frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 16 May 2022
doi: 10.3389/fmars.2022.891080

Hepatic Metabolomics Analysis of Hybrid Grouper (*Epinephelus fuscoguttatus*♀×*Epinephelus polyphekadion*♂) Fed With Quercetin and Sodium Quercetin-5′-Sulfonates

Jun Luo[1], *Eric Amenyogbe*[1], *Jian-sheng Huang*[1,2] *and Gang Chen*[1,2*]

[1] Fishery College, Guangdong Ocean University, Zhanjiang, China, [2] Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Zhanjiang, China

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***Correspondence:**
Gang Chen
cheng@gdou.edu.cn

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Marine Science

Received: 07 March 2022
Accepted: 21 April 2022
Published: 16 May 2022

Citation:
Luo J, Amenyogbe E, Huang J-s and Chen G (2022) Hepatic Metabolomics Analysis of Hybrid Grouper (Epinephelus fuscoguttatus♀×Epinephelus polyphekadion♂) Fed With Quercetin and Sodium Quercetin-5′-Sulfonates. Front. Mar. Sci. 9:891080. doi: 10.3389/fmars.2022.891080

Combining feeding trials and metabolomics analyses of tissues and biofluids could shed light on nutrient effects and changes in feed intake. In the present study, hybrid grouper (F1 hybrid *Epinephelus fuscoguttatus*♀×*Epinephelus polyphekadion*♂) was used as the marine fish model to quantify the impacts of quercetin and sodium quercetin-5′-sulfonates on serum biochemistry and the status of hepatic lipid accumulation and the changes of metabolites in the liver using ultra-performance liquid chromatography-electrospray triple quadrupole mass spectrometry (UPLC-QTRAP/MS/MS). The study shows that total protein, albumin, alkaline phosphatase and glucose were not significantly different among the three groups ($P > 0.05$). Total cholesterol, triglyceride, and alanine aminotransferase of fish fed quercetin, and sodium quercetin-5′-sulfonates were significantly lower than fish fed without additives ($P < 0.05$). Low-density lipoprotein cholesterol and aspartate aminotransferase of fish fed quercetin were significantly lower than that of fish fed without additives and sodium quercetin-5′-sulfonates ($P < 0.05$). The high-density lipoprotein cholesterol of fish fed quercetin, and sodium quercetin-5′-sulfonates were significantly higher than that of fish fed without additives ($P < 0.05$). Lipid accumulation in the quercetin and sodium quercetin-5′-sulfonates groups decreased significantly. Quercetin and sodium quercetin-5′-sulfonates were effective by increasing hypolipidemic and hepatoprotective compounds that are known for reducing blood lipid levels and liver fat accumulation. As a result of this study, we provide international data for metabolic adaptations during the additives feeding using the fish liver as the study model. By understanding the metabolic effects of these feed additives, this study provides a first step toward understanding the molecular mechanisms of these additives and how they function.

Keywords: Quercetin, Sodium quercetin-5′-sulfonates, oxylipins, metabolomics, marine fish

INTRODUCTION

Aquaculture nutrition has evolved into a more efficient and sustainable industry with continuous feed formulation innovation over the last two decades. It usually depends on the fish oil (FO) and fish meal (FM) for fish nutrition at higher trophic levels, such as salmonids and marine species, since these nutrition resources provide adequate nutrition for the fish. Natural prey consumed by wild fish has the most similar nutritional composition to FM and FO diets. FM indeed contains highly digestible, high-quality proteins with an appropriate essential amino acid profile, and FO contains a high fatty acid profile suitable for many fish species, especially marine fish (Oliva-Teles et al., 2015; Roques et al., 2020). A shortage of marine resources for aquaculture will result from increased aquaculture production and other markets for pharmaceutics, cosmetics, and human nutrition (FAO, 2016; Roques et al., 2020). As a result, the aquaculture industry's FM and FO market values will not be sustainable in the future. As a result of these constraints, FM and FO in fish feed need to be replaced by more sustainable raw materials. In the past decade, incorporating plant ingredients to replace marine ingredients has resulted in many enhancements in the formulation of sustainable diets. Since plant resources are now being used as feed for fish, persistent organic pollutants found in feed and fish have decreased (Berntssen et al., 2015). FO substitution is largely dependent on the supply of essential fatty acids. In fact, a number of marine species (Bell and Koppe, 2011; Roques et al., 2020) are unable to produce long-chain PUFAs from the nutritional poly-unsaturated fatty acids (PUFAs) linolenic and linoleic acid because of their enzymatic limitations. Furthermore, a decrease in the docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) content in fish flesh results from substituting vegetable oils, while the other qualities of fish flesh remain the same (Oliva-Teles et al., 2015).

FM and FO are only about 10–15% of the total FM, and FO needed for fish feed (Medale et al., 2013; Ytrestøyl et al., 2015) to meet carnivorous and marine species' nutritional needs. In this regard, replacing fish feed with fish resources is complicated for carnivorous fishes, and earlier researches have resulted in decreased growth rates and metabolic changes which are not compatible with the production of aquaculture (Geay et al., 2011; Collins et al., 2013). Hence, there is a need to include new feedstuffs in fish diets to compensate for these shortcomings, but researchers must first accurately characterise them to fully comprehend their impacts on fish metabolism.

Known as the most abundant flavonoid in foods (Petersen et al., 2016), quercetin (QE) has been linked to healthy and functional diets (Spagnuolo et al., 2012; Bigliardi and Galati, 2013). According to previous studies, QE mostly occurs in its glycosylated form in fruits and vegetables (Wang et al., 2020), exhibiting anti-cancer antiviral and anti-bacterial properties, neuroprotective effects, and anti-inflammatory properties beneficial to human health (Wang et al., 2020). Selective sulfonation of QE has been shown to exert liver-protective effects against heavy metal and carbon tetrachloride-induced liver damage in rodents and has potential as a chemopreventive and chemotherapeutic agent for liver disease (Cui et al., 2014). Notably, compared with the parent compound QE, sodium quercetin-5′-sulfonates (QS) showed stronger biological activity than Czerwonka et al. (2020) reported. Furthermore, QS was shown to have antioxidant (Robak and Kopacz, 1989), anti-bacterial (Woznicka et al., 2013) and antitumor activities (Krol et al., 2002) and exhibited hepatoprotective activity in rodent models stimulated by heavy metals (Szelag et al., 2003; Magdalan et al., 2007; Chlebda et al., 2010; Trocha et al., 2012). However, no studies have examined the impact of QE and QS on hybrid grouper (F1 hybrid *Epinephelus fuscoguttatus*♀×*Epinephelus polyphekadion*♂) metabolism, which needs to be studied further.

