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Branched-Chain Amino Acids Supplementation Mitigates Mitochondrial Damage Caused by High Glucose or High Fat Loads in Hepatocytes of Lateolabrax maculatus

Huijuan Ren , Yixiong Ke , Xueshan Li , Lin Wang , Kai Song , Guardiola Francisco A , Chunxiao Zhang , Kangle Lu * , Samad Rahimnejad *

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Article

Application of Branched-Chain Amino Acids Mitigates Mitochondrial Damage to Spotted Seabass (*Lateolabrax maculatus*) Hepatocytes Cultured in High-Glucose and High-Fat Media

Huijuan Ren [1], Yixiong Ke [1], Xueshan Li [1], Lin Wang [1], Kai Song [1], Francisco A Guardiola [2], Chunxiao Zhang [1] Kangle Lu [1,*] and Samad Rahimnejad [2,*]

[1] State Key Laboratory of Mariculture Breeding, Fisheries college of Jimei University, Xiamen 361021

[2] Immunobiology for Aquaculture Group, Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 3 0100 Murcia, Spain

* Correspondence: lukangle@jmu.edu.cn (K.L.); s.rahimnejad@um.es (S.R.)

Simple Summary: Mitochondria play a vital role in cellular metabolism, energy production, and survival. In aquaculture, high- glucose or high-fat diets can cause oxidative stress, metabolic disorders, and mitochondrial damage. Branched-chain amino acids (BCAAs), which are essential for animal growth, offer a range of biological benefits. This study demonstrated that BCAAs supplementation enhances mitochondrial function in spotted seabass (*Lateolabrax maculatus*) hepatocytes, effectively mitigating the damage caused by glucose or fat loads on these cells.

Abstract: This study explored the metabolic effects of branched-chain amino acids (BCAAs) on the hepatocytes of spotted seabass (*Lateolabrax maculatus*) under high-glucose (HG) or high-fat (HF) conditions. Hepatocytes were cultured under five different conditions: control, high glucose (HG), HG + BCAAs (Leu 0.8 mM, Ile 0.4 mM, Val 0.8 mM), high fat (HF), and HF + BCAAs (Leu 0.8 mM, Ile 0.8 mM, Val 0.8 mM). After 72 hours of culture, cells and cell supernatants were collected to measure relevant indicators. The results revealed that BCAAs supplementation significantly reduced glycogen and lipid accumulation in hepatocytes exposed to high-glucose or high-fat conditions ($P<0.05$). Additionally, alanine aminotransferase and aspartate aminotransferase activities in the supernatant were significantly decreased, indicating that BCAAs alleviated hepatocyte damage induced by these conditions. Furthermore, BCAAs addition markedly enhanced antioxidant activity by increasing superoxide dismutase and catalase activity, improving total antioxidant capacity, and reducing malondialdehyde levels. These findings suggest that BCAAs improve antioxidant defenses in hepatocytes under high-glucose or high-fat loads. Metabolic enzyme activity analysis revealed that BCAAs significantly increased the activities of citrate synthase (CS), alpha-ketoglutarate dehydrogenase complex (α-KGDHC), succinate dehydrogenase (SDH), phosphoenolpyruvate carboxykinase (PEPCK) and liver pyruvate kinase (LPS), while significantly decreasing fatty acid synthase (FAS) activity. Gene expression analysis further demonstrated that BCAAs supplementation downregulated the expression of lipogenic genes (*fas* and *srebp-1c*), and upregulating the expression of lipolytic genes (*ppaα* and *atgl*) as well as glucose metabolism-related genes (*g6pd*, *hk*, *pfk*, *pk*, *fbp*, and *g6pase*). Under high-glucose or high-fat conditions, hepatocytes exhibited decreased adenosine triphosphate (ATP) content, increased reactive oxygen species (ROS) levels, and reduced mitochondrial membrane potential. These adverse effects were mitigated by BCAAs supplementation. In conclusion, BCAAs supplementation alleviated hepatocyte damage caused by high-glucose or high-fat conditions, enhanced antioxidant defenses, and protected mitochondrial activity and function by promoting glucose and lipid metabolism.

