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Original Research Article

Gut microbiota-bile acid crosstalk and metabolic fatty liver in spotted seabass (*Lateolabrax maculatus*): The role of a cholesterol, taurine and glycine supplement

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ABSTRACT

The prevalent practice of substituting fishmeal with plant protein frequently leads to disturbances in bile acid metabolism, subsequently increasing the incidence of metabolic liver diseases. Bile acid nutrients such as cholesterol, taurine and glycine have been shown to enhance bile acid synthesis and confer beneficial effects on growth. Therefore, this study aimed to investigate the effects of cholesterol-taurine-glycine (Ch-Tau-Gly) supplement on bile acid metabolism and liver health in spotted seabass (*Lateolabrax maculatus*) fed a plant-based diet. Two isonitrogenous and isolipidic diets were formulated: (1) plant protein-based diet (PP); (2) PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine (CTG). Each experimental diet was randomly fed to quadruplicate groups of 30 feed-trained spotted seabass in each tank. The results revealed that supplementing plant-based diet with Ch-Tau-Gly supplement led to an increase in carcass ratio (meat yield) in spotted seabass ($P < 0.05$), indirectly contributing positively to their growth. The dietary supplement effectively suppressed endogenous cholesterol synthesis in the liver, promoted the expression of bile acid synthesis enzyme synthesis, and simultaneously the expression of intestinal *fxr* and its downstream genes, including *hnf4α* and *shp* ($P < 0.05$). The reduction in *Lactobacillus_salivarius* and bile salt hydrolase (BSH) were observed in CTG group with concurrently increased conjugated chenodeoxycholic acid (CDCA) bile acids ($P < 0.05$), suggesting the enhancement of the hydrophilicity of the bile acid pool. In CTG group, fatty liver was alleviated with a corresponding increase in lipid metabolism, characterized by a downregulation of genes associated with lipogenesis and lipid droplet deposition, along with an upregulation of genes related to lipolysis. Our study underscored the ability of Ch-Tau-Gly supplement to influence the gut microbiota, leading to an increase in the levels of conjugated CDCA ($P < 0.05$) in the bile acid pool of spotted seabass. The interplay between the gut microbiota and bile acids might constitute a crucial pathway in the promotion of liver health. These findings offer a promising solution, suggesting that Ch-Tau-Gly supplement have the potential to promote the growth of aquatic species and livestock fed on plant-based diets while addressing issues related to metabolic fatty liver.

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1. Introduction

The continuous growth of global population has led to an ever-increasing demand for sustainable food sources, including meat and fish products, resulting in rapid expansion of the livestock and aquaculture industries. Meeting the industry's focus on producing a large quantity of high-quality livestock and aquatic products in a short timeframe has become a paramount concern. However, under short-cycle, intensive farming conditions, there is a higher occurrence of fatty liver syndrome, especially in poultry, which is

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detrimental to the overall health of the animals (Khosravinia et al., 2015). In aquaculture, the use of plant-based ingredients in fish feed has become common due to their cost-effectiveness and availability. However, this transition to plant-based diets has posed challenges, particularly for carnivorous fish, like the spotted seabass (*Lateolabrax maculatus*), widely cultivated in China and Southeast Asia, which have protein and lipid requirements of around 40% and 12%, respectively (Zhang et al., 2019). The spotted seabass, through extensive domestication efforts, has shown remarkable adaptability, transitioning from initial extreme anorexia to compensatory intake and ultimately embracing full plant protein diets (Liang et al., 2019, 2017; Zhang et al., 2019). Notably, studies focused on the spotted seabass have revealed that the incorporation of plant-based protein not only had a negative effect on growth and immunity but also decreased bile acids (BAs) production, consequently exacerbating the occurrence of fatty liver (Zhang et al., 2019).

To effectively address the disruptions of bile acid status and lipid metabolism induced by plant protein-based diets, one promising strategy is to directly incorporate BAs (Romano et al., 2020). The incorporation of BAs held the potential to diminish liver and whole-body lipid content by upregulating the expression of genes responsible for lipid hydrolysis and fatty acid oxidation. It's important to note that the results of these studies can vary depending on factors such as species, types, and levels of BAs used (Romano et al., 2020; Zhou et al., 2018). It is noteworthy to mention that certain exogenous BAs can exert adverse effects. For instance, the administration of bovine salts has been demonstrated to induce enteritis in distal intestinal of Atlantic salmon (Kortner et al., 2016).

Primary BAs are produced in the liver from cholesterol through a series of enzymatic reactions (Hofmann et al., 2010). Fish, similar as mammals, possess the ability to synthesize cholesterol from acetates. However, dietary cholesterol primarily originates from animal-based ingredients such as fish meals, while plant protein ingredients are inherently deficient in cholesterol (Cheng and Hardy, 2004). In particular, the non-starch polysaccharides present in plant-based diets have been implicated in contributing to hypocholesterolemia, although this effect could be alleviated through the addition of exogenous cholesterol (Deng et al., 2010). Similarly, consumption of high plant protein-based diet by spotted seabass has been found to induce hypocholesterolemia, consequently leading to heightened endogenous cholesterol synthesis and placing an increased burden on the liver (Zhang et al., 2019). Therefore, the supplementation of cholesterol in plant-based diets becomes imperative, and its positive impact on growth has been observed in carnivorous fish, such as rainbow trout and turbot (Deng et al., 2014; Yun et al., 2012). It is worth highlighting that the ability to synthesize BAs, particularly through the chenodeoxycholic acid (CDCA) synthesis pathway, is enhanced after cholesterol diet (Li et al., 2023a), thereby suggesting that dietary cholesterol may further influence the composition of BAs.

Additionally, free BAs primarily combine with taurine and infrequently with glycine in fish (Hofmann et al., 2010). There is limited research on glycine in aquaculture. Recent studies indicate that supplementing aquafeeds based on soybean meal with 1% to 2% glycine significantly enhanced the growth rate and feed efficiency of hybrid striped bass (Li et al., 2023b). Similarly, in large-mouth bass, research has demonstrated that replacing 90% of fishmeal with soybean meal in the diet and adding 2% glycine could substantially improve growth performance (Rossi et al., 2021). These findings suggest that supplementing aquafeeds with glycine in plant protein diets can benefit aquaculture growth. Likewise, glycine at different levels has also been found to have positive effects in broiler chicken production (Abdelfatah et al., 2023). Although the specific mechanisms driving the positive effects of glycine remain incompletely understood, there is indisputable evidence that glycine at suitable levels exerts a favorable influence on animal growth.

While fish possess the ability to internally synthesize taurine, most of them still rely on external sources to fulfill their taurine requirements (El-Sayed, 2014). Given this relatively limited capacity, coupled with the deficiency of taurine in plant-based diets, the supplementation of taurine in high plant protein-based diets became crucial to maintain optimal bile acid status (Romano et al., 2020). Dietary taurine has been reported to enhance the utilization of plant-based diets in carnivorous fish (Biasato et al., 2022; Hongmanee et al., 2022) and elevate the activity of cytochrome P450 7A1 (CYP7A1), the rate-limiting enzyme for bile acid synthesis (Li et al., 2023a). This, in turn, promotes bile acid synthesis and exerts a hypocholesterolaemic effect (Xu et al., 2020). Additionally, fish fed taurine-enriched diets exhibited an expansion of bile acids in the gallbladder and improved lipid digestibility, owing to the emulsifying properties of taurine, thus mitigating the adverse effects of dietary plant protein on lipid digestion (Kim et al., 2015). Similarly, in broilers, a comparable phenomenon has been noticed, wherein dietary supplementation of taurine alleviated hepatic liver accumulation under heat stress conditions (Lu et al., 2019).

Therefore, with the goal of optimizing plant-based diets for carnivorous fish, the present study endeavors to investigate the profound impact of incorporating cholesterol-taurine-glycine (Ch-Tau-Gly) supplement on bile acid metabolism and liver health in spotted seabass subjected to a plant-based diet. By delving into this exploration, we aim to uncover intricate mechanisms and provide potential nutritional strategies for addressing fatty liver diseases in aquatic species and other animals, benefiting their overall health and productivity.

2. Materials and methods

Throughout the duration of the feeding period, meticulous adherence to the Laboratory Animal Welfare Guidelines of China (Decree No. 2 Ministry of Science and Technology, issued in 2021) was strictly observed.

2.1. Experimental diets

Here, 2 isonitrogenous and isolipidic experimental diets were formulated as shown in Table 1. The control diet was formulated with 10% fishmeal and 52% mixed plant protein (including 15% cottonseed protein concentrate, 10% soybean meal and 27% soybean protein concentrate), named PP. The experimental diet (named CTG) was supplemented with Ch-Tau-Gly supplement (including 0.5% cholesterol, 0.5% taurine and 1.3% glycine), on the basis of PP. Both diets were made into dry floating pellets with a diameter of 3 mm using a Twin-screwed extruder (EXT50A, YANGGONG MACHINE, China) under the following extrusion conditions: feeding section (120 °C/5 s), compression section (130 °C/5 s) and metering section (150 °C/4 s). After pelletization and oil spraying, the crude protein for the 2 feed groups on a dry matter basis was 45.1% and 44.6% respectively, while the crude lipid was 11.8% and 11.5% respectively. All diets were air-dried naturally and stored at −20 °C until used.

