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Metabolic disorder induces fatty liver in Japanese seabass, *Lateolabrax japonicas* fed a full plant protein diet and regulated by cAMP-JNK/NF-kB-caspase signal pathway

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ABSTRACT

A 10-week growth trial was conducted to investigate the effects of replacing dietary fishmeal with plant proteins on nutrition metabolism, immunity, inflammation and apoptosis responses in liver tissues of Japanese seabass, *Lateolabrax japonicas* (initial body weight = 10.42 ± 0.01 g). Two isonitrogenous and isoenergetic diets were formulated. A basal diet containing 54% fishmeal (FM), whereas another diet was prepared by totally replacing FM with a plant protein blend (PP) composed with soybean protein concentrate and cottonseed protein concentrate. Although essential amino acids, fatty acids, and available phosphorus had been balanced according to the FM diet profile, the significantly lower growth performance, metabolic disorder, and fatty liver symptom were observed in the PP group. Compared with the FM group, fish in the PP group showed significantly lower plasma free EAA level and PPV. Glucose metabolism disorder was expressed as the uncontrollable fasting glycolysis and pyruvate aerobic oxidation at postprandial 24 h with significantly up-regulated GK, PK and PDH genes expression, which potentially over-produced acetyl-CoA as the substrate for protein and lipid synthesis. Significantly reduced plasma GLU, but increased GC level, along with very significantly reduced liver GLY storage could be observed in the PP group. Plasma TG and hepatic NEFA contents were significantly decreased, but the hepatic TC content was very significantly increased in the PP group, in addition, hepatocyte vacuolation appeared. The significantly up-regulated cholesterol synthesis gene (HMGCR) expression but down-regulated bile acid synthesis gene (CYP7A1) expression could be the main reason for the fatty liver induced by cholesterol accumulation. The reduced plasma IgM content accompanied by the up-regulated mRNA levels of pro-inflammatory cytokines (TNFα and IL1β) and activated apoptosis signals of liver tissues were found in the PP group. The hyperthyroidism (higher plasma T3 and T4) and the accelerated energy metabolism rate decreased the growth performance in the PP group. The activated p65NF-kB may promote the hepatocytes apoptosis via the extrinsic pathway (caspase8/caspase3). Simultaneously, a "self-saving" response could be observed that activated cAMP promoted the lipolysis/β-oxidation process and up-regulated gene expression of anti-inflammatory cytokine IL10 via promoting CREB expression, further inhibited the over-phosphorylation of JNK protein, which might impede the intrinsic apoptosis pathway (caspase9/caspase3). In conclusion, the nutrient and energy metabolic disorder induced fatty liver related to the cholesterol accumulation in Japanese seabass fed full PP diet, which was under the regulation by cAMP-JNK/NF-kB-caspase signaling pathway. The hemostasis phosphorylation of JNK protein protected the liver tissues from more serious damage.

1. Introduction

With the rapid expansion of global aquaculture production, the demand for fish meal (FM) has increased because of its ideal nutritional quality of fish feeds [1]. However, the resource of fishmeal is limited and the price is rising steadily. Finding an alternative protein source has become an inevitable requirement for sustainable aquaculture [2]. Plant proteins (PP) have been used as the alternative protein sources because of relatively abundant supplementation and lower price. Although full plant protein diets had been successfully utilized in some

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omnivorous and herbivorous fish species [3–5], and even can be accepted by several carnivorous fish [6–9], it is much more sensitive for most carnivorous species to the negative factors of PP, including poor palatability, essential nutrients deficiency and anti-nutritional factors, etc. [10,11].

Japanese seabass (*Lateolabrax japonicus*) is a euryhaline fish species and is widely cultured in China and Southeast Asia [1]. Ai et al. suggested that the diet containing 41% protein and 12% lipid with P/E of 25.9 mg protein/kJ is optimal for Japanese seabass [12]. But as a carnivorous fish species, it is fishmeal-reliant and less likely to accept PP than herbivorous or omnivorous species [13]. Previous studies in our laboratory found that the anorexia, higher mortality and poor growth performance were shown in Japanese seabass responding to a high PP diet [14,15]. However, it could adapt to the PP diet during 4-8w, according to the feeding history during acclimation stage before the feeding trial [14,15]. The longest adaptation time required for 8w was observed in Liang et al., in which fish was fed trash fish before the feeding trial [14], and then Liang et al. found at most 4w was required when fish was fed the high FM diet during the acclimation stage [15]. In the recent study, we observed that Japanese seabass could well accept a PP diet in 1w when the fish was pre-fed a low FM (25% FM) commercial diet on the farm. Therefore, in the present study, the nutrient metabolism and related health status of fish are concerned when Japanese seabass could fully intake a PP diet.

Regarding fish immune dysfunction problems attributed to plant protein diets, most studies were focused on intestinal health by rupturing the mechanical, chemical, biological and the immune barriers of the intestine [16,17]. However, the liver represents a frontline organ critically involved in the regulation of metabolic processes, hormone production, detoxification, and immunological responses [18,19]. The hepatobiliary disease has become a stubborn problem plaguing intensive fish culture, which was often related to non-integrated nutrition in commercial feed [19]. Compared with FM, the limiting factors of PP can induce metabolic disorder, chronic inflammation, apoptosis and immunity inhibition of fish and finally, lead to the liver damage [20–22]. Fatty liver could be performed with triglyceride (TG), non-esterified fatty acid (NEFA) and cholesterol accumulation, which had been reported on Atlantic salmon [22], European seabass [9], and an omnivorous species, Hybrid tilapia [21]. c-Jun N-terminal protein kinase (JNK) is a subfamily of the mitogen-activated protein kinase (MAPK) superfamily [23]. JNK is a key regulator of many cellular events, including injury in toxic, metabolic, immune and cell death (apoptosis) [24]. Prolonged activation of JNK could promote apoptosis, and the activity of JNK itself is tightly controlled by other intracellular signaling pathways, such as cAMP and NF-kB signaling pathway, which play the key roles in interacting with the JNK cascades [25,26]. As a key second messenger molecule, cAMP regulates various cellular functions including lipid metabolism, inflammation, cell differentiation by affecting downstream genes function [25]. While transcription factor NF-kB is a regulator of cell apoptosis, immunity and could be acting as a "break" to the JNK activation [26].

Until now, little work has been conducted to map the regulatory mechanisms related to the liver immunological responses of fish fed a full PP diet. The objectives of the present study were to investigate growth performance, liver health and the underlying metabolism regulation mechanism in Japanese seabass with normally ingested FM or PP diets.

2. Materials and methods

During the feeding period, the experimental fish were maintained in compliance with the Laboratory Animal Welfare Guidelines of China (Decree No. 2 of Ministry of Science and Technology, issued in 1988).

Table 1
Formulation and compositions of experimental diets (g/kg).

Ingredients	FM	PP
Fish meal[1]	540	
Wheat gluten	180	180
Cottonseed protein concentrate		382
Soybean protein concentrate		230
Fish oil	30	50
Soybean oil	20	40
Wheat middling	100	
Tapioca flour	30	30
Microcrystalline cellulose	51	
$Ca(H_2PO_4)_2$	5	22
L-Lysine HCL (78%) [2]		12
DL-Met (98%)[2]		6
L-Thr (98%) [2]		4
Lecithin oil	20	20
Yeast extract	10	10
Vitamin and mineral premix[3]	14	14
Total	1000	1000
Nutrients compositions (g/kg, in dry matter basis) and feed hardness		
Crude protein	407.9	406.0
Crude lipid	117.7	116.5
Crude ash	98.9	69.0
Crude fiber	17.8	31.5
Moisture	95.9	103.6
Nitrogen free extract[4]	261.8	273.4
Starch	143.0	108.0
Available phosphorus, AP[5]	9.0	9.0
Gross energy(MJ/kg)	22.6	22.3
Feed hardness(N)	17.96 ± 1.60[a]	30.18 ± 1.60[b]