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Keywords: Spotted seabass (*Lateolabrax maculatus*); Hepatocytes; High-Glucose or High-Fat Load; BCAAs; Glucose and Lipid Metabolism; Mitochondrial Function

1. Introduction

Branched-chain amino acids (BCAAs), comprising leucine (Leu), isoleucine (Ile), and valine (Val), are essential amino acids that must be obtained through dietary sources [1,2]. BCAAs are among the most abundant amino acids in proteins and play pivotal roles in protein synthesis, as well as key serving as carbon and nitrogen sources in metabolic pathways [3]. Maintaining a precise balance between BCAAs intake and metabolism is critical for physiological health.

Aberrant metabolism or incomplete oxidation of BCAAs, particularly Ile and Val, has been associated with catabolic stress and impaired mitochondrial function [4]. Multiple studies conducted on mammals, including humans, have shown that feeding high-fructose diets combined with elevated BCAAs levels increased cardiac myocyte apoptosis in mice [5]. Similarly, excessive BCAAs intake has been linked to adverse effects such as insulin resistance and mitochondrial dysfunction [4,6]. Conversely, other studies have highlighted the beneficial effects of BCAAs supplementation, including reduced muscle damage biomarkers and alleviated muscle soreness [7–14]. Diets with appropriate BCAAs levels have also been shown to promote metabolic health in rodents and humans by enhancing hepatic insulin sensitivity, inhibiting hepatic fat production, reducing protein degradation, and increasing energy expenditure [15–18].

Spotted seabass (*Lateolabrax maculatus*) is a carnivorous marine fish of significant economic value in China. However, feeding carnivorous fish high-carbohydrate diets has been shown to increase liver glycogen accumulation, lipid deposition, liver damage, and disrupt in glucose metabolism [19–21]. Similarly, prolonged high-fat diets intake impair lipid transport, compromise liver function, and can lead to fatty liver disease, threatening the physiological health of fish [22–24].

Given these challenges, spotted seabass serves as an excellent model for studying amino acid metabolism in fish. To better understand the metabolic effects of BCAAs in aquaculture species, this study investigated the impacts of BCAAs on the mitochondria of spotted seabass hepatocytes under high-glucose or high-fat conditions. Using isolated hepatocytes, the study aimed to elucidate the metabolic response of farmed fish to BCAAs, providing valuable insights into the regulation of BCAAs metabolism in marine fish.

2. Materials and Methods

2.1. Model Establishment

The cells used in this study were derived from a spotted seabass hepatocyte cell line, which was established through tissue block migration and multiple passages during the initial culture stages and maintained in our laboratory. Before the experiment, the cells were cultured in DMEM/F12 medium supplemented with 20% fetal bovine serum and 1% penicillin-streptomycin at 28°C under 5% CO_2 conditions. Prior to experimental treatments, the cells were grown to 70-80% confluence.

The experiment consisted of six experimental groups: control, high glucose (HG), HG + BCAAs (Leu 0.8 mM, Ile 0.4 mM, Val 0.8 mM), high fat (HF), and HF + BCAAs (Leu 0.8 mM, Ile 0.8 mM, Val 0.8 mM) groups, with three replicates per group. The control group was cultured in standard DMEM/F12 medium. The HG group was cultured in DMEM/F12 medium supplemented with 40 mM glucose [25], while the HF group was cultured in DMEM/F12 medium containing 0.1 mM fatty acids (prepared by mixing oleic acid and palmitic acid at a ratio of 1:1 from a 10 mM stock solution) [26] for 72 h. Following the treatments, the supernatant was collected. The cells were then washed twice with PBS, harvested using trypsin digestion, and washed again twice with PBS before further analysis.

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2.2. Measurement of Indicators

2.2.1. Cellular Biochemical Analyses

Hepatocytes were seeded in 6-well cell culture plates and cultured until stable adhesion was achieved before initiating the experimental treatments. After treatments, cells were collected for subsequent analyses. Glycogen content in each group was measured using Nanjing Jiancheng reagent kits (A043-1-1), following the manufacturer's instructions.

Cell culture supernatants were collected and centrifuged at 4°C at 1000 r/min for 10 minutes to separate the supernatant. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were quantified using Nanjing Jiancheng reagent kits following the provided protocols.