2.2. Experimental fish, feeding and sampling

The feeding trial was conducted in an indoor recirculated tank system comprising 256 L concrete tanks at Nankou base of the Chinese Academy of Agricultural Sciences, Beijing, China. These tanks were equipped with mechanical and biological filtration and

Table 1
Formulation and nutrient composition of the experimental diets.

Item	PP	CTG
Ingredients, % (as-is basis)		
Fish meal[1]	10	10
Cottonseed protein concentrate[2]	15	15
Soybean meal[3]	10	10
Soybean protein concentrate[3]	27	27
Tapioca starch	5	5
Wheat flour	15	14.1
Wheat middling	1.43	0
Fish oil	5.2	5.2
Soybean oil	4.5	4.5
Lecithin oil	2	2
Cholesterol		0.5
Taurine		0.5
Glycine		1.3
Vitamin and mineral premix[4]	1.4	1.4
$Ca(H_2PO_4)_2$	2.6	2.6
L-Lys·HCl (78%)	0.45	0.45
DL-Met (98%)	0.3	0.3
L-Thr (98%)	0.12	0.12
Total	100	100
Analyzed chemical composition, % (dry matter basis)		
Moisture	2.59	2.42
Crude ash	8.06	8.27
Crude protein	45.1	44.6
Crude lipid	11.8	11.5

PP: plant protein-based diet; CTG: PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine.

[1] Fish meal (protein 67.07%) was purchased from Tecnológica de Alimentos S.A., Ltd., Peru.

[2] Cottonseed protein concentrate (protein 65.52%) was purchased from Xinjiang Jinlan Vegetable Protein Co. Ltd., China.

[3] Soybean meal (protein 45.97%) and soybean protein concentrate (protein 64.0%) were purchased from Yihai Kerry Investment Co. Ltd., China.

[4] Vitamin and mineral premix (mg/kg diet): vitamin A 20; vitamin B_1 10; vitamin B_2 15; vitamin B_6 15; vitamin B_{12} (1%) 8; niacinamide 100; vitamin C phosphate (35%) 1000; calcium pantothenate 40; biotin (2%) 2; folic acid 10; vitamin E (50%) 400; vitamin K_3 20; vitamin D_3 10; inositol 200; choline chloride 4000; corn protein powder 150; $CuSO_4·5H_2O$ 10; $FeSO_4·H_2O$ 300; $ZnSO_4·H_2O$ 200; $MnSO_4·H_2O$ 100; KI (10%) 80; Na_2SeO_3 (10% Se) 10; $CoCl_2·6H_2O$ (10% Co) 5; NaCl 100; $MgSO_4·5H_2O$ 2000; zeolite 4995.

supplied with surface freshwater at a 10% renewal weekly. It is important to note that this study constitutes a workshop-level aquaculture trial. While it may not capture the full complexity of the environment, it does offer a higher level of precision in investigating causal relationships. Juvenile spotted seabass were purchased from a commercial producer located in Tianjin, China and acclimatized for 2 weeks using a commercial diet before the feeding trial. After 24 h of starvation, fish with an initial body weight of 41.70 ± 0.01 g were randomly selected and distributed into 8 tanks with 30 fish per tank and 4 tanks per treatment. Fish were hand-fed twice daily (08:00 and 17:00) to satiation for 56 d. Throughout the feeding period, water parameters were maintained with optimal ranges, with a water temperature of 24 ± 1 °C, pH ranging from 7.4 to 8.0, dissolved oxygen saturation level above 6.0 mg/L and ammonia nitrogen level below 0.3 mg/L.

At the beginning and end of the feeding trial, fish in each tank were batch-weighted and counted. After the initial 2 weeks of experiment, fecal samples were collected from each tank. To be more specific, fresh feces were collected following a 3-h feeding period of spotted seabass. These collected fecal samples were subsequently freeze-dried and stored at −20 °C for bile acid composition analysis. At the end of the trial, 16 fish per treatment (4 fish from each tank, 4 tanks in each treatment) were anesthetized with chlorobutanol (0.3 g/mL).

After 4 h of starvation, 4 fish were randomly chosen from each tank and anesthetized to collect digesta from the intestine for microbiota analysis. The entire intestine was divided into 2 parts, with the anterior segment being the proximal intestine (PI) and the posterior segment being distal intestine (DI). The proximal and distal intestinal digesta (PID and DID) were collected from each fish, the entire operation was performed in a sterile environment. The procedures were as follows: with 75% ethanol cleaning the exterior of each fish, the intestine was removed aseptically from the abdominal cavity and placed in sterile Petri dishes. The whole intestine was sectioned from the middle with scissors, separated into proximal and distal intestines, and opened individually lengthwise. The intestinal digesta was gently removed with a spatula and transferred to sterile cryotubes. Following sufficient mixing of the digesta in the tube, it was rapidly transferred to liquid nitrogen and then stored at −80 °C for digesta-related gut microbiota profiling and BAs analysis.

After 24 h of starvation, following anesthesia, the spotted seabass were first weighed and measured for their body length. Subsequently, blood samples were drawn through the caudal vein and promptly centrifuged to obtain plasma (supernatant) for blood biochemical analysis. Following the abdominal cavity dissection, measurements of visceral weight, liver weight, gallbladder weight, abdominal fat weight and carcass weight (removed of head, viscera, fins and tail) were taken for the calculation of viscerosomatic index (VSI), hepatosomatic index (HSI), gallbladder index (GBI), abdominal fat rate (AFT) and carcass rate (CR), respectively. Liver and distal intestinal samples were dissected, immediately frozen in liquid nitrogen, and kept at −80 °C for mRNA isolation and tissue homogenate analysis. Two samples near the bile duct in each fish were fixed in 4% paraformaldehyde for paraffin and frozen sections, respectively. Livers from another 3 fish in each tank were pooled into zip-lock bags and then stored at −20 °C for crude lipid assay. After weighing, the gallbladder was placed in a centrifuge tube and stored at −20 °C for BAs analysis.

2.3. Chemical compositions analysis of diets and lipid content of liver

Duplicate analyses of the dry matter, crude protein, crude lipid and crude ash of diets, as well as the crude lipid of liver were conducted following standard protocol (AOAC, 1995). The dry matter was determined by drying the samples at 105 °C to a constant weight. Crude ash was obtained by combustion using muffle furnace (CWF1100, Carbolite, Derbyshire, UK) under 550 °C for 16 h. Crude protein was measured with Kjeldahl method by Kjeltec 2300 Unit (Foss Tecator, Hillerød, Denmark). Crude lipid was determined by acid hydrolysis with ANKOM^{HCl} Hydrolysis System (HCli, ANKOM Technology, Macedon, USA) followed by extraction with ANKOM^{XT15} Extractor (XT15i, ANKOM Technology, Macedon, USA).

2.4. Blood biochemical analysis and liver homogenate parameters of liver functions and metabolism

Plasma alanine aminotransferase (ALT, C009-2-1), aspartate aminotransferase (AST, C010-2-1), alkaline phosphatase (AKP, A059-2-2), triglyceride (TG, A110-2-1), total cholesterol (TC, A111-2-1), high-density lipoprotein cholesterol (LDL-C, A113-2-1), high-density lipoprotein cholesterol (HDL-C, A112-2-1), as well as liver TG, TC, LDL-C, total protein (TP, A045-2-2) were measured using commercial assay kits (Nanjing Jiancheng Co., Nanjing, China) following the manufacturer's instructions. The standard curves for AST, ALT, and AKP all met the following criteria: $R^2 \geq 0.99$. The linear ranges for the detection of TG, TC, LDL-C, HDL-C, and TP are 0 to 9 mmol/L, 0 to 19.9 mmol/L, 0 to 10.4 mmol/L, 0 to 5.16 mmol/L, and 0.2 to 1.3 mg/mL, respectively.

The contents of cAMP (MM-3259101), CYP7A1 (MM-9171302), cytochrome P450 7B1 (CYP7B1, MM-92648301) and cytochrome P450 8B1 (CYP8B1, MM-92647201) were determined with enzyme-linked immunosorbent assay (ELISA, Jiangsu Meimian Industrial Co., Ltd., Yancheng, China), according to the manufacturer's recommendations.

2.5. Proximal intestinal digesta lipase and bile salt hydrolase content

A homogenate sample was prepared by mixing the proximal intestinal digesta with normal saline (1:9). The supernatant was obtained by centrifugation at 4 °C, 845 RCF for 20 min. Then the supernatant was assayed for lipase (LPS, A054-2-1, Nanjing Jiancheng Co., Nanjing, China) and bile salt hydrolase (BSH, MM9117001, Jiangsu Meimian Industrial Co., Ltd., Yancheng, China) by enzyme-linked immunosorbent assay, based on the manufacturer's instructions.