Typically, new feed formulae are evaluated by determining their analytic composition and digestibility and then evaluating their influences on growth performance, food intake, and further zootechnical indices. In spite of this, these techniques may not be complete enough to understand the effects of fish feed on the metabolism of fish. Chemical analyses of fish diets give a rough idea of the composition; nonetheless, they do not offer data about the profiles of small compounds, like non-essential nutrients such as creatine and taurine and other nutrition-related factors, and several under nutrition-related factors like phytic acid, polyphenols, or mycotoxins found in plant feeds (Glencross et al., 2007; Roques et al., 2020). An extensive and expensive analysis is needed for each of these factors and compounds. In metabolomics, the global set of metabolites in a biological system is investigated in order to gain insight into the system's metabolic state. Combining feeding trials and metabolomics analyses of tissues and biofluids could shed light on nutrient effects and changes in feed intake.

Hepatic is a multifunctional organ with a high metabolic rate and biotransformation capacity, making it highly susceptible to drugs, feed, pollutants and toxins (Cuykx et al., 2018; Song et al., 2019; Zhou et al., 2019). Interestingly, hepatic is involved in the metabolism of QE and its derivatives and represents one of the essential metabolic organs (Wang et al., 2016b). Hybrid grouper is an essential marine aquaculture fish-breed developed through hybridisation technology and widely cultured in the southeast coast of China and other countries (Amenyogbe et al., 2020; Xie et al., 2021). The *in vivo* absorption, distribution, metabolism, and bioavailability of QE have been extensively studied in animal models and humans (Wang et al., 2016b). After digestion and absorption by the digestive system in vertebrates, QE forms glucuronic acid or sulfate derivatives in the liver (Wang et al., 2016b). The content and structure type of QE derivatives play a key role in their absorption and distribution (Spencer et al., 2008). Based on high performance liquid chromatography (HPLC) detection method, it was found that QE was mainly deposited in the body in the form of aglycone after being absorbed by tilapia, and the changes of QE concentration were found in plasma, liver and whole-body homogenate (Park et al., 2009). For a better insight into metabolic adjustment, serum biochemical parameters, and liver fat accumulation through

feeding additives (QE and QS) to hybrid groupers, the liver hepatic metabolic profile was examined after 56 days of feeding.

MATERIALS AND METHODS

Chemical Synthesis of QS

All chemicals and reagents are analytically pure and used without further purification. QE (97%, *w/w*) was purchased from Macklin Biochemical Co., Ltd (Shanghai, China). ^{1}H NMR and ^{13}C NMR spectra were measured at room temperature on a Bruker Avance III 400 MHz NMR spectrometer. Tetramethylsilane (TMS) served as the internal standard.

QS were prepared using the method outlined by Czerwonka et al. (2020). This method includes the following two steps: synthesis of quercetin-5′-sulfonic acid was carried out; the product of sulfonic acid was neutralised with NaOH solution to get the targeted material QS. In brief, the QE powder was added to the appropriate 98% sulfuric acid and put in an 80°C water bath for 2 h to heated with stirring. After the reaction, the mixture was cooled down to room temperature (25°C), and purified water was added while stirring continuously. The quercetin-5′-sulfonic acid precipitate was collected by filtration and then double crystalised from the saturated aqueous solution. The final product of QS was obtained by neutralising the synthesised acid in NaOH aqueous solution and double crystallising the resulting precipitate from the saturated water solution.

^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.47 (1H, s, 5-OH), 10.98 (1H, s, 4'-OH), 10.78 (1H, s, 7-OH), 9.46 (1H, s, 3-OH), 9.26 (1H, s, 3'-OH), 7.87(1H, d, H-2'), 7.62(1H, d, H-6'), 6.40 (1H, d, H-8), 6.19(1H, d, H-6).

^{13}C NMR (101 MHz, DMSO-d_6) δ: 175.84 (C-4), 163.99 (C-7), 160.76 (C-5), 156.12 (C-9), 145.96 (C-2), 145.46 (C-3'), 144.20 (C-4'), 136.10 (C-3), 131.19 (C-5'), 120.81 (C-1'), 117.76 (C-2'), 115.37 (C-6'), 103.04 (C-10), 98.22 (C-6), 93.26 (C-8).

The molecular structure of the compound is shown in **Figure 1**.

Experimental Design and Diets

Referring to the study of grouper (Ye et al., 2020; Xie et al., 2021), the ingredients and proximate composition of the primary feed are presented in **Tables 1**, **2**. We opted for QE dosage based on a previous study (Shin et al., 2010; Zhai and Liu, 2013; Xu et al., 2019). Group CT was fed a non-additive (QE or QS) diet; group QE was fed a diet containing 0.8 mmol/kg of QE; group QS was fed a diet containing 0.8 mmol/kg of QS. Before the feed was prepared, all the raw materials had to be crushed, sieved and weighed precisely according to the formula. Mixed all the powder ingredients, and then added oil and water mixed thoroughly with

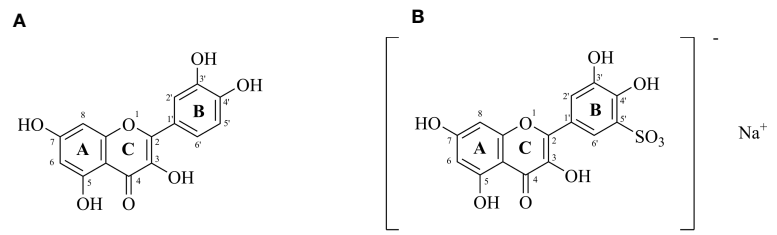

FIGURE 1 | **(A)** Structure of quercetin (3,3′,4′,5,7-pentahydroxyflavone); **(B)** Sodium Quercetin -5'-Sulfonates (sodium 3,3',4',5,7-pentahydroxyflavon-5'-sulfonate) (Czerwonka et al., 2020).

TABLE 1 | Ingredients and proximate composition analysis (on dry weight basis) of the experimental diets.