[1] Fishmeal (crude protein content was 68.8%) and fish oil were supplied by Triple Nine Fish Product Co., Esbjerg, Denmark; CPC (crude protein content was 61.5%, low free gossypol and raffinose) was supplied by Sino-Leader Biotech Co. Ltd. Beijing, China; SPC (crude protein content was 65.2%) and soy oil were supplied by Yihai Kerry Investment Co. Ltd. Shandong, China.
[2] The addition levels of CAA (*L*-Lys-HCl, *DL*-Met and *L*-Thr) were calculated by the ideal AA model.
[3] Vitamin premix (mg/kg diet): Vitamin A 20; Vitamin D3 10; Vitamin K3 20; Vitamin E 400; Vitamin B1 10; Vitamin B2 15; Vitamin B6 15; Vitamin B12 (1%) 8; Vitamin C 1000; Niacinaminde 100; Calcium pantothenate 40; Biotin (2%) 2; Folic acid 10; Inositol 200; Choline chloride (50%) 4000; Corn gluten meal 150; Mineral premix (mg/kg diet): $CuSO_4 \cdot 5H_2O$ 10; $FeSO_4 \cdot H_2O$ 300; $ZnSO_4 \cdot H_2O$ 200; $MnSO_4 \cdot H_2O$ 100; KI (10%) 80; $CoCl_2 \cdot 6H_2O$(10%Co) 5; Na_2SeO_3 (10% Se) 10; NaCl 100; Zeolite 695; Others (mg/kg diet): $MgSO_4 \cdot 5H_2O$ 2000; Antioxidant 200; Zeolite 4300.
Nitrogen free extract = 100- (moisture + crude protein + crude lipid + crude ash + crude fiber).
[5] AP was calculated according to the ingredients digestibility database on Japanese seabass in our lab (unpublished data).
[6] Antinutritional factors, including free gossypol (mg/kg), trypsin inhibitor (ng/mL) and β-conglycinin (ng/mL) didn't reach the limits of detection in PP diet.
[7] The letters "a" and "b" indicate a significant difference ($P < 0.05$) between the two groups.

2.1. Experimental diets

Two diets were formulated to be isonitrogenous and isoenergetic. A basal diet was used as the control containing 54% low-temperature steam-dried fishmeal (named as FM), whereas another diet was prepared by replacing 100% of the fishmeal with a plant protein blend (soybean protein concentrate (SPC): cottonseed protein concentrate (CPC) = 1:1.66 and named as PP). Adding the crystallized amino acids (CAAs, including *L*-Lys-HCl, *DL*-Met, and *L*-Thr), fish oil and monocalcium phosphate to the PP diet to balance essential amino acid (EAA) Lys, Met and Thr, essential fatty acids (EFA) and available phosphorus (AP), respectively following the profile of FM diet. Each diet was extruded into 2 mm diameter pellets using a twin-screwed extruder (EXT50A, Yang gong Machine, China). All diets were dried in natural conditions and stored at −20 °C. The diet formulation, chemical compositions, and pellet hardness were shown in Table 1. The analyzed

Table 2
Amino acid composition of experimental diets (g/kg dry material).

Feeds	FM	PP
Essential amino acid		
Lysine	30.1	27.7
Threonine	16.7	16.8
Valine	20.5	18.1
Isoleucine	17.6	16
Leucine	28.9	25.8
Phenylalanine	16.4	20.4
Methionine	11.9	11.0
Tryptophan	4.7	5.0
Arginine	23	38.1
Histidine	9.2	10.6
ΣEAA[a]	179	189.5
Conditionally Essential Amino Acid		
Proline	17.6	18.7
Glycine	20.9	15.9
Cystine	3.8	6.1
ΣCEAA[b]	58.0	57.7
Non-essential amino acid		
Alanine	23.6	16.4
Serine	16.4	18.7
Aspartic acid	36.9	40.2
Glutamic acid	58.0	79.2
ΣNEAA[c]	134.9	154.5
ΣAA[d]	356.2	384.7
ΣEAA/ΣAA	0.50	0.49

[a] ΣEAA: sum of essential amino acids.
[b] ΣCEAA: sum of conditionally essential amino acid.
[c] ΣNEAA: sum of non-essential amino acids.
[d] ΣAA: sum of total amino acids.

amino acid composition of experimental diets was shown in Table 2.

2.2. Experimental fish, feeding protocol and sampling

Juvenile Japanese seabass were obtained from Weihai Yulong Aquafarm, Shandong, China. Desalinated and acclimated gradually to freshwater over a period of 2 weeks. Before the formal feeding trial, fish were acclimatized and adapted to the PP diet for 2 weeks in the system to make sure fish can well ingest both of diets. Fish (initial body weight = 10.42 ± 0.01 g) were randomly selected and distributed into eight conical fiberglass tanks (256 L, water depth: 80 cm; volume: 0.25 m^3) in a recirculation system after 24 h starvation with 30 fish per tank and four tanks per treatment. The water temperature was maintained at 26 ± 2 °C, pH = 7.5–8.5, dissolved oxygen (DO) was 6.8–7.8 mg/L, ammonia nitrogen content < 0.5 mg/L and NO_2^- < 0.1 mg/L. Aeration was supplied to each tank 24 h per day and fluorescent light was separately designed above the tanks and kept on from 8:00 to 21:00 for a photoperiod of 13L:11D. Fish were fed to apparent satiation twice a day at 8:00 and 20:00 for 10 weeks. Food intake was measured daily.

Thirty fish at the initiation of feeding trial and three fish per tank at termination were randomly collected and stored frozen (−80 °C) for determination of proximate analysis. Growth performance including final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR) were detected by batch weighing the fish at the end of the 10 weeks. Food intake was recorded per tank for feed conversion rate (FCR), feeding rate (FR), productive protein value (PPV) and productive lipid value (PLV). After 24 h of starvation at the end of the growth trial, twelve fish for each treatment (3 fish from each tank) were randomly selected and anesthetized with chlorobutanol (300 mg/L). The body weight, body length, viscera, liver, and abdominal adipose weight were recorded individually to calculate condition factor (CF), hepatosomatic index (HSI) and visceral adipose index (VAI). Blood samples were drawn from the caudal part of these sedated fish using anticoagulant syringes with 2% NaF and 4% potassium oxalate and centrifuged at 4,000 rpm for 10 min at 4 °C to obtain plasma. Two liver samples near to the bile duct were collected for histology examination (fixed in 4% paraformaldehyde solution), the rest of the liver were rapidly collected and frozen in liquid nitrogen for biochemical criterion analysis and RNA isolation. All samples were stored at −80 °C until analysis.

2.3. Chemical analysis

All chemical analyses were carried out in duplicate according to AOAC (2006). The dry matter was analyzed by drying the samples to constant weight at 105 °C. Crude protein was determined with a Kjeltec™ 2300 Unit (FOSS, Denmark) using the Kjeldahl method. Crude lipid was analyzed by acid hydrolysis with a Soxtex System HT 1047 Hydrolyzing Unit (Foss, Hillerød, Denmark), followed by Soxhlet extraction using a Soxtex System 1043 (Foss, Hillerød, Denmark). Ash was analyzed by combustion in a CWF 1100 muffle furnace (Carbolite, UK) at 550 °C for 6 h. Starch content was analyzed by colorimetric using 3,5-dinitrosalicylic acid at a wavelength of 540 nm (TU-1900, PERSEE, China). Free gossypol in the experimental diets was determined by high-performance lipid chromatography (HPLC) (LC-15C, SHIMADZU, Japan) and the detection limit was 1.0 mg kg^{-1}. Trypsin inhibitor and β-conglycinin in the feed were determined using the ELISA method from commercial kits (Beijing Longkefangzhou Bioengineering Institute, China, No. EA03 and EA01) and the detection limit was 30 ng mL^{-1} and 800 ng mL^{-1}, respectively. The hardness of feed was determined by the Texture Analyzer (TA-XY2i, Stable Micro, England). The amino acid contents in the diets were analyzed in the lab of Evonik Degussa (Beijing, China) Co., Ltd. Plasma free amino acid were analyzed by Sykam S-433D automatic amino acid analyzer (SYKAM Corporation, Germany).

2.4. Hematological and liver homogenate parameters of liver functions and nutrient metabolism

Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), alkaline phosphatase (AKP), total bile acid (TBA), glucose (GLU), glucagon (GC), triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and immunoglobulin M (IgM); hepatic total antioxidative capability (T-AOC), anti-superoxide anion free radical (Anti-$O_2^{\cdot-}$), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), malondialdehyde (MDA) 4-hydroxynonenal (4-HNE) and glycogen (GLY) were determined by assay kits (Nanjing Jiancheng Co.,Nanjing, China) following the protocols given by the supplier. Non-esterified fatty acid (NEFA) was determined by the assay kit (Wako Pure Chemical Industries, Ltd. Japan). Cyclic-adenosine monophosphate (cAMP) was determined by the assay kit (Meimian, China). Thyroid hormones T3 and T4 were determined by radioimmunoassay (RIA) kit (No. HY-001 and No. HY-002) at the Beijing Sino-UK Institute of Biological Technology.