2.6. Liver histopathological

After 24 to 48 h immersion in 4% paraformaldehyde, a portion of the liver was dehydrated, embedded in paraffin wax, sectioned at 5 μm thickness, followed by hematoxylin–eosin (HE) staining to assess lipid accumulation. The other part was embedded in O.C.T. compound and frozen, cut into 10 μm sections, followed by oil red O staining to visualize lipid droplets in the liver. All the processes were performed according to standard procedures, followed by microscopic examination (TissueFAXS, TissueGnostics, Vienna, Austria). Moreover, the lipid droplet area in liver was analyzed by Image J software (1.8.0_112, National Institutes of Health), primarily by quantifying the red area in slices stained with oil red O.

2.7. Quantitative real-time PCR

Total RNA quantification, reverse transcription and mRNA quantification were performed according to the protocols described by Zhang et al. (2019). All the genes of spotted seabass were obtained from an RNA-seq database. The elongation factor 1 alpha (*ef-1α*) (GenBank accession no. JQ995144) was used as an endogenous reference to normalize the template amount. The $2^{-\Delta\Delta CT}$ method was used to present RT-qPCR data (Livak and Schmittgen, 2001). The primer sequences in our study were shown in Table S1.

2.8. Western blot

Liver tissues were homogenized using RIPA buffer (P0013B, Beyotime, Shanghai, China) with added phosphatase inhibitor cocktail (5870, Cell Signaling Technology, United States). The protein concentration was measured using a BCA Protein Quantification Kit (5000201, Bio-Rad, United States). Approximately 20 μg of protein was separated by electrophoresis on polyacrylamide gels and subsequently transferred to nitrocellulose membranes. After blocking for 30 min at room temperature, the membranes were incubated overnight at 4 °C with the following primary antibodies: anti-fatty acid binding protein 1 (FABP1) (1:1000; ab171739, Abcom, UK) and anti-GAPDH (1:1000; ABPR001, Goodhere Biotech, Hangzhou, China). Subsequently, the membranes were incubated for 1 h in goat anti-rabbit IgG-HRP secondary antibody (1:10,000, SC-2054, Santa Cruz Biotechnology, United States). Proteins were detected using enhanced chemiluminescence (ELC), and the quantification was performed using Image J software.

2.9. Bile acids pool analysis

All samples, including intestinal digesta, plasma, liver, gallbladder and feces, were pretreated with the following procedures: (1) Bile: Firstly, 5 μL bile was added to 1995 μL of 80% methanol. Then, 400 μL of the supernatant was taken into a new 2-mL centrifuge tube, 200 μL of 50% methanol and 400 μL of 20% methanol were added, vortexed and mixed, and then passed through a 0.2-μm filter membrane to the injection bottle. (2) Plasma: 100 μL plasma was added to 900 μL of 75% ethanol and vortexed for plasma purification. The main process include activation, lavage, elution and blowing with nitrogen, and finally 200 μL of 75% ethanol was added to re-dissolve, then passed through a 0.2-μm filter membrane to the injection bottle. (3) Intestinal digesta, liver and feces: 0.100 g samples were weighed accurately into a 2-mL centrifuge tube, 1 mL 75% ethanol was added, mixed with sharp shaking, and centrifuged at 4 °C, 21,130 × *g* for 10 min. A volume of 500 μL of supernatant was removed, adding 500 μL pre-cooled acetonitrile, then passed through a 0.2-μm filter membrane to the injection bottle.

After pretreatment, all the samples were detected by high-throughput target-based ultra-high-performance liquid tandem chromatography-Q-TOF-MS/MS (UHPLC-QTOF-MS/MS; UHPLC, Agilent 1290, Agilent; Q-TOF, SCIEX 6600, SCIEX). The raw data were processed by Agilent Mass Hunter Workstation Software (version B.08.00) with default parameters and manual inspection to ensure the qualitative and quantitative accuracy of each compound. The target compound's peak areas were integrated and the output was used for quantitative calculation.

The quantification curve of BAs involved in the experiment was well linear with recoveries in the range of 60% to 125%, which already satisfied the requirements for the quantitative analysis of endogenous substances, with specific data mentioned in Wei et al. (2021). There were 22 reference standards of bile acid involved in the method, including cholic acid (CA), CDCA, deoxycholic acid (DCA), lithocholic acid (LCA), α-muricholic acid (α MCA), β-muricholic acid (β MCA), ω-muricholic acid (ω MCA), ursodeoxycholic acid (UDCA), hyodeoxycholic acid (HDCA), hyocholic acid (HCA), tauro-cholic acid (TCA), tauro-chenodeoxycholic acid (TCDCA), tauro-deoxycholic acid (TDCA), tauro-α-muricholic acid (Tα MCA), tauro-β-muricholic acid (Tβ MCA), tauro-ω- muricholic acid (Tω MCA), tauro-ursodeoxycholic acid (TUDCA), tauro-hyodeoxycholic acid (THDCA), glycol-cholic acid (GCA), glycol-chenodeoxycholic acid (GCDCA), glycol-deoxycholic acid (GDCA) and glycol-ursodeoxycholic acid (GUDCA).

2.10. DNA extraction, separation and high-throughput sequencing of microbiota

Intestinal digesta DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration and integrity were assessed by a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. Then they were fragmented by S220 Focused-ultrasonicators (Covaris, USA) and cleaned up with Agencourt AMPure XP beads (Beckman Coulter Co., USA). The TruSeq Nano DNA LT Sample Preparation Kit (Illumina, San Diego, CA, USA) was used to construct the library and Illumina Novaseq 6000 platform was used to sequence, then generated 150 bp paired-end reads. The raw downstream data (FastQ file) were processed by Trimmomatic (v 0.36) to trim adapters, filter out low-quality bases, and remove reads containing "N" bases (ambiguous bases). Subsequently, the data were aligned to the host genome using Bowtie2 (v 2.2.9), and

host sequences were removed. After obtaining valid reads, the metagenome was assembled by MEGAHIT (v 1.1.2). The assembled scaffolds were subjected to Open Reading Frame (ORF) prediction using Prodigal (v 2.6.3), and the ORFs were translated into amino acid sequences. The non-redundant gene sets were constructed using CDHIT (v 4.5.7) for predicted genes in all samples, with clustering parameters of 95% identity and 90% coverage. The longest gene was selected as representative sequence of each gene set. The clean reads were compared to the non-redundant genes (set at 95% identity) using Bowtie2 (v 2.2.9), followed by statistical information on the abundance of genes in the corresponding samples. Species taxonomy was obtained from the corresponding taxonomic database of the NR library, and the abundance of the species was derived from the corresponding gene abundance. To create abundance profiles at different taxonomic levels, including domain, kingdom, phylum, class, order, family, genus, and species, the relative abundance of species within each sample is tallied, resulting in taxonomic-level-specific abundance profiles. The gene set representative sequence was annotated with KEGG database with an e-value of 1e-5 using DIAMOND (v 0.9.7). The gene sets were matched against the CAZy database using hmmscan (v 3.1) to obtain the information on carbohydrate-active enzymes. The entire metagenome sequencing was bioinformatic analyzed by Shanghai OE Biotech Co., Ltd (Shanghai, China), with specific parameters referenced from Du and Liu (2021).

2.11. Calculations

Total primary BAs = sum up of all primary BAs concentration;

Total secondary BAs = sum up of all secondary BAs concentration;

CA_/CDCA_BAs = (CA + TCA + GCA)/(CDCA + TCDCA + GCDCA);

Free_/conjugated_BAs = (CA + CDCA + LCA + DCA + UDCA)/(TCA + GCA + TCDCA + GCDCA + TDCA + GDCA + TUDCA + TβMCA);

Tauro_BAs = sum up of taurine conjugated BAs concentrations;

Glyco_BAs = sum up of glycine conjugated BAs concentrations.

All the BAs were categorized based on experience in mammals.

2.12. Statistical analysis

All statistical data were analyzed using SPSS 22.0 and evaluated for variances using Levene's test. Comparisons between 2 groups were performed using 2-tailed unpaired Student's *t*-tests (normal distribution) or Mann–Whitney U-tests (non-normal distribution). All results were presented as means ± standard errors of the mean (SEM). The level of significant difference was set at $P < 0.05$. The graphics were drawn by GraphPad Prism 8.3 (GraphPad Software Inc. USA).

3. Results

3.1. Growth performance and morphometric parameters

In the present study, fish fed CTG exhibited a lower survival rate compared with PP ($P < 0.05$, Table 2), but the survival remained above 95% in both groups. Although fish fed PP diet had a higher FR ($P < 0.05$), SGR and FCR had no significant difference between groups ($P > 0.05$). Meanwhile, VSI, AFT and CF were not significantly influenced by dietary treatments ($P > 0.05$). It is worth noting that CTG diet significantly increased the carcass ratio of spotted seabass ($P < 0.05$).

Table 2
Growth performance and morphometric parameters of spotted seabass under dietary treatment.