Ingredients	Percentage	Proximate composition	Percentage
Fish meal	50.00	Moisture	9.70
Vital wheat gluten	12.00	Crude protein	48.74
Wheat flour	17.47	Crude fat	10.60
Corn gluten meal	8.00	Crude ash	9.90
Fish oil	4.00		
soybean oil	1.50		
Soybean lecithin	1.50		
Microcrystalline cellulose	1.00		
Calcium monophosphate	2.00		
Choline chloride	0.50		
Ethoxyquin	0.03		
Vitamin and mineral premix	2.00		
Total	100.00		

Two kg premix contained the following: VB_1, 20.00 g; VB_2, 20.50g; VB_6, 45.00 g; VB_{12}, 0.10 g; VK_3, 4.55 g; VE, 90.00 g; VA, 10 g; VD_3, 40.00 g; nicotinic acid, 82 g; D–calcium pantothenate, 60.67 g; biotin, 2.0 g; folic acid, 6.17 g; inositol, 140.04 g; cellulose, 478.97g; ferric citrate, 14.35g; $ZnSO_4{\cdot}7H_2O$, 29.12g; $MgSO_4{\cdot}H_2O$, 12.19g; $MnSO_4{\cdot}7H_2O$, 0.11g; $CuSO_4{\cdot}5H_2O$, 18.85 g; $CoCl_2{\cdot}6H_2O$, 4.35 g; $Ca(H_2PO_4)_2{\cdot}H_2O$, 50 g; KIO_3, 0.03 g; KCl, 17.74 g; Na_2SeO_3, 2.0 g; zeolite powder, 851.26 g.

TABLE 2 | Preparation of quercetin mixture for the different additive levels used in experimental diets.

Groups	Additive levels (mmol/kg diet)	QE or QS (g/kg diet)	Microcellulose (g/kg diet)
CT	0.0	0.000	10.000
QE	0.8	0.271	9.729
QS	0.8	0.381	9.619

a groove-type mixer (CDH-100, Sichuan Chuanda Drying Technology Engineering Co., Ltd., China). In this study, dough with a diameter of 4 mm was wet extruded with a pelletiser (F-26, South China University of Technology, Guangzhou, China) and dried under air conditioning at 16°C, stored in a sealed bag at -20°C until feeding.

Experimental Conditions and Fish

Experimental fish were taken from the self-breeding and self-raising seedlings of Zhanjiang Ocean High-tech Park, Guangdong Ocean University. The fish were selected and transported with a special fish transportation vehicle to the breeding base of Guangdong Evergreen Feed Industry Co., Ltd., Zhanjiang, China. They were given the basal diet (crude protein ≥ 40%, crude fibre ≤ 5.0%, crude ash ≤ 16%, crude lipid ≥ 6%, moisture ≤ 12%, total phosphorus 0.90-1.60 and lysine ≥ 2.10) for two weeks to become accustomed to the experimental diets and farming conditions. After 24 hours of fasting, 225 fish (weighed 10.10 ± 0.02 g) were distributed evenly among 9 fibreglass tanks (500L). 25 fish per tank, 3 replicates per treatment. The experiment lasted eight weeks; fish were fed twice a day at 9:00 and 16:00 at 5 - 8% of their body weight. During this experimentation, the rearing water (flow-through natural seawater processed with sand filtration and sedimentation) was constantly aerated and maintained under the following conditions: temperature 25 - 29°C, pH 7.5 - 8.0, salinity 28.0 - 32.0g/L, dissolved oxygen 6 - 8 mg/L, and ammonia stayed 0.03 ± 0.01 mg/L. The fish were reared in a continuous 24-h ventilated flowing water culture system.

Sample Collection

Before sampling, the fish fasted for 24 hours at the end of the feeding trial and were weighed with the average fish size (54.36 ± 3.95 g); then, they were anesthetised with 8 mg/L methane-sulfonate (MS-222, Sigma-Aldrich). Blood samples were collected from the caudal vein of fish (n = 6 per tank) randomly selected from each tank and pooled together. The collected blood (approximately 0.7 mL per fish) was transferred into 1.5 mL anticoagulant-free centrifuge tubes and then stored at 4°C for 12 h. After centrifugation at 3500 rpm and 4°C for 15 min, the separated serum was obtained and immediately stored at -80°C.

Eighteen individuals of hybrid grouper were randomly selected for each treatment to collect livers for metabolomic analysis. Six (n = 6 per tank) liver samples from each replicate tank were pooled together in two tubes each. For histological analysis (Oil red O staining in the liver), the livers of two fishes from each tank were sampled. We immediately froze liver tissues in liquid nitrogen and stored them at -80°C. During sample collection, low temperatures and rapidity were maintained.

Biochemical Parameters in Serum

Cryopreserved serum samples were thawed on ice. The serum total protein (TP, A045-4), albumin (ALB, A028-2-1), total cholesterol (T-CHO, A111-1-1), triglyceride (TG, A110-1-1), alkaline phosphatase (AKP, A059-2), glucose (GLU, A154-1-1), low-density lipoprotein cholesterol (LDL, A113-1-1), high-density lipoprotein cholesterol (HDL, A112-1-1), aspartate aminotransferase (AST, C010-2-1) and alanine aminotransferase (ALT, C009-2-1) levels were measured using the methods described in the detection kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) through the use of a VICTOR Nivo Multimode Microplate Reader (PerkinElmer).

Oil Red O Staining

The trimmed liver tissue was embedded with an embedding agent (OCT) and then placed in a frozen sectioning machine for cryofixation. The sectioning was started after waiting for solidification. Frozen sections were stained in fresh oil red O staining solution for 8-10 min at room temperature, rinsed in tap water, and counterstained with hematoxylin until the desired degree of staining (Servicebio, China). Finally, glycerol jelly mounting medium was added to the sections, and the slides were sealed with cover glass. Lipid droplets were examined with the Nikon Eclipse microscope (Nikon, Japan). Quantification of the relative area of oil red O solution staining was performed using Image-Pro Plus 6.0 software. Six images of each sample were randomly selected, and the average relative area of Oil Red O staining was calculated to determine the relative content of lipid droplets.