2.5. RNA isolation, reverse transcription and real-time quantitative PCR (RT-qPCR)

Total RNA from the liver tissues were extracted using RNAiso Plus reagent (Takara, Japan), spectrophotometrically quantified using a NanoDrop 2000 (Thermo, USA) and electrophoresed on a 1% denaturing agarose gel to evaluate the integrity. For each reverse transcription reaction, 1.0 μg of total RNA was first treated with gDNA Eraser to remove genomic DNA contaminants and then subjected to cDNA synthesis by reverse transcription in a 20 μL volume using the PrimeScript RT reagent Kit (Takara, Japan).

The genes of the Japanese seabass (*Lateolabrax japonicus*) were obtained from an RNA-seq database. EF1α (GenBank accession no. JQ995144), a housekeeping gene whose expression was not affected by the treatment in the present experiment, was used as an endogenous reference to normalize the template amount. The gene-specific primers

Table 3
Primer sequences for real-time PCR.

Gene	Primers	Sequence 5′–3′	Target size (bp)	TM (°C)	E-Values (%)
TNFα	F[a]	GACTCCATAGGCAGCAAAGC	205	60.8	103.2
	R[a]	AGAAAGTCTTGCCCTCGTCA			
IL1β	F	CTGAACATCAAGGGCACAGA	192	60.8	92.8
	R	GTTGAAGGGGACAGACCTGA			
TGFβ1	F	GCAGGCAGTGAAAGAAAAGG	200	63.9	96.3
	R	CTAATGGCCTCAATGCGTTT			
IL10	F	TTCAAAACTCCGTTCGCCTG	161	61	97.1
	R	TCACTCTTGAGCTCGTCGAA			
CREB	F	ATGGAGGACGGACAGACAAC	151	56.7	99.4
	R	GACACACGTGAAGCTGCATT			
caspase8	F	AAGACGCATCTGTTCGCTTCCTG	116	61.6	98.9
	R	GCGACAGCTTCAGCCTATCCATC			
caspase9	F	TGCGGAGGAGGTGAACGAGAC	138	62.8	90.5
	R	CGGTTCGTCGGACATGCTCAG			
caspase3	F	ATCACAGCAACTACGCCTCATTCG	176	61.6	98.9
	R	GCCTCTGCAAGCCTGGATGAAG			
FASN	F	AGGCATTGTGGAGGGTGTAG	233	56.8	97.1
	R	CCAGTCCACCAGTGATGATG			
ACC1	F	AATCAACATCCGCCTGACTCCAAC	176	59	90.2
	R	CCTGCTTGTCTCCGTATGCTTGG			
PPARα	F	GACAACGATGCCCTCAGCTC	193	58	92.7
	R	CGGCACAAACTCGACACTCA			
CPT1α	F	TCCGTGGCAGTCTTCTGAGGTC	115	60.4	92.9
	R	GCAGCAGCAGACATACACCTACAG			
PPARγ	F	AGGCCTGCTGAATGTGAAGC	170	58	93.3
	R	GCTGGATGAAGTGGACGTGG			
ATGL	F	CTTCCTCTCCGCAACAAGTC	211	55.8	100
	R	TGGTGCTGTCTGGAGTGTTC			
HMGCR	F	GGAAGAGGAAGAGGACAACAAGCC	80	56.6	101.2
	R	GAACCATGACCAGGCCAAGCG			
APOA1	F	TGCAGAAGCACATTGACGAGTACC	175	56.6	102.6
	R	ATTGGCACGAGCTTGGACTTGG			
APOB	F	CGCTGGAGGATGGAATGTCTGC	176	57.7	97.3
	R	ACGCACAGATACTTGGCTCTTGAC			
CYP7A1	F	ACCCCACACTGAACCTCACT	92	57.7	92
	R	GGAAGCACTGGAAAGACGCA			
FXR	F	CACAATCCTCTCACCAGACAGACC	89	56.6	105.5
	R	CCTTCCTCAGCACCTCCAACATG			
RXRα	F	GGGCATGAAGAGAGAAGCCG	175	59.3	90.6
	R	CACGCCAAGGTTGGTCTCAA			
SHP	F	CGGTCGTCACTGTTAAGGCACTG	126	59.3	95.5
	R	TGGTCCGAGCAGGATCTGTCTAAG			
PEPCK	F	ACGCCAATGTGCCTTTTTAC	128	55.8	92
	R	GCTCCCTTCACAAACTCAGC			
FBP	F	CACGTCACGTCTGGTGCTAAGC	180	61.9	90.5
	R	TGGCATAGTGTGAGGCGGACTG			
G6P	F	TCTGTGTCCTACCTGGTGGTGTTC	164	60.7	93.5
	R	GAGATGAATCCTGCTGCGAGACTG			
GK	F	GGTGAAGAAGCGAATGAGTGAGGAG	119	60.7	97.1
	R	CATGCTCTGTTCCGTCTGGTGTG			
PK	F	CTCTCGTTCTGTGGATATGCTGAAGG	105	60.7	104.4
	R	AGGATGGTCTCTGCGTGGTACTC			
PFK1	F	GATGAACGCAGCGGTGAGGTC	132	61.9	92.4
	R	CCAGCCACATTGTGCCAGTCC			
PDH	F	AAGTGCCGCCATATTGTAGG	225	56.7	92.9
	R	ACCGGCAGAAAAGGAGGTAT			

[a] F: forward primer; R: reverse primer.

used for mRNA quantification by RT-PCR were designed by Primer Premier 5.0 and showed in Table 2. The RT-qPCR analysis was performed using a CFX96™ Real-Time System (Bio-Rad, USA) in a 20 μL reaction volume containing iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA).

Serial dilutions of cDNA generated from liver tissues at a suitable level were used to generate a standard curve to determine the amplification efficiency (E-values) of target and reference genes. The E-values ranged from 90.5% to 105.5% (Table 3). The RT-qPCR temperature profile for all genes was 95 °C for 30 s followed by 36 cycles of 10 s at 95 °C, 30 s at Tm (Table 3) and 40 s at 72 °C. After the final cycle of PCR, the melting curves were systematically monitored (65 °C temperature gradient at 0.05 °C/10 s from 65 to 95 °C). During the detection, each sample was run in three replicates. PCR-grade water in place of the template served as the negative control. $2^{-\Delta\Delta Ct}$ method [27] was used to analyze RT-qPCR data. Expressions of mRNA of target genes are shown as the n-fold difference relative to the calibrator.

2.6. Histopathological and immunohistochemical examination of the liver tissues

All fixed liver samples were dehydrated by the standard procedures, and the samples were embedded in paraffin and cut to 6 μm sections. Liver sections were stained following the protocols of hematoxylin and eosin (H & E) staining and observed by light microscopy (Leica DM2500, Leica, Solms, Germany).

The immunofluorescence co-staining test for p65 NF-kB and cleaved-caspase3 were as follows. Liver sections were deparaffinized, rehydrated and rinsed with PBST (0.1% Tween-20 in phosphate buffered saline, PBS). Antigen retrieval was obtained by maintaining slides in citrate antigen retrieval solution (pH 6.0) in a pressure cooker for 10 min. Sections were blocked 30 min by serum-free blocking buffer (Dako, USA). Sections were incubated with polyclonal p65 NF-kB and cleaved-caspase3 antibody (Abcam, USA) overnight at 4 °C. After washing with PBST, sections were incubated at room temperature for 1 h with Alexa Flour 488 goat anti-mouse antibody for p65 NF-kB and 555 goats anti-rabbit antibody for cleaved-caspase3, respectively (Life Technology, USA). After 3 washes with PBST, sections were mounted with an anti-fade mounting medium that contains DAPI (VECTASHIELD, Vector Laboratories, USA) for nuclei staining. The fluorescent signal was captured using a confocal microscope (Zeiss LSM700, Germany) in merge format.

2.7. Detection of JNK phosphorylation by western blotting

Liver tissues were homogenized in RIPA buffer (Beyotime, China) with an added phosphatase inhibitor cocktail (Roche, Germany). The protein concentration was measured using a BCA Protein Quantification Kit (Beyotime, China). Protein extracts were run on Criterion gels (Bio-Rad, USA) and blotted onto nitrocellulose membranes (Millipore, USA). After blocking for 30 min at room temperature, immunoblots were incubated overnight at 4 °C in primary antibodies, namely, anti-JNK, anti-P-JNK (Thr183/Tyr185) (Cell Signaling Technology, USA), and anti-GAPDH (ImmunoWay, USA). JNK, P-JNK, and GAPDH blots were then incubated for 1 h in goat anti-rabbit IgG-HRP (Beijing TDY Biotech, China) secondary antibody. Proteins were detected using enhanced chemiluminescence (ECL) (Biorad, USA). Quantification was performed using ImageJ software.