Item	PP	CTG	SEM
Growth performance			
SR,[1] %	98	95	1.0
BWi, g/fish	41.7	41.7	0.01
BWf, g/fish	152.8	143.5	3.55
FI, g/tank	3047	2730**	100.2
FR,[2] %	1.9	1.8**	0.03
WGR,[3] %	262.1	233.9*	9.69
SGR,[4] %/day	2.32	2.21	0.044
FCR[5]	0.93	0.94	0.009
CR,[6] %	67.07	68.36**	0.222
Morphometric parameters			
VSI,[7] %	11.20	10.93	0.163
AFT,[8] %	6.02	5.92	0.117
CF,[9] %	1.33	1.33	0.017

PP: plant protein-based diet; CTG: PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine.
SR = survival rate; BWi = initial body weight; BWf = final body weight; FI = feed intake; FR = feeding rate; WGR = weight gain rate; SGR = specific growth rate; FCR = feed conversion rate; CR = carcass rate; VSI = viscerosomatic index; AFT = abdominal fat rate; CF = condition factor. "*" means $P < 0.05$, "**" means $P < 0.01$.
[1] SR = 100 × final fish number/initial fish number.
[2] FR = 100 × FI/[(Wf + Wd − Wi)/2]/days, where Wf is the final total weight, Wd is the total weight of dead fish, Wi is the initial total weight.
[3] WGR = 100 × (Wf + Wd − Wi)/Wi.
[4] SGR = 100 × [Ln (BWf/BWi)]/days.
[5] FCR = FI/(BWf- BWi).
[6] CR = 100 × (carcass weight)/(body weight).
[7] VSI = 100 × (viscera weight)/(body weight).
[8] AFT = 100 × (abdominal fat weight)/(body weight).
[9] CF = 100 × (body weight, g)/(body length, cm)^3.

3.2. Dietary cholesterol-taurine-glycine supplement improved liver functions and reduced the accumulation of lipid droplets

Lower AST and ALT of plasma were observed in fish fed CTG compared with PP ($P < 0.05$, Fig. 1A), while AKP was not significantly influenced ($P > 0.05$). Dietary CTG significantly decreased the HSI of fish ($P < 0.05$, Fig. 1B). Moreover, histological characteristics of HE and Oil Red O staining results revealed that lower proportion of fatty liver in fish fed CTG compared to fish fed PP (Fig. 1C and D). The relative area of lipid droplets in the liver significantly decreased in CTG ($P < 0.05$, Fig. 1E), with concurrently decreased mRNA expression of perilipin 2 (*plin2*) in liver, a gene related to lipid droplet deposition ($P < 0.05$, Fig. 1F). As expected, fish fed CTG had lower lipid content in their livers ($P < 0.01$, Fig. 1G).

3.3. Dietary cholesterol-taurine-glycine supplement improved lipid metabolism

Lower TG level was observed in the plasma of CTG group fish compared to PP group ($P < 0.01$, Fig. 2A), while no statistical difference in liver ($P > 0.05$, Fig. 2B). The fish fed CTG significantly decreased the mRNA expression of lipogenesis genes (acetyl CoA carboxylase 1, *acc1*; fatty acid synthase, *fasn*; $P < 0.01$, Fig. 2C), while increased expression levels of genes related to lipolysis, i.e., adipose triglyceride lipase (*atgl*) and monoacylglycerol esterase (*magl*) ($P < 0.01$, Fig. 2D). Another lipolysis gene, i.e., hormone-sensitive triglyceride lipase (*hsl*), showed a lower expression level in fish fed CTG. The expression levels of peroxisome proliferator activated receptor alpha (*pparα*) and apoptotic cysteine protease (*c/ebpα*) were down-regulated in CTG group ($P < 0.05$, Fig. 2D), but no statistical differences in carnitine palmitoyltransferase 1 alpha (*cpt1α*)

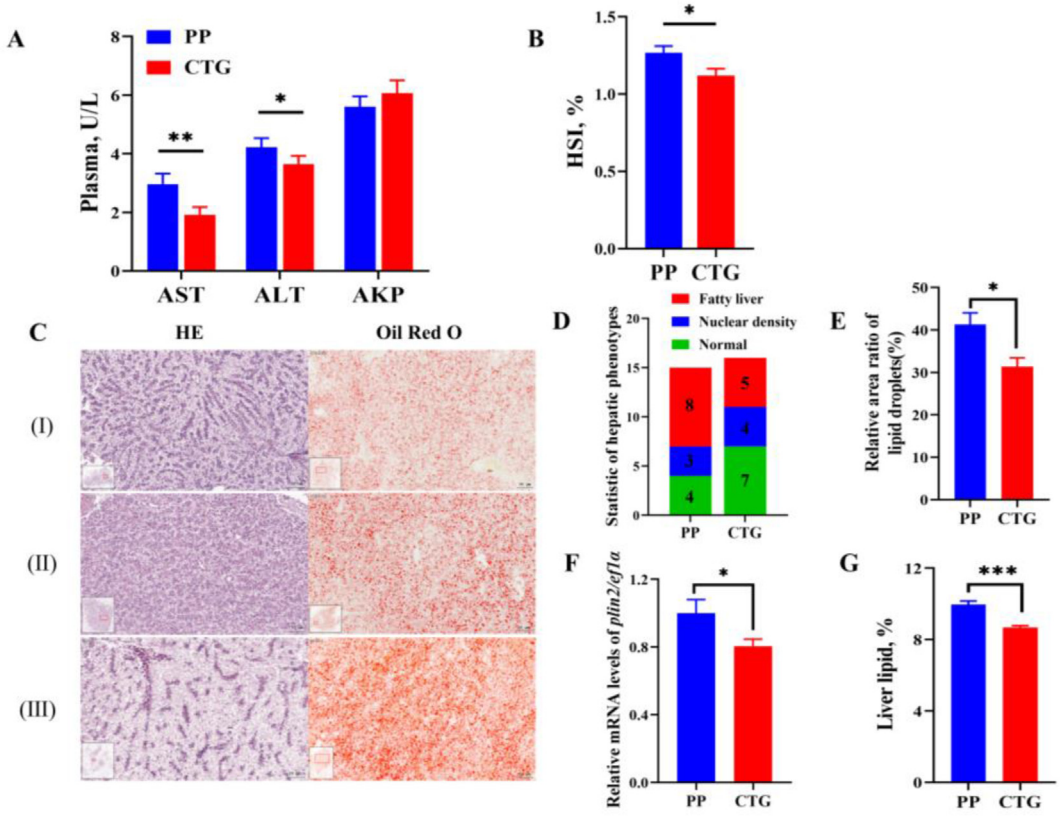

Fig. 1. Supplementing cholesterol-taurine-glycine supplement in plant protein-based diet reduced the accumulation of lipid droplets in the liver of spotted seabass. (A) Indicators of liver function (AST = aspartate aminotransferase; ALT = alanine aminotransferase; AKP = alkaline phosphatase) in plasma. (B) Hepatosomatic index (HSI). (C) The images (bar = 50 μm) of hematoxylin–eosin (HE) staining and oil red O staining in liver. I: Normal (no obvious abnormality); II: nuclear dense liver; III: fatty liver. (D) Statistical results of hepatic histopathological phenotypes. (E) The relative area ratio of lipid droplets according to the results of oil red O staining. (F) Relative mRNA expression of *plin2*, a marker of lipid droplet deposition. (G) Lipid content in liver. Data are presented as means ± SEM. PP: plant protein-based diet; CTG: PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine. "*" means $P < 0.05$, "**" means $P < 0.01$ and "***" means $P < 0.001$.

and peroxisome proliferator activated receptor γ (*pparγ*) ($P > 0.05$). Our results showed that fish fed CTG showed lower gene and protein expression levels of FABP1 in liver compared to PP ($P < 0.01$, Fig. 2E to G). The increased lipase activity in PID was observed in fish fed CTG diet ($P < 0.05$, Fig. 2H). Regarding the hepatic cAMP content, supplementary Ch-Tau-Gly supplement in plant-based diet significantly reduced cAMP content in liver ($P < 0.05$, Fig. 2I).

3.4. Dietary cholesterol-taurine-glycine supplement inhibited cholesterol synthesis and enhanced bile acid metabolism

Fish fed CTG diet exhibited increased levels of TC, HDL-C and LDL-C in plasma and TC in liver ($P < 0.05$, Fig. 3A and B). The mRNA expression of genes related to cholesterol synthesis 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr*) and sterol regulation related genes Sterol regulatory element binding protein 1 and 2 (*srebp1* and *srebp2*) in liver was down-regulated in fish fed CTG ($P < 0.05$, Fig. 3C), while the gene related to cholesterol transport apolipoprotein B α (*apobα*) was up-regulated ($P < 0.001$, Fig. 3C).

