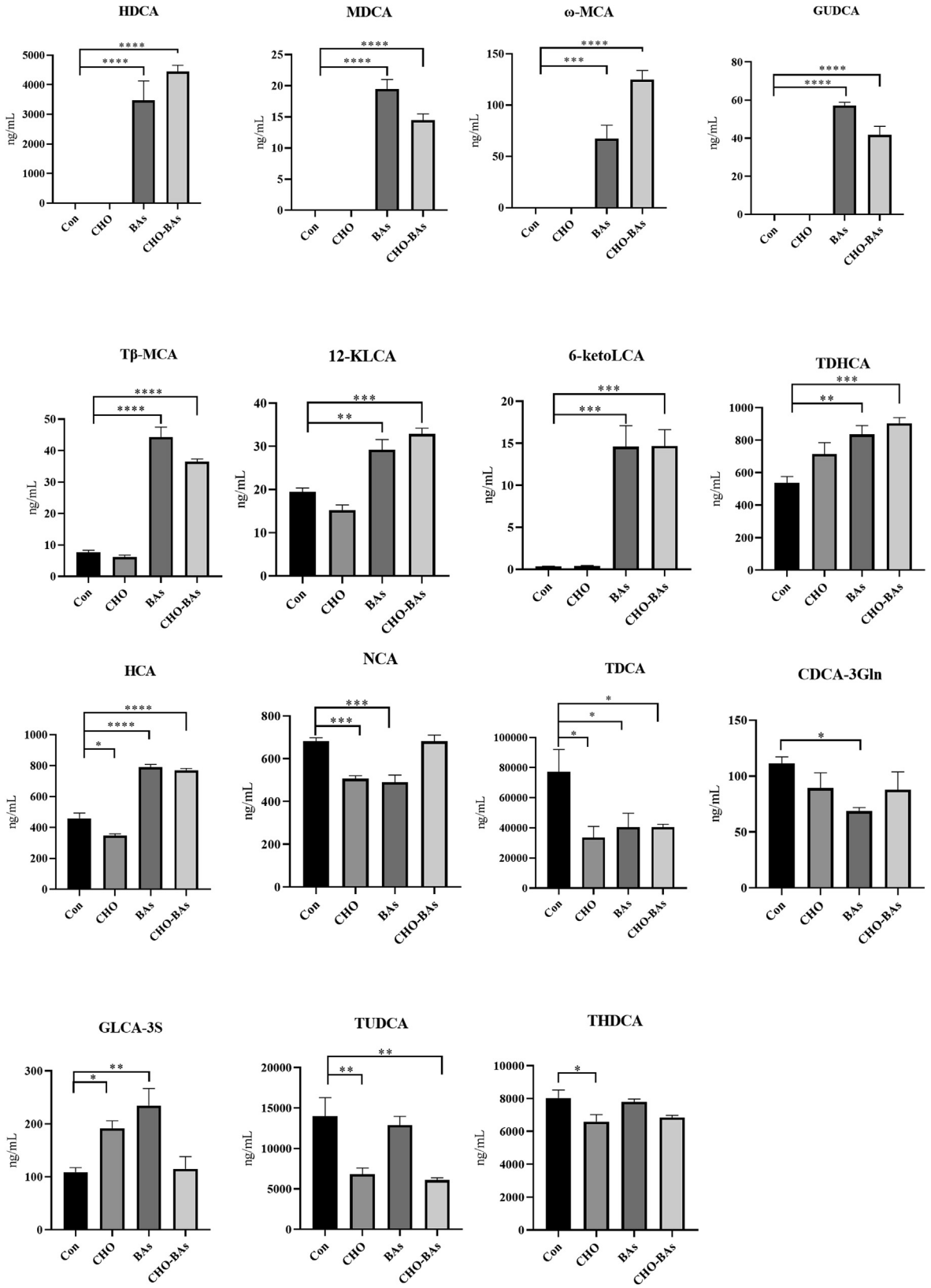

Fig. 5. Significantly different content of various bile acids (ng/mL) in tilapia bile between Con and CHO group, or BAs group or CHO–BAs group, respectively. HDCA = hyodeoxycholic acid; MDCA = murideoxycholic acid; ω-MCA = ω-muricholic acid; GUDCA = glycoursodeoxycholic acid; Tβ-MCA = tauro β-muricholic acid; 12-KLCA = 12-ketolithocholic acid; 6-ketoLCA = 5-β-cholanic acid-3α-ol-6-one; TDHCA = taurodehydrocholic acid; HCA = hyocholic acid; NCA = norcholic acid; TDCA = taurodeoxycholic acid; CDCA-3Gln = chenodeoxycholic acid-3-β-D-glucuronide; GLCA-3S = chenodeoxycholic acid-3-β-D-glucuronide; TUDCA = tauroursodeoxycholic acid; THDCA = taurohyodeoxycholic acid (sodium salt). The values are expressed as mean ± SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, $n = 3$). GIFT = genetically improved farmed tilapia; Con = control, the basal diet; CHO = basal diet supplemented with 12 g/kg cholesterol; BAs = basal diet supplemented with 0.2 g/kg bile acids; CHO–BAs = basal diet supplemented with a combination of 12 g/kg cholesterol and 0.2 g/kg bile acids.

MCA by the 7α-dehydrohydroxyl conversion of intestinal microbiota in mice (Zheng et al., 2021). The ω-MCA is also yielded from β-MCA via intestinal microbial 6β-hydroxyl oxidation and 6α-reductive isomerization (Eyssen et al., 1983), and UDCA is formed through *Clostridium*-mediated 7α/β-isomerization in human (Wahlström et al., 2016). These data suggest that there are more types of bile acids in the BAs group in comparison with the Con group and the cholesterol group. It is shown that bile acids regulate their enterohepatic circulation via the modulation of FXR and TGR5 signaling pathways. FXR, a member of the nuclear receptor superfamily, is predominantly expressed in the liver, kidney, and intestines (Teodoro et al., 2011). Both conjugated bile acids (GUDCA, TCDCA, Tα-MCA, Tβ-MCA) and unconjugated bile acids (CA, CDCA) could act as ligands for FXR. Research on mice has demonstrated that the activation of intestinal FXR enhanced the secretion of fibroblast growth factor 15 (FGF15, human ortholog FGF19) (Zaborska and Cummings, 2018). After circulation through the blood vessels into the liver, the FGF15 is reported to bind fibroblast growth factor receptor 4 (FGFR4), suppressing hepatic CYP7A1 expression by activating the JNK signaling pathway, inhibiting bile acid synthesis. Moreover, FXR induces the expression of bile salt export pump (BSEP), which stimulates the efflux of accumulating bile acids and bilirubin, ameliorating liver injury (Wagner et al., 2011). In the present study, dietary