2.8. Statistical analysis

The normality of the data was assessed using SPSS Statistics 22.0. All data were reported as mean value ± standard error of the mean (S.E.M). Values between the two groups were compared with independent *t*-test, $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered statistically very significant. The graphics were drawn by GraphPad Prism 7.0 (GraphPad Software Inc. USA).

3. Results

3.1. Growth performance

The results of growth performance are presented in Table 4. Both FM and PP groups showed high survival (≥99%) and there was no significant difference between the two groups. Compared with the FM group, fish in the PP group showed the significantly lower FBW, WGR SGR and PPV ($P < 0.05$) but significantly higher FCR and FR ($P < 0.05$). The PLV between the two groups had no significant difference. At postprandial 24 h, the completely empty gut in fish of the FM group was observed, but undigested chyme existed in the intestinal tract of fish in the PP group (S. Fig). The slow gastric emptying rate in fish fed PP diet could be related to the much higher hardness of PP diet.

3.2. Morphometric parameters and whole body composition

As shown in Table 5, the HSI of fish in the PP group was significantly higher than that in the FM group ($P < 0.05$), but the CF, VSI, and VAI had no significant difference between the two groups. The whole body ash of fish in the PP group was significantly lower than that in the FM group ($P < 0.05$). The whole body moisture, protein and lipid content had no significant difference between the two groups.

3.3. Hepatic antioxidant responds and hematological liver functions and immune parameters

The results of hepatic peroxidation parameters are presented in Table 6. In liver tissues, T-AOC and GSH-Px of fish in the PP group were significantly decreased ($P < 0.05$). The other antioxidant enzymes including SOD and CAT, Anti-O_2^- and the products of lipid peroxidation including MDA and 4-HNE had no significant difference between the two groups.

The results of hematological liver function and immune parameters were listed in Table 7. Compared with fish in the FM group, the increased plasma ALT, AST, and TP contents were observed in fish of the PP group, but plasma AKP and IgM contents were significantly decreased ($P < 0.05$). Plasma TBA had no significant difference between the two groups ($P > 0.05$).

3.4. Plasma free amino acid profiles

The plasma free amino acid (FAA) profiles of Japanese seabass are shown in Table 8. Compared with the FM group, plasma EAA, including lysine, threonine, valine, isoleucine and leucine of fish in the PP group were significantly decreased ($P < 0.05$), and plasma arginine and histidine were abundant in fish fed PP diet. The proline, one of the conditional essential amino acids (CEAA) was also reduced significantly in the PP group, but other CEAA had no significant difference between the two groups. Fish in the PP group showed significantly higher plasma glutamic acid, carbamide, and ornithine, but lower for plasma alanine. In general, although there was no significant difference in plasma total AA between the two groups, fish in the PP group showed lower plasma EAA, but higher non-essential amino acid (NEAA)

Table 4
Effects of totally replacing fishmeal with dietary plant protein on the growth performance in *Lateolabrax japonicas* (means ± SEM).

	SR(%)[2]	FBW(g)[3]	WGR(%)[4]	SGR(%)[5]	FCR[6]	FR(%)[7]	PPV(%)[8]	PLV(%)[9]
FM	100.0 ± 0.00	110.0 ± 0.94[b]	954.0 ± 8.48[b]	3.56 ± 0.01[b]	0.94 ± 0.01[a]	2.35 ± 0.02[a]	40.38 ± 0.34[b]	83.49 ± 0.80
PP	99.0 ± 1.00	78.1 ± 1.19[a]	643.0 ± 13.58[a]	3.05 ± 0.02[a]	1.11 ± 0.02[b]	2.57 ± 0.05[b]	33.83 ± 0.60[a]	80.55 ± 1.51

[1]The letters "a" and "b" indicate a significant difference ($P < 0.05$) between the two groups.
[2]SR (survival, %) = 100 × final fish number/initial fish number, n = 4.
[3] FBW: final body weight, n = 4.
[4] WGR (weight gain rate, %) = 100 × (final fish weight - initial fish weight + dead fish weight)/initial fish weight, n = 4.
[5] SGR (specific growth rate, %) = 100 × [ln (FBW/initial body weight)]/days, n = 4.
[6] FCR (feed conversion ratio) = feed intake/(Wf + Wd - Wi). Wf is the final total weight, Wd is the total weight of dead fish, Wi is the initial total weight, n = 4.
[7] FR (feeding rate, %) = 100 × total feed consumption/[(initial fish weight + initial fish weight + dead fish weight)/2]/days, n = 4.
[8] PPV (productive protein value, %) = 100 × (FBW × Cfp - IBW × C0 + Wd × C0)/feed protein consumption. Cfp (%) is final protein content in whole fish body, C0 (%) is initial protein content in whole fish body, and Wd is total body weight of dead fish during experiment.
[9] PLV (productive lipid value, %) = 100 × (FBW × Cff - IBW × C0 + Wd × C0)/feed lipid consumption. Cff (%) is final lipid content in whole fish body, C0 (%) is initial lipid content in whole fish body, and Wd is total body weight of dead fish during experiment.

Table 5
Effects of totally replacing fishmeal with dietary plant protein on the morphometric parameters and whole body composition in *Lateolabrax japonicas* (means ± SEM).

	CF[2]	VSI(%)[3]	HSI(%)[4]	VAI(%)[5]	Moisture (%)	Ash(%)	Crude protein (%)	Crude lipid (%)
FM	1.40 ± 0.00	10.68 ± 0.34	1.29 ± 0.13[a]	6.59 ± 0.49	69.03 ± 0.13	3.79 ± 0.10[b]	15.41 ± 0.04	10.36 ± 0.23
PP	1.40 ± 0.00	11.28 ± 0.28	1.73 ± 0.10[b]	6.06 ± 0.24	69.64 ± 0.31	3.10 ± 0.11[a]	15.87 ± 0.22	10.61 ± 0.19

[1]The letters “a” and “b” indicate a significant difference ($P < 0.05$) between the two groups.
[2] CF (condition factor) = 100 × (body weight, g)/(body length, cm) [3], n = 12.
[3] VSI (viscerasomatic index, %) = 100 × viscera weight/whole body weight, n = 12.
[4] HSI (hepatosomatic index, %) = 100 × liver weight/whole body weight, n = 12.
[5] VAI (visceral adipose index, %) = 100 × visceral adipose weight/whole body weight, n = 12.

($P < 0.05$).

3.5. Glucose metabolism

Compared with fish in the FM group, the glucose metabolism disorder in Japanese seabass of PP group was observed with uncontrollable fasting glycolysis and pyruvate aerobic oxidation at postprandial 24 h (Fig. 1). The significantly reduced plasma glucose, but increased GC level ($P < 0.05$), along with very significantly reduced liver glycogen storage appeared in the PP group ($P < 0.01$) (Fig. 1A). The mRNA levels of gluconeogenesis genes including phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBP) and glucose-6-phosphatase (G6P) had no significant difference between the two groups, but the expression of glycolysis-related genes including glucokinase (GK) and pyruvate kinase (PK), and pyruvate aerobic oxidation gene pyruvate dehydrogenase (PDH) were very significantly upregulated in PP group ($P < 0.01$) (Fig. 1B).

3.6. Lipid metabolism

As shown in Fig. 2, lipid metabolism disorder appeared in Japanese seabass fed the PP diet with the symptom of cholesterol accumulation and ultra-lipolysis/β-oxidation in liver tissues. Compared with FM group, very significantly lower plasma TC, HDL-C, LDL-C levels ($P < 0.01$), but significantly higher plasma HDL-C/TC ratio and hepatic TC accumulation were observed in PP group ($P < 0.05$) (Fig. 2A). The hepatic gene expression of cholesterol synthesis gene 3-hydroxy-3-methylglutaryl-coA reductase (HMGCR) was very significantly upregulated ($P < 0.01$), while bile acid synthesis gene cholesterol 7 alpha-hydroxylase (CYP7A1) and small heterodimer partner (SHP) were significantly down-regulated ($P < 0.05$) (Fig. 2B), which could be the main reason for cholesterol accumulation in liver tissues. The significantly reduced plasma TG and hepatic NEFA content could be observed in the PP group ($P < 0.05$) (Fig. 2C). Both the mRNA levels of lipogenesis/proliferation-related genes, including fatty acid synthetase (FASN) and peroxisome proliferator-activated receptor gamma (PPARγ), and lipolysis/β-oxidation-related genes, including hormone-sensitive lipase (HSL), peroxisome proliferator-activated receptor alpha (PPARα) and carnitine palmitoyltransferase 1alpha (CPT1α) in liver tissues were up-regulated at varying degree in PP group ($P < 0.05$) (Fig. 2D), in which mRNA level of CPT1α was much higher upregulated (3.20 fold) than other genes.