Hepatocytes were seeded in 24-well cell culture plates and cultured until stable adhesion was achieved prior to experimental treatments. After treatment, the culture medium was removed, and the cells were washed twice with PBS. The cells were then fixed with Oil Red O fixative for 20-30 minutes. Following fixation, the fixative was discarded, and the cells were washed twice with distilled water. To prepare for staining, 60% isopropanol was added for 20-30 seconds and then discarded. Freshly prepared Oil Red O staining solution was applied, and the cells were stained for 10-20 minutes. After removing the staining solution, the cells were washed with 60% isopropanol for 20-30 seconds until the interstitial spaces were clear. This was followed by 2-5 washes with distilled water. For nuclear counterstaining, Mayer's hematoxylin staining solution was applied for 1-2 minutes. The staining solution was then discarded, and the cells were washed 2-5 times with distilled water. Subsequently, Oil Red O buffer was applied for 1 minute and discarded. Finally, distilled water was added to cover the cells, and they were observed under an optical microscope (DM5000B, Leica).

2.2.2. Oxidative Stress-Related Indicators

2.2.2.1. Measurement of Antioxidant and Oxidative Stress Markers

Superoxide dismutase (SOD, A001-3-2) and catalase (CAT, A007-1-1) activities, malondialdehyde (MDA, A03-1-2) concentration, and total antioxidant capacity (T-AOC, A015-2-1) in hepatocytes were assessed using Nanjing Jiancheng reagent kits, according to the manufacturer's instructions.

2.2.2.2. Measurement of Reactive Oxygen Species (ROS) Concentration

After cell collection, the samples were washed twice with PBS. A DCFH-DA probe, diluted 1:1000 in serum-free culture medium, was added to the cells. The samples were incubated at 37°C for 20 minutes, followed by three washes with serum-free culture medium. Finally the cells were analyzed quantitatively using a flow cytometer (CytoFLEX, Beckman Coulter).

2.2.3. Key Enzymatic Activities in Hepatocyte Metabolism

The activities of citrate synthase (CS, BC1065), α-ketoglutarate dehydrogenase (α-KGDHC, BC0715), succinate dehydrogenase (SDH, BC0955), phosphoenolpyruvate carboxykinase (PEPCK, BC3315), and lipase (LPS, BC2345) in hepatocytes were measured using reagent kits from Beijing Solebao Technology Co., Ltd.

Fatty acid synthase (FAS, H231-1-1) activity and ATP (S0027) content in hepatocytes were assessed using reagent kits from Nanjing Jiancheng Co., Ltd. and Biyun Tian Biotechnology Co., Ltd., respectively.

2.2.4. Mitochondrial Status

2.2.4.1. Mitochondrial Membrane Potential Detection

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Cells were cultured in 6-well plates and allowed to adhere stably before undergoing experimental treatments. At the end of the experiment, the cells were collected and resuspended in 0.5 ml of cell culture medium. An equal volume (0.5 ml) of JC-1 staining working solution was added, and the mixture was gently inverted several times to ensure thorough mixing. The cells were incubated at 37°C in a cell culture incubator for 20 minutes. Following incubation, the cells were centrifuged at 600 ×g for 3-4 minutes at 4°C, and the pellet was collected. The cells were washed twice with JC-1 staining buffer and resuspended in 1 ml of the same buffer. This process was repeated twice, involving centrifuging at 600 ×g for 3-4 minutes at 4°C, discarding the supernatant, and resuspending the cells in JC-1 staining buffer. Finally, the cells were resuspended in an appropriate volume of JC-1 staining buffer and analyzed using a flow cytometer (CytoFLEX, Beckman Coulter).

2.2.4.2. Mitochondrial Activity Staining

Cells were cultured on glass coverslips (cell climbing slices). After the experimental treatments, the cell culture medium was removed, and Mito-Tracker Red CMXRos (C1035, Beyotime) working solution was added. The cells were incubated at 37°C for 15-30 minutes. Following incubation, the Mito-Tracker Red CMXRos working solution was removed, and the cells were stained with culture medium containing 1X Hoechst 33342 (C1027, Beyotime) live-cell staining solution. The cells were incubated at room temperature for 10 minutes. The staining solution was aspirated, and the cells were washed 2-3 times with PBS. An appropriate amount of anti-fluorescence quenching mounting medium was then applied. A coverslip was placed over the cells, which were subsequently observed using a laser confocal microscope (TCSSP8, Leica).