Metabolomics Analysis

Extraction and Preparation of Samples

Hepatic samples were thawed on ice. Take 50 ± 2 mg of one sample, add cold steel balls to the mixture, and homogenate at 30 Hz for 3 min. Add 1 mL 70% methanol with internal standard extract to the homogenised centrifuge tube, whirl the mixture for 5 min, and then centrifuge it with 12,000 rpm at 4°C for 10 min. After centrifugation, draw 400 uL of supernatant into the corresponding EP tube and store in a -20°C refrigerator overnight, centrifuge at 12000 r/min at 4°C for 3 min, and take 200 uL of supernatant in the liner of the corresponding injection bottle for on-board analysis.

HPLC Conditions

Liver sample extracts were analyzed using an LC-ESI-MS/MS system (UPLC, ExionLC AD, https://sciex.com.cn/; MS, QTRAP® System, https://sciex.com/).The analytical conditions

were as follows (Zhang et al., 2021), UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 mm*100 mm); column temperature, 40°C; flow rate, 0.4 mL/min; injection volume, 2μL; solvent system, water (0.1% formic acid): acetonitrile (0.1% formic acid); gradient program, 95:5 V/V at 0 min, 10:90 V/V at 11.0 min, 10:90 V/V at 12.0 min, 95:5 V/V at 12.1 min, 95:5 V/V at 14.0 min.

ESI-QTRAP-MS/MS

LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP), QTRAP® LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (Sciex). The ESI source operation parameters were as follows (Li et al., 2022): source temperature 500°C; ion spray voltage (IS) 5500 V (positive), -4500 V (negative); ion source gas I (GSI), gas II (GSII), curtain gas (CUR) were set at 55, 60, and 25.0 psi, respectively; the collision gas (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

Data Processing and Statistical Analysis

MS data acquisition and processing were performed previously described (Chen et al., 2013). Metabolites were annotated using the Metware in-house MS2 spectral tag (MS2T) library (Wuhan Metware Biotechnology Co., Ltd.; http://www.metware.cn, Wuhan, China). Differential variables of the metabolites were analysed using orthogonal partial least squares-discriminant analysis (OPLS-DA). VIP ≥ 1 with fold change ≥ 2 or fold change ≤ 0.5 was considered statistically significant. The results of the biochemical indexes were presented as the mean ± standard error (SEM). The experimental data were subjected to one-way ANOVA. Multiple treatment groups were compared by Duncan's honestly significant difference test at $P \leq 0.05$. Statistical analysis of all data was performed using SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Serum Biochemical Indices

Table 3 presents the effects of different diets on serum biochemical parameters in grouper. The result shows that TP, ALB, AKP and GLU were not significantly different among the three groups ($P > 0.05$). T-CHO, TG and ALT of fish fed QE and QS were significantly lower than those of fish fed CT ($P < 0.05$). LDL and AST of fish fed QE were significantly lower than that of fish fed CT and QS ($P < 0.05$). The HDL of fish fed QE and QS was significantly higher than those fish fed CT ($P < 0.05$).

Lipid Accumulation in the Liver of Fish Fed Different Diets

For the comparison of lipid accumulation in the liver, oil red O staining was performed, shown in **Figure 2**. Lipid droplets and nuclei were stained red and blue for oil red O staining, respectively. The CT group showed more red spots and lipid droplets than the QE and QS groups in the present study. Compared to the CT group (25.67%), lipid accumulation in the QE and QS groups decreased significantly to 19.55% and 19.32%, respectively ($P < 0.05$).

Differential Metabolites in the Liver in Response to QE Treatment

To investigate changes in liver metabolites in QE-treated hybrid grouper, livers from the CT and QE groups were collected for an extensive targeted metabolomic analysis. OPLS-DA model was used to determine metabolite differences between the CT and QE groups. OPLS-DA combines orthogonal signal correction (OSC) and PLS-DA methods to screen for differential variables by removing uncorrelated differences, allowing further indication of differences between groups. OPLS-DA scores clearly classify samples into distinct clusters, indicating significant differences in metabolites between the CT and QE groups. The number of differential metabolites is clearly shown in the OPLS-DA S plot. A total of 91 differential metabolites (**Supplementary Table S1**) were detected in the CT and QE groups (VIP ≥ 1, fold change ≥ 2 or ≤ 0.5). After qualitative and quantitative analysis of the detected differential metabolites, the results of the metabolites with the top-ranked changes are presented in **Figure 3C**.

TABLE 3 | Serum biochemical indices of hybrid grouper fed with experimental diets.

Parameters	Experimental diets		
	CT	QE	QS
TP (g L^{-1})	30.03 ± 0.78	30.28 ± 1.18	30.55 ± 0.73
ALB (g L^{-1})	5.67 ± 0.27	5.67 ± 0.36	5.72 ± 0.28
T-CHO (mmol L^{-1})	3.49 ± 0.16^{a}	2.66 ± 0.08^{b}	2.71 ± 0.10^{b}
TG (mmol L^{-1})	0.37 ± 0.03^{a}	0.24 ± 0.03^{b}	0.26 ± 0.02^{b}
AKP (U L^{-1})	40.65 ± 4.84	40.77 ± 1.90	53.05 ± 5.51
GLU (mmol L^{-1})	4.85 ± 0.50	4.43 ± 0.28	4.84 ± 0.15
LDL (mmol L^{-1})	0.65 ± 0.02^{a}	0.35 ± 0.03^{b}	0.57 ± 0.01^{a}
HDL (mmol L^{-1})	2.35 ± 0.15^{b}	3.54 ± 0.11^{a}	3.49 ± 0.19^{a}
AST (U L^{-1})	73.06 ± 1.03^{a}	50.57 ± 3.10^{b}	65.86 ± 4.82^{a}
ALT (U L^{-1})	165.26 ± 8.32^{a}	133.84 ± 2.61^{b}	138.10 ± 3.36^{b}

Values with no letter or the same letter superscripts mean no significant difference (P > 0.05), while with different small letter superscripts mean significant difference (P < 0.05).