3.7. Energy metabolism

Accelerated energy metabolism rate in Japanese seabass of PP group was observed and shown in Fig. 3. The extremely increased hepatic cAMP content ($P < 0.01$) (Fig. 3A) and the significantly up-regulated mRNA level of the cAMP response element-binding protein (CREB) ($P < 0.05$) promoted the energy metabolism by accelerating the lipolysis/β-oxidation processes in PP group (Fig. 3B). The hyperthyroidism symptom appeared with significantly higher plasma thyroid hormones T3 and T4 levels ($P < 0.05$) (Fig. 3C).

3.8. Hepatic pathological examination - histology, inflammation and apoptosis

Fish liver sections were examined after Hematoxylin and eosin (H & E) staining and immunofluorescence signaling for p65 NF-kB and cleaved-caspase3 antibody, which are specific for cellular apoptosis. Eight samples were observed in each group. Two typical phenotypes are shown in Fig. 4. Phenotype I (Fig. 4A) showed normal hepatocytes with the well-shaped cell, higher and mainly cytoplasmic expressed p65 NF-kB (green signal) and less cleaved-caspase3 (red signal). Phenotype II (Fig. 4B) defined the fatty liver tissues with enlarged and vacuolation cells, less and mainly nuclear (marked with DAPI in blue color) expressed p65 NF-kB, which co-expressed with cleaved-caspase3. The yellow "□" indicates the signals of p65 NF-kB co-stained with cleaved-caspase3 on the nucleus; the purple “O” indicates the cytoplasm expressed p65 NF-kB (the inactive form) did not appear together with the cleaved-caspase3. The hepatic histopathological examination results of each group were shown in Fig. 4C. In the FM group, 7 samples had normally shaped hepatocytes and only one sample showed hepatocytes steatosis. In the PP group, 3 samples had normally shaped hepatocytes and 5 samples had hepatic steatosis. The mRNA levels of inflammatory cytokines and apoptosis-related genes in liver tissues were shown in Fig. 4D and E. In PP group, both pro-inflammatory cytokines tumor necrosis factor alpha (TNFα), Interleukin 1 beta (IL1β) and anti-inflammatory cytokines transforming growth factor beta 1 (TGFβ1) and Interleukin 10 (IL10) gene expression were significantly upregulated. The extrinsic apoptosis gene caspase8 mRNA level was significantly increased, but the intrinsic apoptosis gene caspase9 had no difference between the two groups.

The JNK protein and phosphorylation JNK (P-JNK) levels and the ratio of P-JNK(Thr183/Tyr185)/JNK in liver tissues of Japanese seabass between the two groups had no significant difference (Fig. 5).

Table 6
Effects of totally replacing fishmeal with dietary plant protein on hepatic antioxidant responses of *Lateolabrax japonicas* (means ± SEM. n = 8).

	T- AOC (U/g prot[2])	SOD (U/g prot)	GSH-Px (U/g prot)	CAT (U/g prot)	Anti-O_2^-(U/g prot)	MDA (nmol/g prot)	4- HNE (ng/g prot)
FM	15.22 ± 2.31[b]	183.33 ± 12.83	253.48 ± 25.79[b]	21.39 ± 1.30	165.18 ± 10.61	0.40 ± 0.03	29.93 ± 2.45
PP	7.53 ± 1.99[a]	203.59 ± 20.62	59.02 ± 29.51[a]	21.30 ± 2.14	146.31 ± 19.59	0.40 ± 0.01	29.06 ± 4.48

1 The letters “a” and “b” indicate a significant difference ($P < 0.05$) between the two groups.
[2] The letter “prot” means total protein in liver tissue.

Table 7
Effects of totally replacing fishmeal with dietary plant protein on plasma liver function parameters of *Lateolabrax japonicas* (means ± SEM. n = 8).

	AST (U/L)	ALT (U/L)	TP (g/L)	AKP (U/L)	TBA (μmol/L)	IgM (μg/L)
FM	4.38 ± 0.60	/[a]	32.57 ± 0.64[a]	104.97 ± 6.08[b]	4.54 ± 0.53	238 ± 48.0[b]
PP	7.55 ± 1.63	3.69 ± 1.28[b]	36.01 ± 1.21[b]	62.02 ± 6.92[a]	3.84 ± 0.27	46.3 ± 8.50[a]

[1] The letters "a" and "b" indicate a significant difference ($P < 0.05$) between the two groups.
[2] The symbol "/" means the activity of ALT (U/L) didn't reach the limit of detection in the plasma of *Lateolabrax japonicas* in the FM group.

Table 8
Effects of totally replacing fishmeal with dietary plant protein on plasma free amino acid (μg/g) in *Lateolabrax japonicas* (means ± SEM).

Parameter	FM	PP
Essential amino acid		
Lysine	47.27 ± 4.56[b]	25.78 ± 2.90[a]
Threonine	27.75 ± 2.58[b]	17.18 ± 1.33[a]
Valine	46.98 ± 3.35[b]	28.52 ± 1.75[a]
Isoleucine	27.82 ± 2.08[b]	15.03 ± 1.25[a]
Leucine	46.95 ± 3.74[b]	26.68 ± 1.98[a]
Phenylalanine	21.02 ± 1.63	23.07 ± 2.78
Methionine	20.28 ± 1.51	16.13 ± 1.02
Tryptophan	5.90 ± 0.22	4.93 ± 0.55
Arginine	36.57 ± 3.46[a]	50.25 ± 3.27[b]
Histidine	9.90 ± 0.53[a]	12.45 ± 0.66[b]
ΣEAA[2]	290.43 ± 23.21[b]	220.03 ± 7.87[a]
Conditionally Essential Amino Acid		
Proline	19.03 ± 2.33[b]	10.90 ± 0.34[a]
Taurine	26.78 ± 1.37	22.73 ± 1.45
Glycine	20.03 ± 1.26	19.03 ± 0.65
Cystine	1.47 ± 0.12	1.17 ± 0.07
ΣCEAA[3]	67.32 ± 4.76	55.33 ± 1.66
Non-essential amino acid		
Alanine	43.40 ± 2.87[b]	29.63 ± 2.24[a]
Serine	14.27 ± 1.33	11.15 ± 0.79
Glutamic acid	4.52 ± 0.23[a]	5.85 ± 0.05[b]
Tyrosine	22.37 ± 1.55	27.82 ± 2.54
Phosphoserine	8.55 ± 0.78	7.95 ± 0.54
Carnosine	1.27 ± 0.07[b]	/[a4]
Phosphorus ethanolamine	1.65 ± 0.13	1.55 ± 0.43
Carbamide	36.30 ± 2.33[a]	117.50 ± 5.20[b]
Ornithine	1.92 ± 0.19[a]	4.00 ± 0.36[b]
ΣNEAA[5]	134.23 ± 8.04[a]	205.45 ± 10.93[b]
ΣAA[6]	491.98 ± 35.88	480.82 ± 16.09

[1] The letters "a" and "b" indicate a significant difference ($P < 0.05$) between the two groups.
[2] ΣEAA: sum of essential amino acids.
[3] ΣCEAA: sum of conditionally essential amino acid.
[4] The symbol "/" means the carnosine (μg/g) didn't reach the limit of detection in the plasma of Lateolabrax japonicas in the FM group.
[5] ΣNEAA: sum of non-essential amino acids.
[6] ΣAA: sum of total amino acids.

4. Discussion

In the previous studies of Japanese seabass receiving a diet with high plant protein content, we observed an extreme anorectic response in the short term, followed by feeding adaptation and even compensatory intake in a longer term as 4-12w [14,15]. Anorexia was the main reason for the poor growth performance and higher mortality in response to plant proteins for this species. However, the feeding adaptation could be influenced by the nutritional history of Japanese seabass. In the current study, we found that Japanese seabass could adapt to the PP diet after 2w low FM diet acclimation, compared with the FM group, even a significantly higher FR could be observed in the PP group. Although the survival rate was not affected by PP diet, a lower growth performance, metabolic disorder, and liver disease could be observed in fish of PP group.