For bile acid metabolism, fish fed CTG diet exhibited increased expression levels of genes related to bile acid synthesis, e.g., *cyp7a1* and *cyp7b1* and liver X receptor alpha (*lxrα*), in liver ($P < 0.05$, Fig. 3D and E), while no difference in *cyp8b1* between treatments ($P > 0.05$). Notably, the mRNA levels of farnesoid X receptor (*fxr*) and nuclear receptor subfamily 0 group B member 2 (*shp*) in liver had no statistical difference between the treatments ($P > 0.05$), while the mRNA levels of *fxr*, hepatocyte nuclear factor 4 alpha (*hnf4α*) and *shp* in distal intestinal were significantly decreased in fish fed CTG ($P < 0.05$, Fig. 3F), and *hnf4α* and liver receptor homolog-1 (*lrh1*) in liver and retinoid X receptor (*rxr*) in distal intestinal were increased ($P < 0.05$, Fig. 3E to F). The results of enzyme-linked immunosorbent assay indicated that fish fed CTG diet respectively decreased and increased CYP8B1 and CYP7B1 contents in liver ($P < 0.05$, Fig. 3G), respectively, while the content of CYP7A1 was not influenced ($P > 0.05$).

3.5. Cholesterol-taurine-glycine supplement affected the bile acid pools

Fish fed CTG showed about three times higher levels of the total bile acid (TBA) in gallbladder and feces than these fed PP, while significantly decreased in plasma ($P < 0.01$, Table 3), but no statistical difference in PID, DID and liver ($P > 0.05$). Following 24 h of fasting, the gallbladder of PP group showed light green, while it appeared dark green in CTG group accompanied by an increase in GBI (Fig. 4A and B). Compared with PP group, TCA, TCDCA and TDCA were significantly raised in gallbladder of CTG group, while CA was decreased ($P < 0.01$, Fig. 4C). Further categorization analysis of BAs revealed that fish fed CTG significantly elevated the levels of primary BAs, CDCA-type BAs (CDCA, TCDCA and GCDCA), and conjugated BAs especially taurine-conjugated BAs in gallbladder ($P < 0.001$, Fig. 4E to J). On the contrary, compared with PP group, CA, TCA, CDCA, TCDCA, LCA and DCA were lower in plasma of CTG group, while GCDCA unexpectedly exhibited a highly significant increase, which was even the

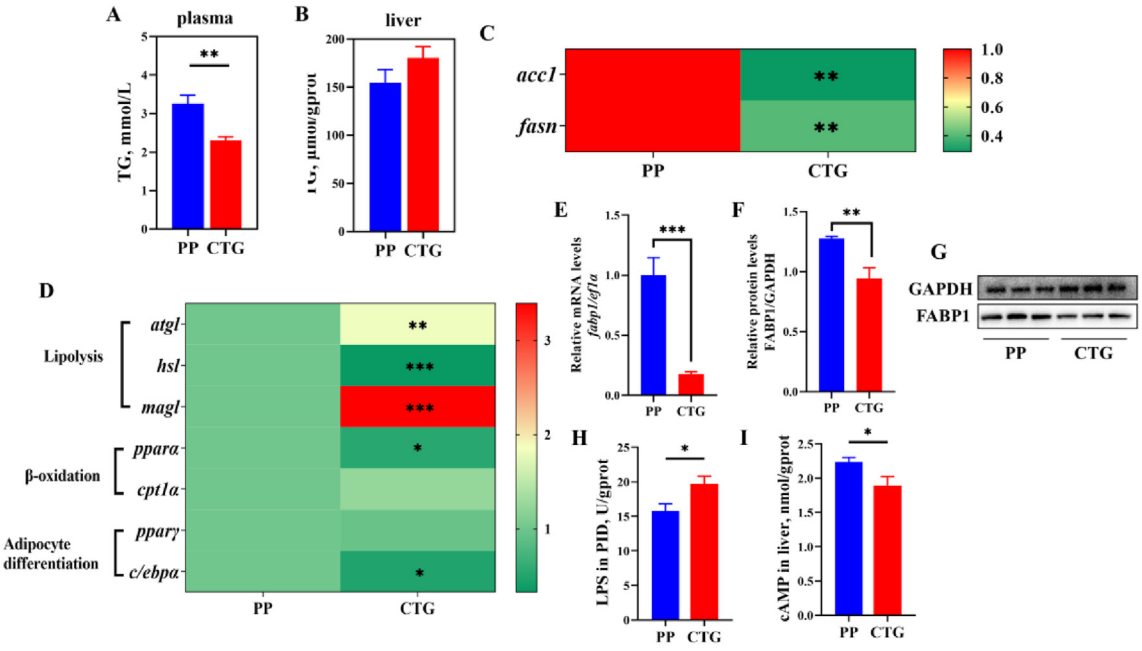

Fig. 2. Supplementing cholesterol-taurine-glycine supplement in plant protein-based diet inhibited lipogenesis and promoted lipolysis in liver of spotted seabass. (A) and (B) Triglyceride (TG) content in plasma and liver, respectively. (C) Transcriptional levels of lipogenesis (*acc1* and *fasn*) related genes. (D) Transcriptional levels of lipolysis (*atgl*, *hsl* and *magl*), β-oxidation (*pparα* and *ctp1α*) and adipocyte differentiation (*pparγ* and *c/ebpα*) related genes. (E), (F) and (G) Transcriptional and protein levels of FABP1. (H) Lipase (LPS) content in proximal intestinal digesta (PID). (I) cAMP content in liver. Data are presented as means ± SEM. PP: plant protein-based diet; CTG: PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine. "*" means $P < 0.05$, "**" means $P < 0.01$, "***" means $P < 0.001$.

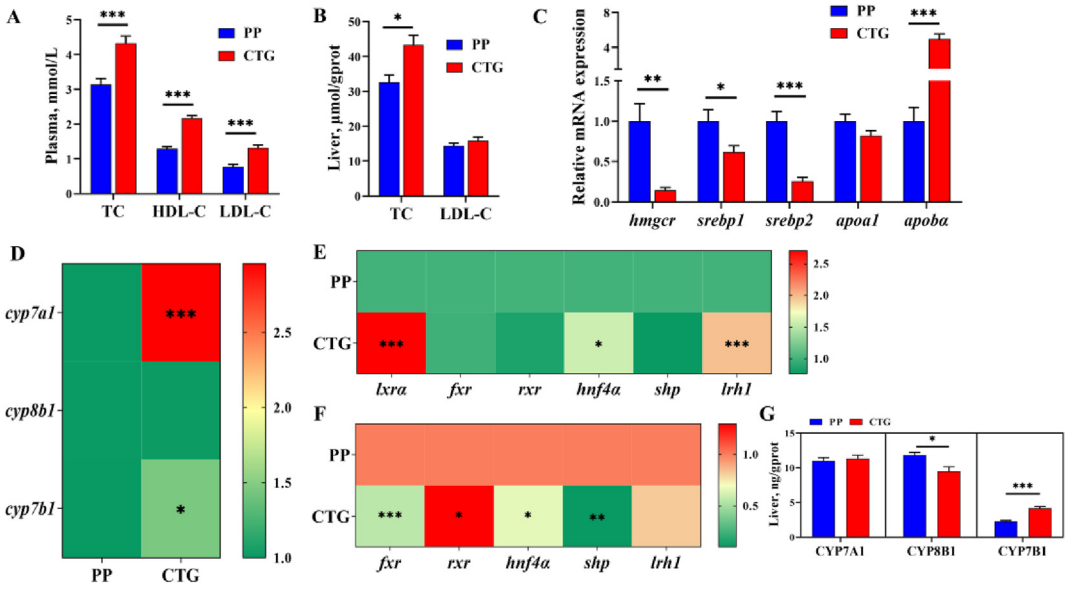

Fig. 3. Effects of cholesterol-taurine-glycine supplement supplemented in plant protein-based diet on cholesterol and bile acid metabolism. (A) TC, HDL-C and LDL-C content in plasma. (B) TC and LDL-C content in liver. (C) Transcriptional levels of cholesterol synthesis (*hmgcr*) and transport (*srebp1*, *srebp2*, *apoa1* and *apobα*) related genes. (D) Transcriptional levels of bile acid synthesis (*cyp7a1*, *cyp8b1* and *cyp7b1*) related genes. (E) and (F) Transcriptional levels of bile acid transport (*lxrα*, *fxr*, *rxr*, *hnf4α*, *shp* and *lrh1*) in liver and distal intestinal, respectively. (G) CYP7A1, CYP8B1 and CYP7B1 content in liver. PP: plant protein-based diet; CTG: PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine. Data are presented as means ± SEM. "*" means $P < 0.05$, "**" means $P < 0.01$, "***" means $P < 0.001$.

highest content of BAs in plasma of CTG group ($P < 0.001$, Fig. 4D). As a result, the primary and secondary BAs in plasma significantly decreased in CTG group ($P < 0.01$, Fig. 4K to L), while CDCA-type BAs and conjugated BAs increased ($P < 0.01$, Fig. 4O to P), compared with PP group. However, in contrast to the results observed in the gallbladder and plasma, CDCA-type BAs in the liver were significantly decreased in the CTG group ($P < 0.01$, Fig. S3A). Fish fed CTG showed decreased levels of secondary BAs in PID and DID ($P < 0.001$, Fig. S2B) and increased levels of conjugated BAs both in PID and DID ($P < 0.01$, Fig. S3B), while BSH, a critical player in the process of bile acid deconjugation, decreased significantly in CTG group ($P < 0.05$, Fig. 5A). In addition, in the CTG group, more BAs profiles exhibited obvious variations in DID compared to PID ($P < 0.05$, Fig. 5B), whereas in the PP group, only

Table 3
Total bile acid content in proximal intestinal digesta (PID), distal intestinal digesta (DID), plasma, liver, gallbladder and feces.