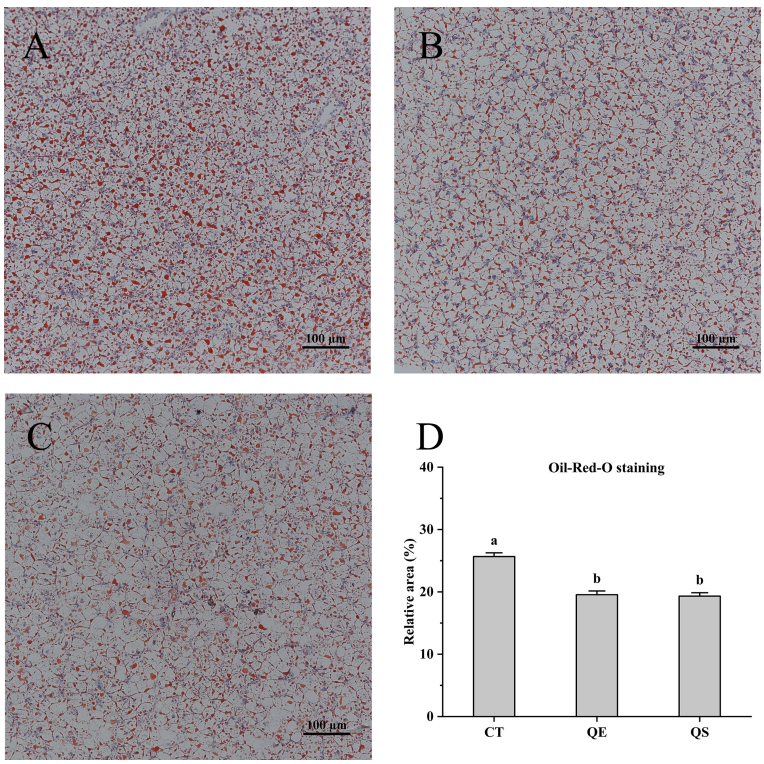

FIGURE 2 | Hepatocyte oil red O (ORO) staining section of hybrid grouper treated with different diets (Magnification × 20). **(A)** CT; **(B)** QE; **(C)** QS; **(D)** The relative area of red lipid droplets in the hepatic section area (%). Values were presented as means ± SEM, and bars with different letters are significantly different ($P < 0.05$).

Differential metabolites from CT vs. QE could be divided into 11 categories, mainly from fatty acyl groups (FA; 42.86%) and amino acids and their metabolites (21.98%). Oxylipins accounted for 71.05% of FA.

Table 4 classifies 28 different oxylipins, with the most derived from arachidonic acid (ARA, 35.71%), docosahexaenoic acid (DHA, 21.43%) derived oxylipins ranking second, and linoleic acid (LA, 14.29%), alpha-linolenic acid (ALA, 10.71%) and eicosapentaenoic acid (EPA, 10.71%) derived oxylipins as follows. Further KEGG analysis showed that arachidonic acid metabolism, inflammatory mediator regulation of TRP channels and linoleic acid metabolism were significantly enriched after QE supplementation (**Figure 3D**).

Effect of QS on Metabolites in the Liver of Hybrid Grouper

To further investigate the effect of QS on the liver metabolites of hybrid grouper, liver tissues from the QS group were also analysed. A total of 83 differential metabolites (**Supplementary Table S2**) were identified (VIP ≥ 1, fold change ≥ 2 or ≤ 0.5), and the results of OPLS-DA and OPLS-DA S plots are shown in **Figures 4A**, **4B**. Among the differential metabolite, 11β-Prostaglandin E2 (11β-PGE2) was the top metabolite that increased the most (**Figure 4C**). The total differential metabolites CT vs. QS could be divided into 10 main categories, of which 42.17% belonged to FA and 36.14% to amino acids and their metabolites. Among the 35 FA species, 29 belonged to oxylipins.

Table 5 classifies 29 different oxylipins, of which the most derived from ARA (31.03%), DHA (27.59%) derived oxylipins ranked second, and LA (13.79%), ALA (10.34%) and EPA (10.34%) derived oxylipins were as follows. Further KEGG analysis showed that arachidonic acid metabolism, inflammatory mediator regulation of TRP channels and linoleic acid metabolism were significantly enriched after QE supplementation (**Figure 4D**).

DISCUSSION

Changes in serum biochemical indicators reflect the physiological and metabolic status of fish, which is helpful for us to determine the response of fish to dietary supplements (Tan et al., 2018; Xie et al., 2021). In general, if lipid parameters including TG, T-CHO, and LDL increase and HDL in fish decreases, is an indication that fish may have some lipid metabolic disorders and liver damage (Zhai and Liu, 2013). In our results, QE and QS decreased serum TG, T-CHO, LDL levels and increased HDL levels in hybrid grouper. Consistent with our findings, Shin et al. (2010) reported that feeding diets supplemented with 0.25% or 0.50% QE significantly reduced T-CHO levels in flounder, with 60 days of feeding better than 30