bile acid supplementation significantly upregulated the expression of *fxr*, *bsep*, and *fgf19* genes. The levels of CA and TCDCA, which acted as activators of the FXR receptor, were increased in the BAs group compared to that in the Con group. Furthermore, Tβ-MCA was obviously elevated in both the CHO–BAs group and BAs group. The inhibition of intestinal-hepatic FXR-FGF15 axis mediated by Tβ-MCA could result in the reduction of blood glucose, and thus serve as molecular targets for the treatment of diabetes (Suocheng et al., 2019). Our finding has shown that dietary bile acids also decreased the level of blood glucose, which is in accordance with this conclusion. Therefore, the addition of bile acids to the diet has positive influence on tilapia health based on the increment of Tβ-MCA in bile, the reduction of hepatic histological damage, and blood glucose level.

5. Conclusion

The present study has shown that a plant-based diet can induce liver injury and weaken cell proliferation in tilapia, but a combined supplementation of cholesterol and bile acid in the diet can alleviate this symptom. Adding exogenous bile acids in the high plant-based diet could alter the bile acid profiles of tilapia. Compared with the control and cholesterol groups, there were more bile acid compositions (such as HDCA, MDCA, ω-MCA, GUDCA) and an increased proportion of TCA in bile. The hepatic expression of *fxr*, *fgf19*, and *bsep* genes as well as Tβ-MCA level were significantly increased, which may be ascribed to reduced fat accumulation in the serum and whole body and may be an indicator of increased hepatopancreatic health. These findings could provide a theoretical basis for the usage of bile acid products in fish, which is of great significance to the sustainable development of aquaculture and has potential contributions to expanding the strategic space for food security.

Author contributions

Jiayuan Jiang: Feeding management, Data analysis, Writing - original draft. **Xing Lu:** Writing - original draft, Writing - review & editing. **Juan Tian**, **Lixue Dong**, **Jianmin Zhang:** Methodology. **Zhongbao Guo**, **Yongju Luo:** Resources. **Zongbin Cui:** Validation. **Hua Wen:** Project administration, Funding acquisition, Resources, Supervision. **Ming Jiang:** Funding acquisition, Conceptualization, Project administration, Data curation, Writing - review & editing.

Data availability statement

All data are available from the corresponding author by request.

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.2024.03.001.

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