Item	PP	CTG	SEM
PID, μmol/g	62.5	58.6	5.81
DID, μmol/g	33.0	24.7	3.91
Plasma, μmol/L	1.43	0.51**	0.145
Liver, μmol/g	1.3	1.7	0.36
Gallbladder, μmol/L	351,019	916,334***	75,434.3
Feces, μmol/g	2.4	7.8**	1.11

PP: plant protein-based diet; CTG: PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine. "*" means $P < 0.05$, "**" means $P < 0.01$ and "***" means $P < 0.001$.

2 BAs underwent changes ($P < 0.05$, Fig. 5C), indicating that fish fed CTG induced stronger microbial processes.

3.6. Dietary cholesterol-taurine-glycine supplement modulated gut microbiota profile

Metagenomic analysis of intestinal digesta of PID and DID revealed the presence of more than 17,000 bacteria at the specie level. The top 15 most abundant bacteria in PID and DID at species level were displayed in Fig. 6A. Overall, the *Aurantimicrobium_sp._MWH-Mo1*, *Actinobacteria_bacterium* and *Acinetobacter_baumannii* were the most abundance species, accounting for 60% of the total abundance. In the top 15 most abundant bacteria, 12/15 were found to be consistent between PID and DID. Differently, *Enterobacter_cloacae_complex_sp._2DZ2F16B1*, *Aestuariivirga_litoralis* and *Actinomycetales_bacterium_mxb001* were enriched in PID, while *Cetobacterium_sp._2G_large*, *Cetobacterium_somerae* and *Brachyspira_aalborgi* were enriched in DID. But there was no statistically significant difference observed in the relative abundance of these predominant bacteria between the 2 groups ($P > 0.05$). Although the alpha diversity (sobs and shannon index) and beta diversity (PCoA analysis) of bacteria in PID and DID didn't show significant differences between the 2 groups ($P > 0.05$, Fig. S4A to D), a large number of statistically different bacteria were detected at species level. Here, we list the top 10 most abundant differential microbiota species (Fig. 6B and C), most of them were significantly lower between different segments after CTG diets, except the genus *Mortierella* (Fig. S5A). The *Lactobacillus*, especially *Lactobacillus_salivarius* and *Lactobacillus_brevis*, showed significantly lower abundance in CTG group, while some pathogenic bacteria, such as *Staphylococcus_hominis*, *Bacillus_kyonggiensis* and *Salmonella_sp._zj-h16*, were also significantly decreased in CTG group.

Spearman correlation analysis found the top 3 abundant bacterial species with significant differences showed a strong correlation with bile acid components, especially in PID. *Bradyrhizobium_sp._MOS002*, *Lactobacillus_salivarius* and *Staphylococcus_hominis* showed significant positive correlations with TCA, LCA, DCA, TUDCA, CA, primary and secondary BAs, taurine conjugated BAs and the ratio of CA_/CDCA_BAs, while *Bradyrhizobium_sp._MOS002* and *Lactobacillus_salivarius* exhibited significant negative correlation with GCDCA in PID ($P < 0.05$, Fig. 6D). Among the DID, the *L. salivarius*, *B. MOS002* and *S. hominis* showed significant positive corrections with LCA, CDCA and secondary BAs ($P < 0.05$, Fig. 6E), and negative corrections with TUDCA, TCDCA and TDCA. Moreover, *L. salivarius* showed a significant negative correction with lipolysis genes such as *atgl* and *magl* ($P < 0.05$, Fig. 6F), while exhibiting a positive correction with genes related to lipogenesis such as *fabp1* and *acc1*. Based on the top 50 of KEGG pathways enrichment, the main bio-metabolic pathways involved in Cellular Processes and Metabolism were identified (Fig. S6A and

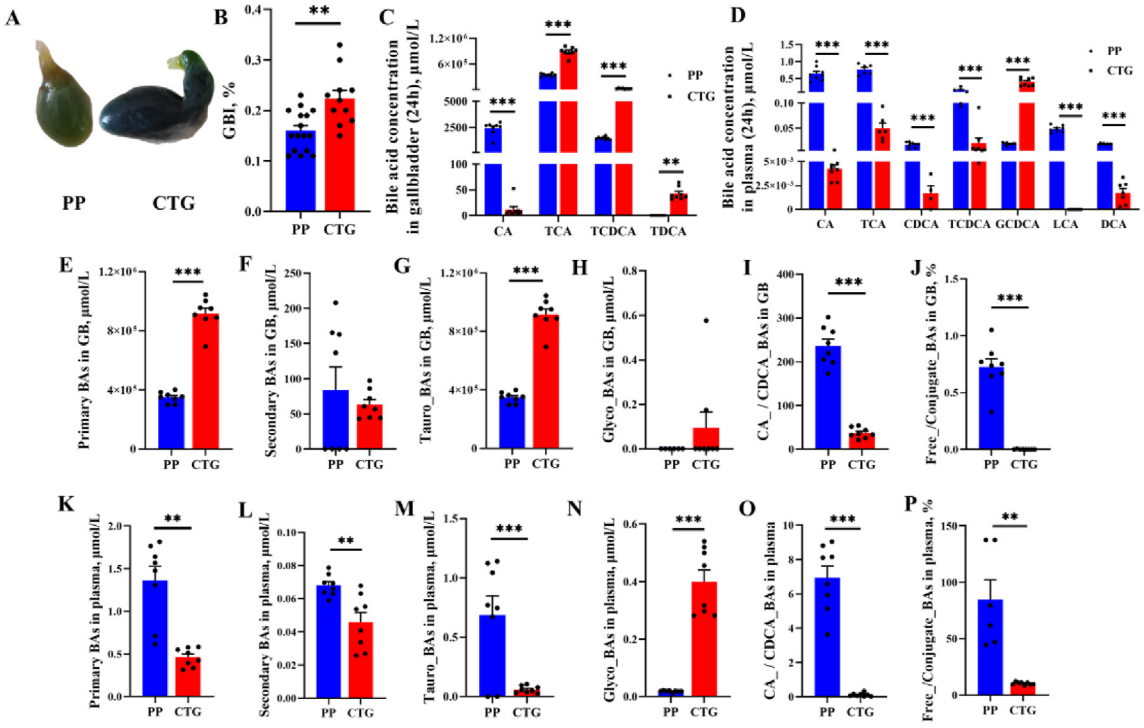

Fig. 4. Supplementing cholesterol-taurine-glycine supplement in plant protein-based diet affected the bile acid pools of spotted seabass. (A) The gallbladder in PP and CTG group. (B) The gallbladder index (GBI). GBI = 100 × (gallbladder weight/body weight). (C) and (D) Bile acid composition in gallbladder and plasma, respectively. (E) to (J) Primary bile acids (BAs), secondary BAs, taurine conjugated BAs (Tauro_BAs), glycine conjugated BAs (Glyco_BAs), CA_/CDCA_ BAs and free_/conjugate_BAs in gallbladder (GB). (K to P) Primary BAs, secondary BAs, Tauro_BAs, Glyco_BAs, CA_/CDCA_ BAs and free_/conjugate_BAs in plasma. Data are presented as means ± SEM. PP: plant protein-based diet; CTG: PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine. "**" means $P < 0.01$, "***" means $P < 0.001$.

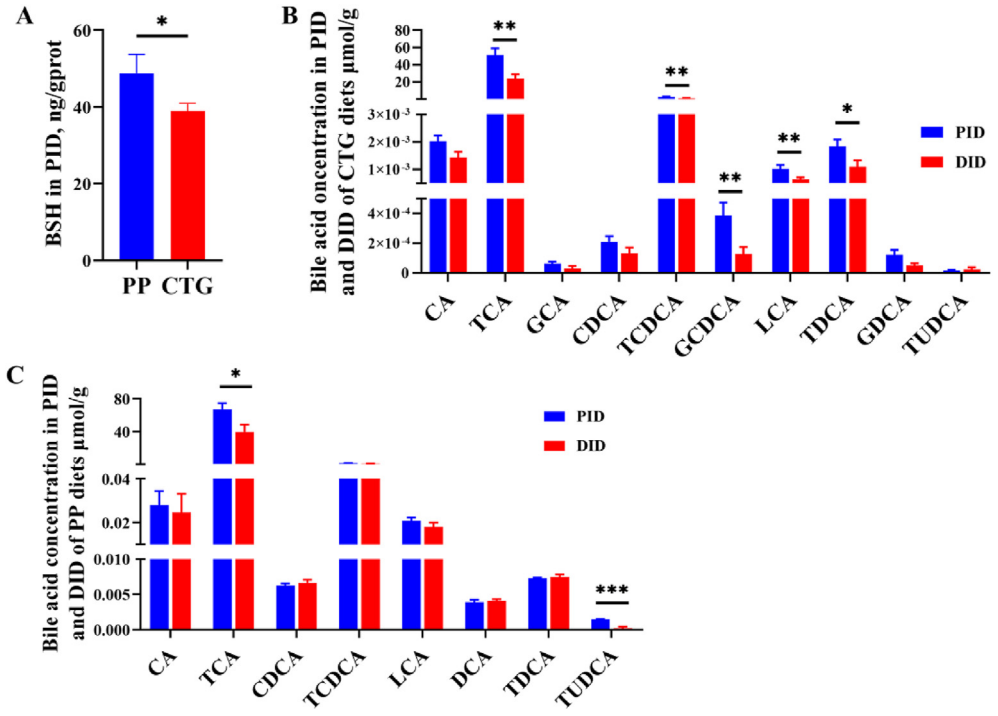

Fig. 5. Cholesterol-taurine-glycine supplement reduced bile salt hydrolase (BSH) content and affected the bile acid profiles of intestinal digesta. (A) BSH content in proximal intestinal digesta (PID). (B) and (C) Bile acid composition in PID and distal intestinal digesta (DID) of PP and CTG diets, respectively. Data are presented as means ± SEM. PP: plant protein-based diet; CTG: PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine. "*" means $P < 0.05$, "**" means $P < 0.01$, "***" means $P < 0.001$.