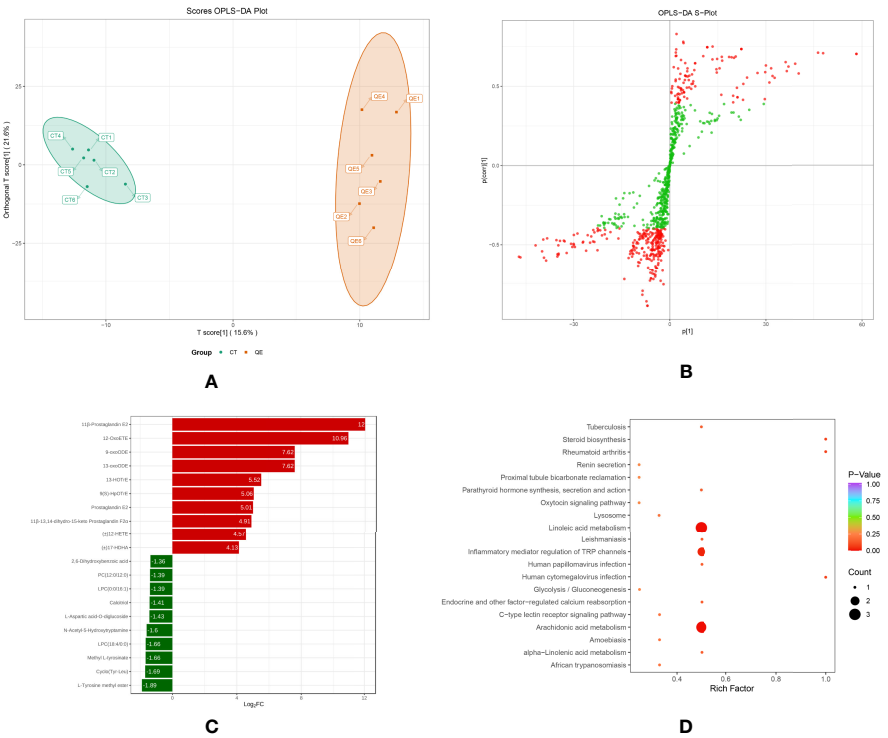

FIGURE 3 | Effect of QE on oxylipins in the liver of hybrid grouper. **(A)** The OPLS-DA scores of the CT group (green) and QE group (red). **(B)** The OPLS-DA S-plot. The X-axis indicates the correlation coefficient between the principal components and oxylipins, and the Y-axis indicates the correlation coefficient between the principal components and oxylipins. The red dot represents VIP ≥ 1, and the green dot represents VIP ≤ 1. **(C)** Bar chart showing the differential oxylipins in the CT group (green) and QE group (red). **(D)** Differential oxylipins enrich KEGG pathways in the CT group and QE group. (VIP ≥ 1, fold change ≥ 2 or ≤ 0.5, n = 6).

days. Repeated use significantly increases the bioavailability of QE (Rangel-Ordonez et al., 2010). Zhai and Liu (2013) indicated that QE could decrease serum TG levels and increase HDL level in tilapia. In addition, QE has a modulating effect on low-density lipoprotein receptor expression (Moon et al., 2012). It reduces blood lipids by increasing the rate of LDL clearance from the blood. Similar results with QE for lowering serum or liver lipids have been reported in rats (Padma et al., 2012), chickens (Qureshi et al., 2011), and rabbits (Kamada et al., 2005), and humans (Egert et al., 2010). In this study, the sulfonated product QS obtained consistent results with QE, indicating that QE and QS showed potential activity in lowering cholesterol and triglycerides and helping to avoid pathological changes in the fatty liver.

QE has preventive and therapeutic effects on hepatic damage, such as fatty liver, cirrhosis and liver fibrosis (Miltonprabu et al., 2017). Cui et al. (2014) revealed that QE significantly reduced the activity of serum AST and ALT in rats. AST and ALT plays an important role in the liver's metabolic function. Increased ALT enzyme activity is an indicator of the degree of liver cell membrane damage, and increased AST level is another indicator of liver mitochondrial damage (Xu et al., 2010; Yang et al., 2011). In this study, QE and QS significantly reduced the levels of AST and ALT in the serum of hybrid grouper and had the effect on protecting the liver. On this basis, it is suggested that both QE and QS can stabilise the liver cell membrane and have a protective effect on mitochondria. Cui et al. (2014) believe that QE and quercetin-5',8-disulfonate (QDS) effectively attenuate the increase of AST and ALT in the serum of mice induced by carbon tetrachloride, and the selective sulfonation of QE increases the hepatoprotection effect. Water-soluble QDS was more effective than QE in reducing AST and ALT release.

In contrast, QE was more potent than QS in our results, probably due to the high dose of QS. Remarkably, Significant increases in markers of hepatotoxicity (AST and ALT) were found in mice injected with high doses of QE (1500 and 2000 mg/kg), mediated in part through oxidative stress (Singh et al., 2022). Related findings were found in acute doses of apigenin (Singh et al., 2012) and genistein (flavonoids) (Singh et al., 2014). From a molecular point of view, the toxic effects of QE are likely related to the oxidation of QE to potentially toxic products during ROS scavenging, namely quercetin-quinine, which is highly reactive with thiols, and GSH might be the principal reactant (Galati et al., 2001; Awad et al., 2002; Boots et al., 2007).

In fish, the liver is the primary site for lipid synthesis and storage (Martin et al., 2017; Zhou et al., 2019). Lipid droplets (LDs) are intracellular organelles dedicated to storing energy in the form of neutral lipids and are involved in lipid metabolism (Welte and Gould, 2017). According to previous studies, QE treatment attenuate inflammation and fibrosis in mice

TABLE 4 | Significant differences in oxylipins between the CT and QE groups.

Compounds	Class II	VIP	Fold Change	Log2FC
(±)15-HETE	ARA	1.55	2.04	1.03
(±)12-HETE	ARA	1.74	23.70	4.57
(±)16-HETE	ARA	1.89	5.00	2.32
(±)17-HETE	ARA	1.89	5.00	2.32
(±)18-HETE	ARA	1.89	5.00	2.32
12-OxoETE	ARA	1.80	1991.47	10.96
11β-Prostaglandin E2	ARA	1.79	4100.47	12.00
11β-13,14-dihydro-15-keto Prostaglandin F2α	ARA	1.55	30.10	4.91
14,15-Leukotriene C4 (ExC4)	ARA	1.11	4.32	2.11
15-deoxy-δ-12,14-PGJ2	ARA	1.51	8.09	3.02
(±)17-HDHA	DHA	1.05	17.52	4.13
(±)4-HDHA	DHA	1.22	3.01	1.59
14 (S)-HDHA	DHA	1.72	14.62	3.87
10-HDoHE	DHA	1.75	3.82	1.93
8-HdoHE	DHA	1.40	12.02	3.59
11-HdoHE	DHA	1.73	14.92	3.90
13-oxoODE	LA	1.78	197.23	7.62
9-oxoODE	LA	1.78	197.23	7.62
13®-HODE	LA	1.74	14.08	3.82
9 (S),12 (S),13 (S)-TriHOME	LA	1.70	10.34	3.37
13-HOTrE	ALA	1.60	45.84	5.52
9 (S)-HpOTrE	ALA	1.43	33.29	5.06
9 (S)-HOTrE	ALA	1.90	6.28	2.65
(±)12-HEPE	EPA	1.86	16.65	4.06
(±)15-HEPE	EPA	1.86	16.65	4.06
(±)18-HEPE	EPA	1.86	16.65	4.06
11-HEDE	Uncategorized	1.63	2.83	1.50
15-HEDE	Uncategorized	1.63	2.83	1.50

nonalcoholic fatty liver disease (NAFLD), alleviate hepatic fat accumulation (Marcolin et al., 2012; Liu et al., 2018). Thus, the current study also observed the lipid accumulation status in hepatic by oil red sections. ORO results show that QE and QS treatments significantly reduced the lipid droplet area in the liver of hybrid grouper, indicating that QE and QS helped to reduce liver fat accumulation. Derivatives of QE have also revealed consistent results in *in vitro* and *in vivo* research (Yu et al., 2016; Qin et al., 2018). Amelioration of fat deposition by QE and its derivatives was probably related to the regulation of adipogenic gene expression and antioxidant function (Jung et al., 2013; Wang et al., 2016a; Liu et al., 2018).