4.1. Growth performance

Compared with the FM group, the lower growth performance of fish in the PP group could be ascribed to the EAA deficiency, poor nutrient absorption, and accelerated basal metabolism rate. Although some essential nutrients, such as EAA, EFA, and AP had been included in PP diet according to the FM diet profile, the treatment is not well-satisfied with the requirement of Japanese seabass. This could be attributed to the leaching loss of CAA and the asynchronous absorption with the combined amino acids which existed in the intestinal tract of fish [28]. The plasma FAA profiles can reflect the amino acid metabolism of fish. In the present study, the significantly decreased plasma EAA, CEAA and lower PPV reflected the EAA deficiency of the PP group. For most fish, CPC and SPC are alternative protein sources with relatively high digestibility. However, the excessive pellet hardness retarded the gastric emptying rate and indirectly reduced digestion rate and nutrients absorption [29]. Besides, the accelerated basal metabolism rate with hyperthyroidism symptom induced the lower growth performance and PPV, although the FR was increased in fish of the PP group.

4.2. Metabolic disorders

Compared with FM, except for the EAA, EFA, and AP deficiency, other important profiles of plant protein are characterized by high carbohydrate and cholesterol deficiency, etc. Carnivorous fish always have a low ability to use carbohydrates as energy sources with the poor ability to control glucose metabolism homeostasis [30]. In the current study, EAA deficiency and accelerated basal metabolism rate restricted the protein synthesis in PP group, thus the fish dissimilated glucose to replenish amino acids through the tricarboxylic acid cycle (TAC), which provides precursors of certain amino acids. However, at postprandial 24 h, glucose requirements should be satisfied by glycogen depletion to glucose (glycogenolysis) or by *de novo* glucose synthesis through gluconeogenesis [31]. But in the present study, the glucose metabolism disorder with uncontrollable fasting glycolysis and pyruvate aerobic oxidation symptom, which could over-produce acetyl-CoA for TAC and release energy.

Acetyl-CoA is the substrate that participates in the biosynthesis reactions of protein, carbohydrate, and lipid [32]. In the current study, the significantly higher hepatic TC content but lower plasma TG and hepatic NEFA contents were observed in PP group, indicating that overproduced acetyl-CoA from glycolysis and pyruvate aerobic oxidation make a high contribution for cholesterol synthesis besides protein synthesis. Fish acquire cholesterol both from the diet (exogenous cholesterol) and *de novo* synthesis (endogenous cholesterol) by the liver [33]. As cholesterol is derived only from animal organisms, dietary cholesterol content decreases or even disappears from a total plant-based diet [34]. Cholesterol catabolism mainly via the synthesis of bile acids, which can regulate the intestinal nutrient absorption and maintain the metabolic balance of lipid and glucose, in addition, prevents the liver and other organs from the accumulation of cholesterol, triglycerides, and toxic metabolites [35]. In the current study, the significantly higher hepatic TC content, plasma HDL-C/TC ratio accompanied with hepatic steatosis phenotype by histological examination could be observed in PP group. It could be largely attributed to the dual influences of compensatory increased synthesis of cholesterol by up-

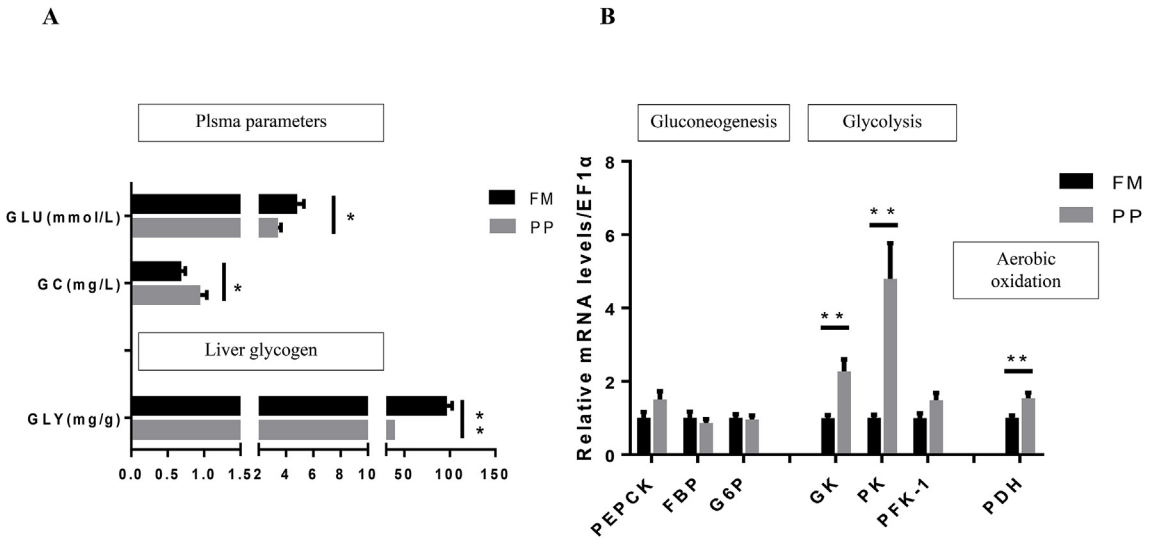

Fig. 1. Uncontrollable fasting glycolysis induced glucose metabolism disorder in Japanese seabass fed the PP diet at postprandial 24 h. (A): The significantly reduced plasma glucose, but increased GC level, and along with very significantly reduced liver glycogen storage indicated a lower energy storage capability in PP group. (B): No significant difference in mRNA levels of gluconeogenesis genes, but very significantly up-regulated the expression of glycolysis and pyruvate aerobic oxidation related-genes. Values marked with "*" are significantly difference ($P < 0.05$), and "**" are very significantly difference ($P < 0.01$) (mean ± SEM, n = 8).

regulating the expression of HMGCR gene as the negative feedback to the dietary cholesterol deficiency, and the decreased bile acids synthesis gene (CYP7A1 and SHP), which indicating that fish fed plant protein based diet have lower efficiency in converting cholesterol into bile acids.

Fish adipocytes are mainly distributed in viscera, liver, and muscle. In the present study, we observed significantly higher HSI and fatty liver symptom in the PP group, but there was generally no significant difference on VAI (6.59% and 6.06%), PLV (83.49% and 80.55%) and whole-body lipid content (10.36% and 10.61%) between FM and PP