B). The abundance of several gene groups involved in fatty acid metabolism, steroid synthesis, cell cycle and apoptosis were significantly reduced in fish fed CTG ($P < 0.05$, Fig. S6C and D). Interestingly, fish fed CTG exhibited stronger resistance than the PP group to 9/30 antibiotic species in PID (Fig. S6E). In DID, the CTG group was more resistant to 23/30 antibiotics compared with PP group (Fig. S6F).

4. Discussion

In the current study, supplementation of Ch-Tau-Gly supplement did not significantly impact the growth performance, estimated as SGR, of spotted seabass. Our findings seemingly contradict several previous studies that supplementing 1% to 2.5% cholesterol or taurine alone to plant protein-based diets could improve growth of fish, including, but not limited to, Atlantic salmon (Zhang et al., 2018), rainbow trout (Deng et al., 2014) and European seabass (Martins et al., 2018). However, fish fed CTG exhibited a significant increase in CR, representing the edible portion of the fish body devoid of the head, viscera, fins and tail (Li et al., 2020). It is worth noting that weight gain is only one aspect of growth and maybe a pathology of increased viscera weight, such as expanded liver, not necessarily increased muscle mass (Du and Turchini, 2021). Furthermore, the consumption of CTG diet by the seabass significantly increased the levels of bile acids. As widely recognized, bile acids play a crucial role in the digestion of nutrients and the absorption of fat-soluble vitamins (Ridlon et al., 2014). Consequently, maintaining an appropriate level of bile acids can promote growth by facilitating the digestion and absorption of nutrients, a phenomenon that has also been validated in rainbow trout (Staessen et al., 2020). Herein, our findings suggested dietary Ch-Tau-Gly supplement benefit growth performance of spotted seabass by reducing the weight of non-edible portions, especially the weight of fats. This provides a viable nutritional strategy for promoting the growth of fish, particularly carnivorous species, in aquaculture production.

Dietary cholesterol plays a pivotal role in regulating both plasma and hepatic cholesterol homeostasis (Van Rooyen et al., 2011). In the present study, supplementation of Ch-Tau-Gly supplement to plant protein-based diet increased plasma and hepatic TC levels, and improved bile acid metabolism. Dietary exogenous cholesterol inhibited the endogenous cholesterol synthesis process (Schoenheimer and Breusch, 1933), in which the cholesterol synthesis rate-limiting enzyme HMGCR contributed substantially, as also supported by our findings of reduced expression of *hmgcr*. Further, during cholesterol biosynthesis, HMGCR was strictly regulated with feedback (Duan et al., 2022). When cholesterol concentrations were low, SREBP2, a master regulator involved in cholesterol synthesis, binds to sterol regulatory element of HMGCR promoter in the proximal region, promoting cholesterol synthesis (Duan et al., 2022; Luo et al., 2020). Similarly, fish fed CTG increased cholesterol concentration, with feedback inhibition of crucial players in cholesterol synthesis, i.e., *srebp2* and *hmgcr*, suppressing the endogenous cholesterol synthesis pathway. Additionally, high levels of cholesterol stimulate the production of hydroxysteroid, which, in turn activate LXR to enhance c cholesterol efflux and BAs synthesis, contributing to maintenance of cellular cholesterol homeostasis (Duan et al., 2022). Our findings confirmed this mechanism, as fish fed CTG increased hepatic cholesterol levels, accompanied by upregulation of LXR expression and enhanced BAs metabolism, to maintain organismal cholesterol homeostasis.

Bile acids are absolutely essential for lipid metabolism, as they emulsify lipids in the intestine and facilitate the absorption of liposoluble nutrients from the diets. Our previous study demonstrated that spotted seabass fed with high levels of plant protein experienced liver cholesterol accumulation and disturbances of bile acid

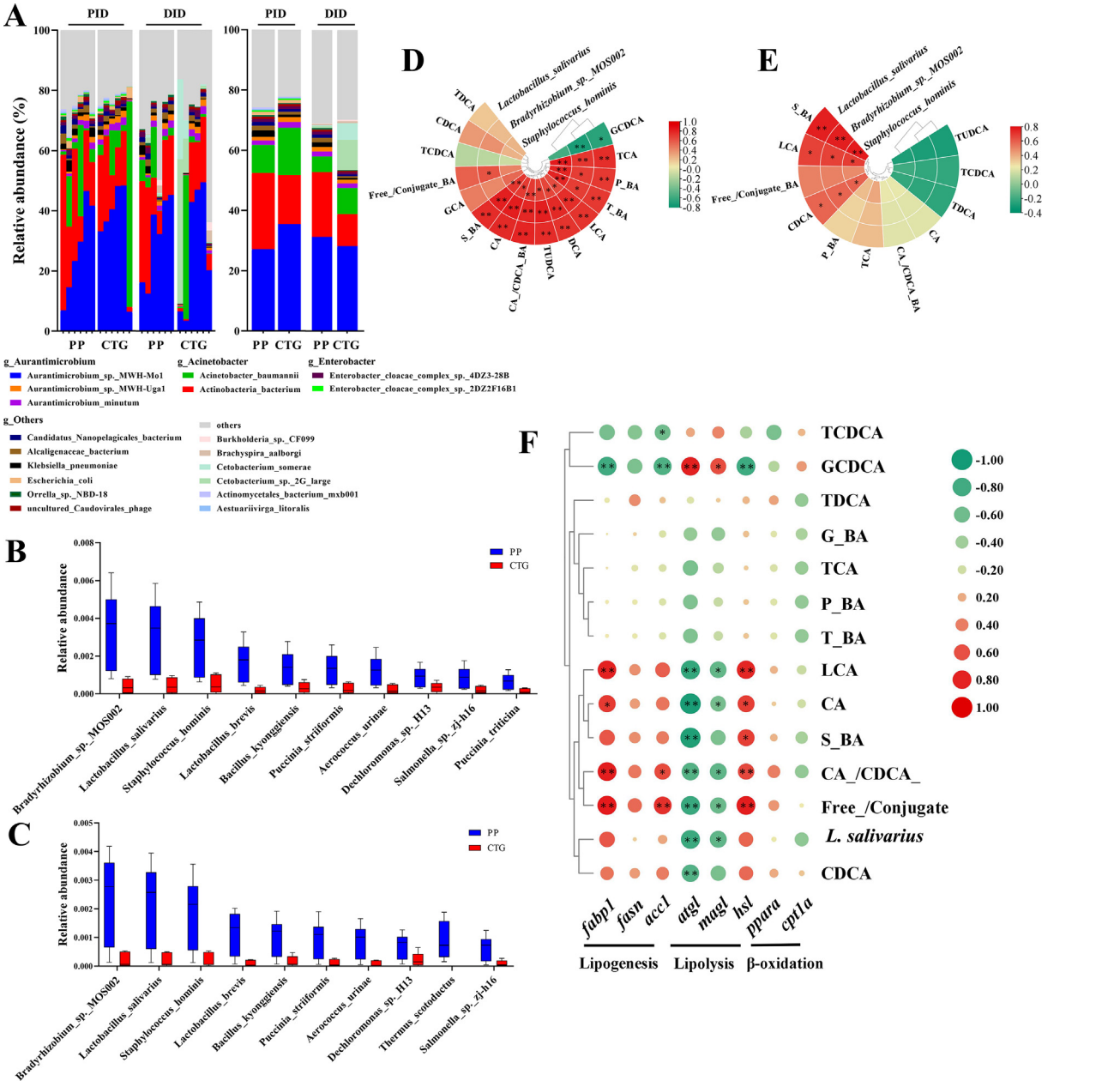

Fig. 6. Cholesterol-taurine-glycine supplement altered the intestinal flora. (A) Top 15 most abundant taxonomic composition in proximal intestinal digesta (PID) and distal intestinal digesta (DID) at species level. (B) to (C) Top 10 most abundance of bacteria with significant differences at species level in PID and DID, respectively. (D) to (E) Correlation analysis of the top 3 microbiota with significant differences in abundance at species level in relation to bile acids composition of PID and DID, respectively. (F) Correlation analysis of bile acid parameters and *Lactobacillus_salivarius* in PID with lipid metabolism. The lipid metabolism indicators involved genes related to lipogenesis, lipolysis and fatty acid β-oxidation. PP: plant protein-based diet; CTG: PP supplemented 0.5% cholesterol, 0.5% taurine and 1.3% glycine. "*" means $P < 0.05$, "**" means $P < 0.01$.