This study provides basic metabolic profiling data for studying the marine fish liver response to QE supplementation by ultra-performance liquid chromatography-electrospray ionization triple quadrupole mass spectrometry (UPLC-QTRAP/MS/MS). These data increased our understanding of QE. Indeed, a study has explored the metabolism of QE (Wang et al., 2016b). On this basis, it has been suggested that high phenolic intake increases intestinal hippurate production (Mullen et al., 2008; de Mello et al., 2017; Ulaszewska et al., 2020). However, only dogs produce hippuric acid primarily in the kidneys; in other species, such as mice and rats, hippuric acid production depends on hepatocytes (Toromanovic et al., 2008; Lees et al., 2013). In particular, most QE-derived metabolites are identified as 3-hydroxyphenylacetic acid, benzoic acid and hippuric acid (Mullen et al., 2008). In this experiment, hepatic hippuric acid was significantly increased after dietary QE and QS

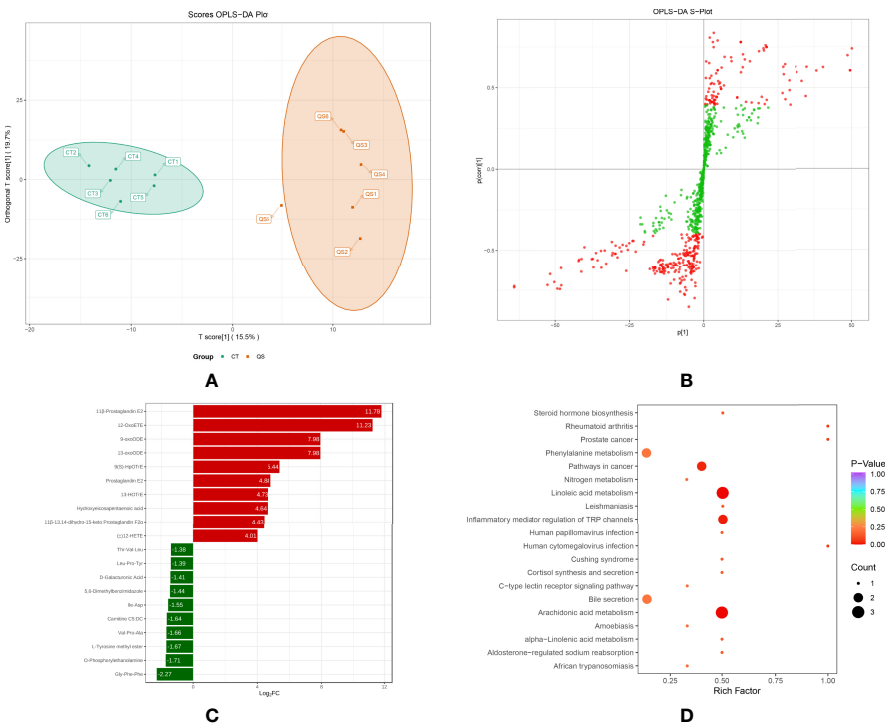

FIGURE 4 | Effect of QS on oxylipins in the liver of hybrid grouper. **(A)** OPLS-DA scores **(B)** OPLS-DA S-plot. **(C)** Bar chart showing the differential oxylipins in the CT group (green) and QS group (red). **(D)** Differential oxylipins enriched KEGG pathways in the CT group and QS group. (VIP ≥ 1, fold change ≥ 2 or ≤ 0.5, n = 6).

TABLE 5 | Significant differences in oxylipins between the CT and QS groups.

Compounds	Class II	VIP	Fold Change	Log2FC
(±)15-HETE	ARA	1.75	2.50	1.32
(±)12-HETE	ARA	1.93	16.13	4.01
(±)16-HETE	ARA	1.98	4.91	2.29
(±)17-HETE	ARA	1.98	4.91	2.29
(±)18-HETE	ARA	1.98	4.91	2.29
12-OxoETE	ARA	1.53	2398.98	11.23
11β-Prostaglandin E2	ARA	1.88	3517.59	11.78
11β-13,14-dihydro-15-keto Prostaglandin F2α	ARA	1.60	21.52	4.43
15-deoxy-δ-12,14-PGJ2	ARA	1.37	12.31	3.62
(±)17-HDHA	DHA	1.48	12.97	3.70
(±)4-HDHA	DHA	1.31	2.66	1.41
14 (S)-HDHA	DHA	1.85	11.65	3.54
10-HdoHE	DHA	1.74	3.08	1.62
8-HdoHE	DHA	1.64	11.22	3.49
11-HdoHE	DHA	1.88	12.92	3.69
16-HdoHE	DHA	1.02	2.14	1.10
20-HdoHE	DHA	1.03	3.78	1.92
13-oxoODE	LA	1.54	252.99	7.98
9-oxoODE	LA	1.54	252.99	7.98
13®-HODE	LA	1.71	9.99	3.32
9 (S),12 (S),13 (S)-TriHOME	LA	1.51	9.47	3.24
13-HOTrE	ALA	1.60	26.49	4.73
9 (S)-HpOTrE	ALA	1.59	43.52	5.44
9 (S)-HOTrE	ALA	1.68	3.43	1.78
(±)12-HEPE	EPA	1.90	11.80	3.56
(±)15-HEPE	EPA	1.90	11.80	3.56
(±)18-HEPE	EPA	1.90	11.80	3.56
11-HEDE	Uncategorized	1.25	2.50	1.32
15-HEDE	Uncategorized	1.25	2.50	1.32

(**Tables S1, S2**), indicating that the liver is directly or indirectly involved in the metabolism of QE and QS in fish.