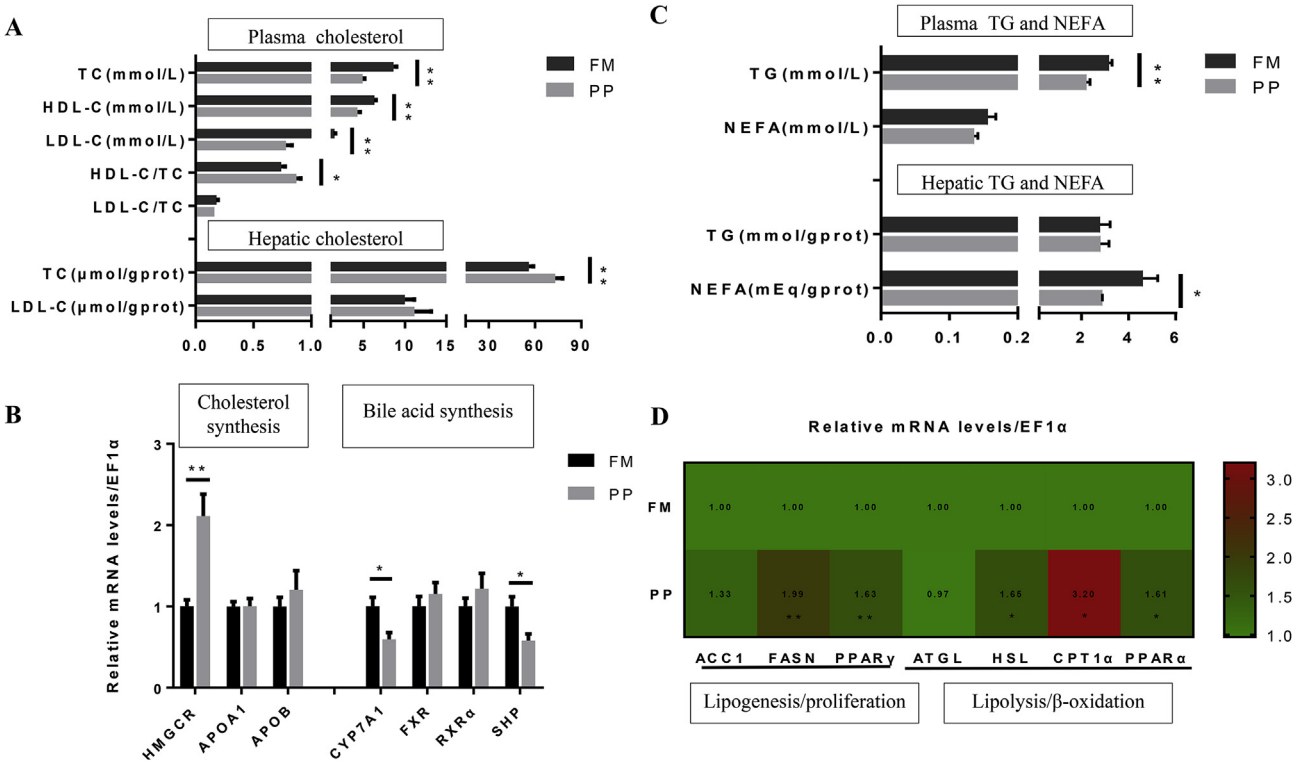

Fig. 2. Lipid metabolism disorder was shown in Japanese seabass fed the PP diet with symptom of cholesterol accumulation in liver tissues. (A): The very significantly lower plasma TC, HDL-C, LDL-C levels, but higher plasma HDL-C/TC ratio and hepatic TC content were observed in PP group. (B): The significantly up-regulated cholesterol synthesis gene, while bile acid synthesis gene were down-regulated indicated a lower efficiency in converting cholesterol into bile acids. (C): The significantly reduced plasma TG and hepatic NEFA contents indicated that the hepatic lipid accumulation in fish of the PP group was not owing to TG and NEFA. (D): In PP group, both the mRNA levels of lipogenesis/proliferation and lipolysis/β-oxidation related genes in liver tissues were up-regulated at varying degree, especially much higher up-regulated β-oxidation gene. Values marked with "*" are significantly difference ($P < 0.05$), and "**" are very significantly difference ($P < 0.01$) (mean ± SEM, n = 8).

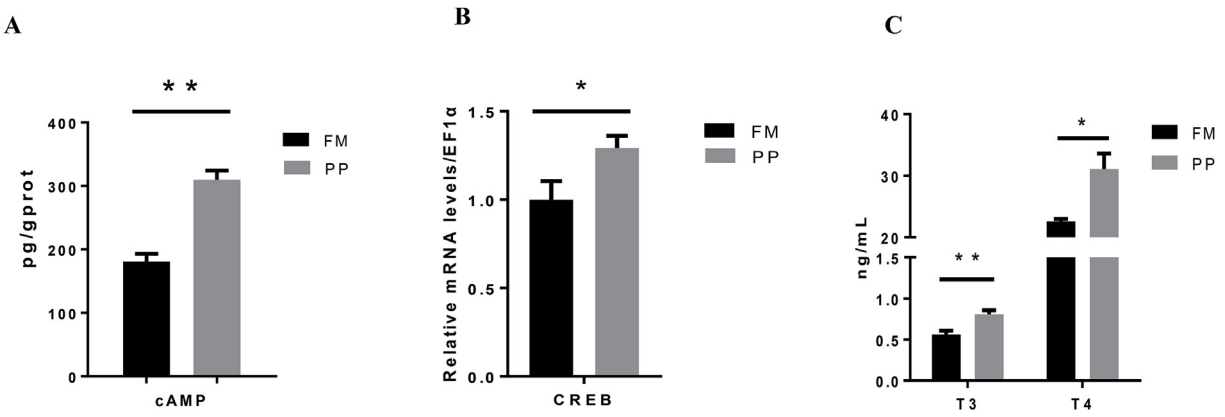

Fig. 3. The accelerated energy metabolism rate and the hyperthyroidism symptom in Japanese seabass fed the PP diet. (A): The extremely increased hepatic cAMP content, which promoted the glycogenolysis and fatty acid β-oxidation in the PP group. (B): The significantly up-regulated CREB mRNA level. (C): The hyperthyroidism symptom with higher plasma thyroid hormones T3 and T4 levels in the PP group. Values marked with "*" are significantly difference ($P < 0.05$), and "**" are very significantly difference ($P < 0.01$) (mean ± SEM, n = 8).

group ($P > 0.05$). The cAMP is a second messenger molecule that synthesized from adenosine triphosphate (ATP), which is produced by glycolysis and TAC. CREB is a transcription factor, and the cAMP/CREB pathway could upregulate genes expression of fatty acid β-oxidation [25,36] to protect the liver tissues from lipid over-accumulation. In the current study, the significantly higher hepatic cAMP content and up-regulated CREB mRNA level promoted the lipolysis/β-oxidation processes (HSL, CPT1, and PPARα) in the PP group. Although the mRNA levels of lipogenesis-related genes FASN, PPARγ, were also significantly up-regulated, this in a large extent owing to the feedback of more accelerated lipolysis/β-oxidation and energy metabolism. Many hormones such as T3, T4 and GC act as a promoter to produce cAMP [37]. The accelerated energy metabolism was often related to the hyperthyroidism with the symptom of high plasma T3 and T4 contents,