synthesis, which resulted in liver inflammation and fatty liver (Zhang et al., 2019). The alleviating fatty liver in fish fed CTG could be attributed to enhanced bile acid synthesis, as the majority of excess cholesterol is excreted in the gallbladder through the synthesis of BAs (Duan et al., 2022; Goedeke and Fernández-Hernando, 2012). We found that the bile acid pool of spotted seabass became replenished after consuming the CTG diet, notably reflected in the elevated TBA levels in gallbladder, which was about three folds higher than that of PP. BAs are produced in liver through the classical (approximately 75% of bile acid production, generating both CA and CDCA) and alternative pathway (predominantly generating CDCA) (Thomas et al., 2008). The sterol-27-hydroxylase (CYP7A1), a rate-limiting enzyme of bile acid synthesis, which determines the amount of bile acid production, was greatly increased in fish fed CTG in our study. Of note, CYP8B1 is required for CA production in the classical pathway and determines the ratio between CA and CDCA, whereas CYP7B1 is required for CDCA production in alternative pathway (Li-Hawkins et al., 2002; Wahlström et al., 2016). Fish fed CTG resulted in lower CYP8B1 and higher CYP7B1 activities in liver suggesting an increased efficiency of the alternative pathway for bile acid synthesis. This hypothesis is in agreement with previous studies in tiger puffer (Xu et al., 2020) and Nile tilapia (Li et al., 2023a) that supplementing cholesterol or taurine alone to plant protein-based diets could increase CYP7B1 activities.

Our analysis of the bile acid profiles in the hepatic-intestinal circulation revealed that the ratio of CA_/CDCA_BAs greatly

reduction in intestinal digesta, plasma and gallbladder. This indicated that fish fed CTG strongly contributed to CDCA production, especially TCDCA. However, it is of interest that TBA in plasma was considerably reduced, which is a good sign for liver function since low BAs in plasma imply lower cytotoxicity. This finding can be supported by the lower plasma AST and ALT levels in CTG group. Hydrophobic BAs, such as LCA, DCA, CDCA and CA, are able of disrupting cell membranes causing cytotoxicity, while these BAs conjugated with taurine or glycine greatly enhanced hydrophilic properties (Guzior and Quinn, 2021; Thomas et al., 2008). In CTG treatment, the reduction in hydrophobicity of bile acid pool was mainly attributed to the decrease in secondary BAs and an increase of fecal excretion, followed by the improvement of conjugated BAs. Additionally, Hydrophobic BAs are potential activators of FXR, while taurine-conjugated bile acids act as antagonists of FXR (Guzior and Quinn, 2021). As a result, the decreased hydrophobic BAs but increased taurine-conjugated BAs in CTG might attenuate the feedback regulation of CYP7A1 by FXR (Chiang and Ferrell, 2022), and then stimulate bile acid production.

Dietary supplementation of Ch-Tau-Gly supplement increased the levels of BAs, with the TBA in the gallbladder of CTG group being three times higher than that of PP group. This increase appeared to have an antibacterial effect, as evidenced by a substantial reduction in the abundance of certain bacteria that exhibited notable differences in CTG group. The predominant BAs in the bile acid pool of spotted seabass were taurine-conjugated BAs, mainly TCA and TCDCA, based on the results of our investigation. Intestinal microorganisms deconjugate these conjugated BAs to their free forms through BSH (Chiang, 2009), whereas in CTG group, BSH was reduced and conjugated BAs were elevated. The primary BAs, after being deconjugated by BSH, underwent 7α-dehydroxylation to produce secondary BAs such as DCA and LCA, which were decreased in gallbladder, plasma and liver of the CTG group. *Lactobacillus*, an important bacteria for BSH-producing involved in the bile acid deconjugated process (Jia et al., 2018), was significantly reduced in CTG group, especially in *Lactobacillus_salivarius*. Moreover, it has been reported that most lactic acid bacteria-derived BSH enzymes exhibited a higher catalytic activity for hydrolyzing glycine-conjugated BAs than taurine-conjugated BAs (Begley et al., 2006). This might explain the elevated GCDCA in plasma of CTG group. As expected, *L. salivarius* and GCDCA showed a strong negative correction in PID. Additionally, *L. salivarius* seemed to be capable of completely deconjugating TCDCA (Guban et al., 2006). In our study, fish fed CTG showed a significant increase in TCDCA in the gallbladder, and there was a negative correlation between *L. salivarius* and TCDCA both in PID and DID. Our findings indicated that alterations in *L. salivarius* reshaped bile acid spectrum by increasing the levels of conjugated BAs, particularly TCDCA and GCDCA, while decreasing the levels of secondary BAs such as DCA and LCA.

Due to *lactobacilli*-mediated bacterial hydrolysis, suboptimal levels of conjugated bile acids might exacerbate the weakened ability to absorb lipids (Guban et al., 2006). Our findings demonstrated that conjugated BAs, especially TCDCA and GCDCA, exhibited a strong negative correction with genes related to lipogenesis, but a strong positive correction with genes related to lipolysis, highlighting their significant involvement in lipid metabolism. Pathological analysis revealed an obvious reduction in fatty liver and lipid droplets, as well as an increase in PID lipase activity and a decrease in plasma TG content in CTG group, suggesting dietary Ch-Tau-Gly supplement have distinct effects on lipid metabolism. Our finding is in line with previous reports that dietary certain level of cholesterol or taurine only could actively reduce lipid deposition by lowering plasma and the whole body lipid levels (Hoseini et al., 2017; Martins et al., 2018; Snyder et al., 2012), promoting intestinal lipase activity (Abdel-Tawwab and Monier, 2018) and improving lipid digestibility. Further, in our study, dietary Ch-Tau-Gly supplement down-regulated the expression of lipogenesis-related genes, such as *acc1* and *fasn*, suggesting the inhibition of de novo lipid synthesis. The low gene expression of *srebp1* in liver may further support the suppression of lipogenesis, as the SREBP1 is a master modulator of fatty acid metabolism and plays an essential regulatory role in lipogenesis (Lee et al., 2022). Specifically, on one hand, suppressed FABP1 expression compromised the capacity of liver to uptake and transport fatty acids for glycerolipid synthesis, resulting in depressed liver weight and TG accumulation, which would be effective against nonalcoholic fatty liver disease (NAFLD) (Mukai et al., 2017; Wu et al., 2017), as supported by the alleviating fatty liver and increased expression of lipolysis relevant genes in our study. On the other hand, FABP1 was strongly associated with PPARα, which was responsible for transporting and targeting fatty acids to metabolic sites and participating in fatty acid β-oxidation (Samulin et al., 2008), which could cause the down-regulated expression levels of *pparα* and *c/ebpα* in CTG group. Additionally, the expression of *plin2* was inhibited in liver of spotted seabass fed CTG, which is in line with that PLIN2 was found to be a hub in the network and identified as a target protein of FABP1 (Pi et al., 2019). Overall, we observed that fish fed CTG exhibited reduced *Lactobacillus* abundance and BSH content, increased conjugated BAs level and lipase activity, enhanced lipid metabolism, and mitigated fatty liver, which collectively experienced beneficial effects on growth. Naturally, further validation of these findings is essential through the expansion of pilot trials and extension of the cultivation period.

5. Conclusion

Spotted seabass fed a plant-based diet supplemented with Ch-Tau-Gly supplement could improve the carcass ratio and lower the risk of fatty liver disease. This dietary approach influences the gut microbiota and composition of bile acids. We observed a decrease in the population of *L. salivarius* when bile acid levels were elevated, leading to an increase in conjugated bile acids, specifically TCDCA and GCDCA. These changes have significant implications for lipid metabolism and ultimately promote liver health. Consequently, the incorporation of Ch-Tau-Gly supplement (including cholesterol, taurine and glycine) is expected to effectively reduce visceral, particularly hepatic, fat deposition and improve meat yield through the interaction with gut microbiota and bile acids. These findings not only offer nutritional strategies for mitigating fatty liver in animals but also hold promise for enhancing potential economic benefits across aquaculture and livestock industries.

Author contributions

Tingting Song: Conceptualization; Methodology; Investigation; Formal analysis; Visualization; Writing – Original Draft. **Xiaofang Liang:** Methodology; Formal analysis. **Hao Wang:** Methodology. **Min Xue:** Project administration; Resources; Writing – Review & Editing. **Jie Wang:** Conceptualization; Supervision; Writing – Review & Editing.

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.2024.03.008.

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