Analysing the metabolomic data, supplementation with QE and QS had the greatest effect on oxylipins metabolites *in vivo*. Oxylipins are a class of bioactive lipids that play important roles in regulating the intensity and duration of inflammatory responses as well as tissue repair, blood coagulation, vascular permeability and energy regulation (Dennis and Norris, 2015). Therefore, we focused on further analysis of oxylipins in FA. Oxylipins are oxidised PUFAs; depending on the source of their parent PUFAs, oxylipins can be divided into two categories: oxylipins derived from omega-6 PUFAs, such as ARA and LA; and those derived from omega-3 PUFAs, such as α-Linolenic acid (18:3n-3) (ALA), eicosapentaenoic acid (20:5n-3) (EPA) and docosahexaenoic acid (22:6n-3) (DHA) (Gabbs et al., 2015; Picklo and Newman, 2015). Studies showed that oxylipins are rapidly produced and eliminated in the body, and the detectable oxylipins represent their biologically active form (Shaik et al., 2013; Barquissau et al., 2017). A large number of oxylipins were detected in this experiment, indicating that the basic physiological reactions requiring the participation of oxylipins may have been going on in the fish liver all the time.

ARA-derived oxylipins are more diverse and are thought to play important roles in most tissues and organs (Leng et al., 2018; Gao et al., 2021). In the present study, 28 and 29 oxylipins were found in the livers of QE and QS, respectively, most of which were ARA-derived (**Tables 4**, **5**). KEGG annotation also showed that ARA metabolism was mainly enriched (**Figures 3**, **4**), suggesting that QE and QS may be involved in the body's basal metabolism by regulating ARA levels. Studies on oxylipins in fish are relatively scarce; however, a recent study found 57 targeted lipids of five major n-6 and n-3 PUFAs in the plasma of large yellow croaker and rainbow trout, approximately 42.1% from ARA (Gao et al., 2021). In addition, Leng et al. (2018) identified the 70 oxylipins quantified in the rat liver, about two-thirds of the oxylipins were derived from n-6 PUFAs, and ARA-derived oxylipins accounted for about two-thirds of them. Of the 87 oxylipins present in the rat brain, half (51%) were derived from ARA, accounting for 81-90% of the total oxylipins mass (Ferdouse et al., 2019b). It is speculated that the predominant oxylipins in animals may be roughly similar even with different dietary conditions; that is, ARA-type oxylipins generally have more types and qualities, suggesting that they may play an important role in life activities.

Oxylipins show a wide range of functions, most of which are still being discovered. Interestingly, oxylipins obtained from different PUFA substrates and pathways can have similar or opposite effects so it is important to fully identify the entire oxylipins profile to properly understand their overall biological effects (Deline et al., 2015). In this study, in addition to ARA, EPA and DHA were also significantly increased after QE and QS treatment. Studies have shown that n-3 EPA and DHA have antithrombotic and antiarrhythmic properties (Endo and Arita, 2016; Lee et al., 2016a). In addition, the n-3 fatty acid EPA has a

strong anti-inflammatory effect and can affect the T cell response to infection (Wu and Meydani, 1998; Harris et al., 2000). Based on the significant health benefits of EPA and DHA, many studies have begun using EPA and DHA dietary supplements to increase the content of n-3 PUFA oxylipins. Dietary supplements EPA and DHA increased anti-inflammatory n-3 LC-PUFA oxylipins and decreased pro-inflammatory n-6 oxylipins in plasma, liver, kidney, heart, brain, and other tissues (Leng et al., 2018; Ferdouse et al., 2019a; Rey et al., 2019; Kutzner et al., 2020). As a low-toxic natural flavonoid, QE will find more meaningful value and far-reaching effects if further studies on ARA, EPA, and DHA respond to treatments.

Furthermore, prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α) have inhibitory effects on adipogenesis and can inhibit the early differentiation of adipocytes (Stewart and Fisher, 2015; Barquissau et al., 2017). In the study, PGE2 was significantly increased after QE and QS treatment (**Table S1, S2**). Increased synthesis and release of PGE2 and PGF2α activate prostaglandin membrane receptors that bind to PGE2 and PGF2α, resulting in sustained calcium oscillations that inhibit the expression of PPARγ target genes, including the uncoupling protein 1 (UCP1) gene, and ultimately inhibit the white adipose tissue browning (Stewart and Fisher, 2015). In this study, 11β-PGE2 was significantly increased after treatment, presumably related to the significant increase in PGE2. Due to its properties, including activation of endogenous stem cells, immunoregulation, and angiogenesis, PGE2 plays a vital role in regenerating various organ systems following injury. As a result, PGE2 can help to reduce inflammation, fibrosis, necrosis, and other adverse effects of liver ischemia or reperfusion injury (Cheng et al., 2021). There is convincing evidence *in vitro* and *in vivo* that PGE2 signalling pathways protect various organs from inflammation, oxidative stress, and fibrosis. Hence the present study suggests that QE and QS help to increase the PGE2 production, which could be beneficial to the hybrid grouper, but further studies are needed to elucidate the exact role.

CONCLUSION

In conclusion, UPLC-QTRAP/MS/MS study of hybrid grouper liver samples brought to light universal information concerning the metabolic metabolism of the liver in response to nutritional additives QE and QS. In addition to providing new ideas for studying feeding additives, these data confirmed the existing research results. This study showed that QE and QS effectively increase hypolipidemic and hepatoprotective compounds that are known for reducing blood lipid levels and liver fat accumulation in groupers. Additionally, we found that QE and QS altered oxylipin levels in the liver of grouper fish, suggesting that the QE and QS are partly mediated through the regulation of oxylipins. There are similarities in the biological effects of quercetin and its sulfonated derivatives. It is believed that sulfation in the structural transformation of flavonoids may provide a novel mechanism to regulate their bioavailability and bioactivity, and further research is needed to evaluate the mechanism underlying the hepatoprotective effects of QE and QS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was conducted following the regulations for the administration of laboratory animals in Guangdong province, China, reviewed and approved by the Guangdong Ocean University Research Council for the care and use of laboratory animals (approval number: GDOU-LAE-2021-021).

AUTHOR CONTRIBUTIONS

JL was involved in project administration, data collection, formal analysis, processing, and writing of the original draft. EA was involved in writing – original draft and writing – reviewing and editing. J-SH supervision and involvement in resources. GC: conceptualised, involved in methodology, acquired funding, supervision and involved in resources. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Development Program of China, Blue Granary Technology Innovation Key Special Project (2020YFD0900200) and Guangdong Provincial Science and Technology program (2016B0201009).

ACKNOWLEDGMENTS

Our acknowledgement goes to all the founders of this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.891080/full#supplementary-material

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