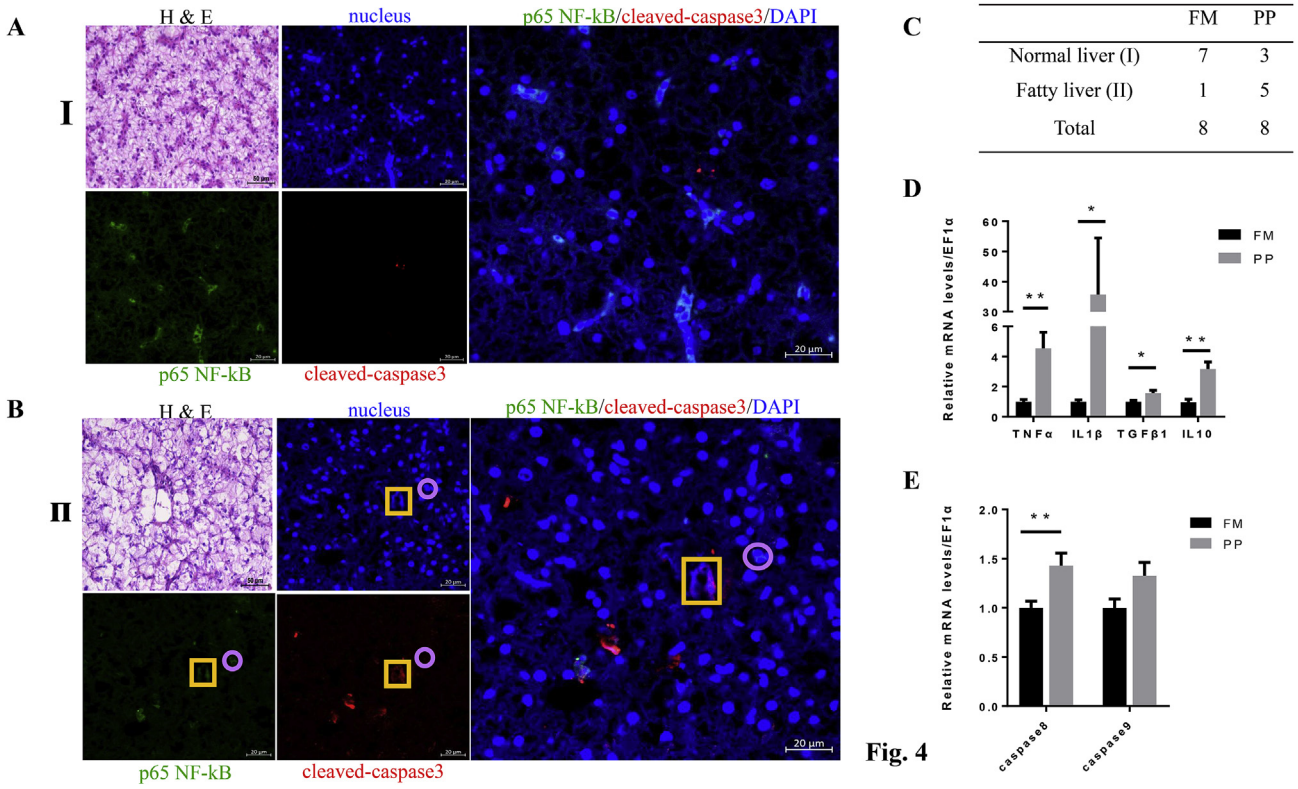

Fig. 4. The fatty liver symptom in Japanese seabass fed the PP diet was related to high expression of inflammatory cytokines and p65 NF-kB/caspase activation. (A): The histological analysis of normal liver tissue by well-shaped cell (H&E staining with bar = 50 μm) and higher and mainly cytoplasmic expressed p65 NF-kB (green signal) and less cleaved-caspase3 (the apoptosis signal in red signal). (B) The histological analysis of fatty liver tissues with enlarged and vacuolation cells (H&E staining) and less and mainly nucleus (marked with DAPI in blue color) expressed p65 NF-kB, which co-expressed with cleaved-caspase3. The yellow "□" indicated the signals of p65 NF-kB co-stained with cleaved-caspase3 on the nucleus; the purple "O" indicated the cytoplasm-expressed p65 NF-kB (the inactive form) without the apoptosis signal of cleaved-caspase3 (bar = 20 μm, n = 8). (C): Phenotype of hepatic histopathological examination in fish fed FM and PP diets. (D): The relatively mRNA levels of pre- and anti-inflammatory cytokines. (E): The relatively mRNA levels of liver apoptosis in Japanese seabass. Values marked with "*" are significantly difference ($P < 0.05$), and "**" are very significantly difference ($P < 0.01$) (mean ± SEM, n = 8). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

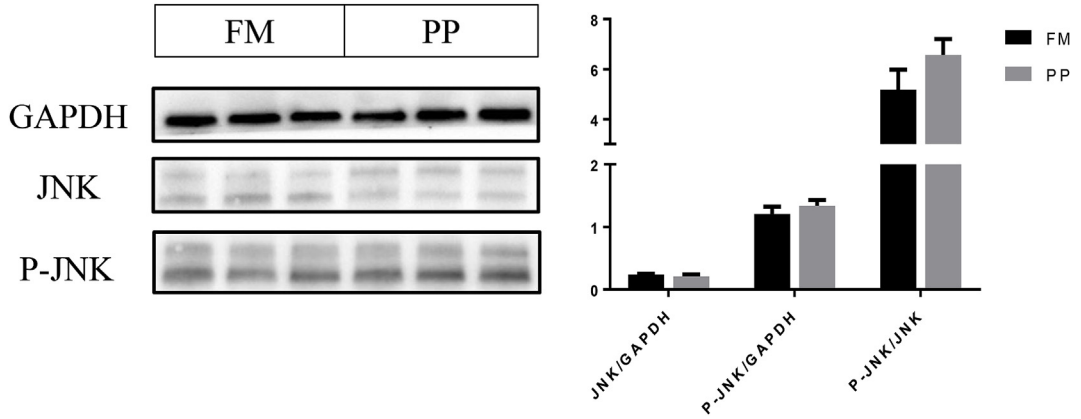

Fig. 5. The western blotting of JNK and phosphorylated JNK (P-JNK) in liver tissues, which indicated the impeded JNK cascade in Japanese seabass fed the PP diet with no significant difference from the FM group in P-JNK expression (mean ± SEM, n = 3).

which is also responsible for the increased food intake, energy consumption, impeded growth and inflammation occurrence in the fish of PP group [38].

4.3. Hepatic inflammation and apoptosis

Growing evidence testify that abnormal cholesterol accumulation in liver tissue, and the products of excessive lipolysis can cause oxidative stress and mitochondrial dysfunction, create more reactive oxygen species (ROS) and pro-inflammatory cytokines, ultimately trigger liver apoptosis [39–41]. T-AOC is a comprehensive index that used to measure the functional status of the antioxidant system [42]. As a member of the anti-oxidase system of fish, GSH-Px is important in combating excessive superoxide radicals and H_2O_2 to protect the cell membrane against oxidative damage [43,44]. In this study, although the significantly lower activity of T-AOC and GSH-Px in liver tissues of fish in PP group were observed, the ROS contents (MDA, 4-HNE) and anti-$O_2^{\cdot-}$ were not significantly different between the two groups. We can infer that although fish in the PP group have a risk of oxidative stress because of the low level of oxidation resistance, the ROS level was still under controllable status. The activities of plasma ALT and AST are proposed as the indicators of liver damage and chronic hepatitis [45], and IgM is the most widely studied immunoglobulin in fish, which could be a good biomarker for evaluating the immune status of fish [46]. In this study, significantly higher plasma AST and ALT activity of Japanese seabass in PP group indicated hepatic dysfunction and hepatotoxicity, in addition, the significantly lower plasma IgM content suggested that PP diet decreased the immunity of Japanese seabass.

TNFα and IL1β are the pro-inflammatory cytokines, whose biological functions are associated with inflammatory processes, hepatocyte proliferation, and liver regeneration in response to liver damage [47,48]. TGFβ1 and IL10 are the anti-inflammatory cytokines, meanwhile, TGFβ1 can also promote fibrosis in liver diseases [49]. In the present study, the both remarkable increased gene expression of pro- and anti-inflammatory cytokines in liver of fish in the PP group showed an obvious inflammatory and “self-saving" reactions [50]. TNFα and IL1β can trigger apoptosis, which is a highly organized and genetically controlled form of cell death that plays an important role in liver fibrosis. However, apoptosis also occurs as a defense mechanism such as killing cells which are damaged by disease or noxious agents [51]. Apoptosis is executed by caspase family protein through activating initiator caspase8 (extrinsic pathway) and caspase9 (intrinsic pathway), then activating executioner caspases3, cutting the different substrates in the cells [52,53]. In the current study, significantly increased hepatic caspase8 mRNA level and cleaved-caspase3 protein signal were observed in the PP group, indicating that TNFα and IL1β activated the exogenous apoptotic pathway in the PP group.

Apoptosis is regulated by cAMP-JNK/NF-kB-caspase signaling pathway [54–56]. As a member of MAPK super-family cascades, the prolonged activation of JNK could promote apoptosis [23,57,58]. The activity of JNK is tightly controlled by upstream cAMP/CREB and downstream NF-kB signaling pathway [55,59]. In addition to regulating lipid metabolism, the cAMP/CREB pathway has been reported to regulate inflammation and cell apoptosis through inhibiting JNK activation by increasing the production of anti-inflammatory cytokine, IL10 [25]. In the present study, the phosphorylation JNK in liver tissues had no significant difference between the two groups, which could be related to the positive “self-saving" regulation by the up-regulated IL10 induced by the high level of cAMP content and CREB gene expression in PP group. NF-kB pathway has been widely reported to prevent prolonged JNK activation in response to TNFα activation that can critically act a function as a “break” of JNK cascade activation. When the NF-kB activation absents, JNK activation was prolonged in response to TNFα, breaking the brake on apoptosis [23,60]. Classically, NF-kB refers to the p65/p50 complex, retaining in the cytoplasm with an inactive form. p65 NF-kB could be activated by some inflammatory cytokines, such as TNFα and IL1β, etc. and enter the nucleus rapidly to act as functions [61,62]. In the present study, higher and mainly cytoplasmic expressed p65 NF-kB could be observed in normal liver tissues, while less and mainly nuclear expressed p65 NF-kB existed in enlarged and vacuolation cells indicating that p65 NF-kB activation plays an important role in the liver apoptosis development. Further, more apoptosis signals (cleaved-caspase3) (red color) were observed in the PP group and clearly co-expressed with p65 NF-kB (green color) on the nucleus (blue color). Conversely, the cytoplasm expressed p65 NF-kB (the inactive form) did not express the signal of cleaved-caspase3. This phenomenon indicated that p65 NF-kB promoted the liver apoptosis, and simultaneously activated-p65 could also shut off the “break” of JNK cascade. The hemostasis phosphorylation of JNK protein protected the liver tissues from more serious damage.

In conclusion, Japanese seabass could well ingest the full plant protein diet, but the growth performance and immunity were significantly reduced and induced the overall metabolic disorder, which further caused the inflammatory reaction and fatty liver. The nutrient and energy metabolic disorder induced fatty liver in Japanese seabass fed full PP diet could be related to the low amino acid efficiency and cholesterol deficiency, which under the regulation by cAMP-JNK/NF-kB-caspase signaling pathway. The hemostasis phosphorylation of JNK protein protected the liver tissues from more serious damage.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.060.

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