2.2.4.3. DNA Damage Detection

Cells were cultured on glass coverslip. Following experimental treatment, the medium was removed, and the cells were washed once with PBS. Then, 1 ml of fixing solution was added, and the cells were fixed for 5-15 minutes. After fixation, the fixing solution was aspirated, and the cells were washed three times with washing solution, with each wash lasting 3-5 minutes. Subsequently, 1 ml of immunostaining blocking solution was added, and the cells were blocked at room temperature for 10-20 minutes. The blocking solution was aspirated, and 1 ml of γ-H2AX mouse monoclonal antibody was added. The cells were incubated at room temperature for 1 hour. After incubation, the antibody solution was carefully removed, and the cells were washed three times with washing solution, each wash lasting 5-10 minutes. Next, 1 ml of anti-mouse 488 was added, and the cells were incubated at room temperature for 1 hour. Following this, the secondary antibody was aspirated, and the cells were washed twice with washing solution, with each wash lasting 5-10 minutes. For Nuclear staining, 1 ml of DAPI staining solution was added, and the cells were stained at room temperature for approximately 5 minutes. The staining solution was then aspirated, and the cells were washed three times with washing solution, with each wash lasting 3-5 minutes. Finally, an appropriate amount of anti-fluorescence quenching mounting medium was applied. A coverslip was placed over the cells, which were then observed under a laser confocal microscope (TCSSP8, Leica).

2.2.5. Fluorescence Quantification

Total RNA was extracted using the Trizol method, following the manufacturer's instructions. RNA concentration and purity were assessed using a microplate spectrophotometer at an absorbance ratio of 260/280 nm. The integrity of the RNA was confirmed through 1% agarose gel electrophoresis. To eliminate genomic DNA contamination, RNA samples were treated with RQ1 RNase-Free DNase. cDNA synthesis was performed using the Novozyme kit, in accordance with the manufacturer's protocol.

Real-time fluorescence quantitative PCR (qPCR) was conducted using the SYBR Green I fluorescence method on a QuantStudio Flex real-time PCR system (Thermo Scientific). The cycling conditions were set according to the kit instructions. Primer sequences were designed using Primer

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5.0 software and synthesed by Shanghai Shenggong Biological Engineering Co., Ltd. The relative expression of target genes was calculated using the 2−ΔΔCt method, with β-actin as the reference gene. The amplification efficiency of primers was validated for both the target and the reference genes. Each reaction was performed in triplicate to ensure accuracy. The primer sequences used in this study are listed in Table 1.

Table 1. Sequences of the primers used for real-time PCR.

target gene	forward sequence (5'–3')	reversed sequence (5'–3')	annealing temperature
g6pd	ATGCTCTGTTTGGTCGCCAT	ACATCCGACAGAGCAACAGG	60
hk	CTGGCTTGTGGGGACAGATT	GAGGCTGGCCCTCTTTATCC	60
pfk-1	CGAGGGGCTAAATGTCAGGG	AAGGGGCATTCCGGTGATTT	60
pk	GTGGCCCAATCCAAATGTCC	GCAAGAGTGAGAGTTGGGGT	60
fbp	AACTGAGAAAGTCCCCCGAC	CCGGCCAAAACCTCGTATCT	60
g6pase	CAGGTCATGGGGTACTGCTC	TTCCCGCTTTGGTTTCACCT	60
fas	AAACTGAAGCCCTGTGTGCC	CACCCTGCCTATTACATTGCTC	60
srebp-1c	CCTCACTCTGCAGCCAATCA	CGTAGTCCCACCCTCAAACC	60
ppaα	CCGTGCGTGTTTTCACCATT	AGACCAAATACATCGCCCCC	60
cpt-1α	CCTCAATGATACATCGGAACCC	CTGCGGCTCATCATCTAACG	60
hsl	CGAAACACAGAGACGGTCCA	TCATGACATCTACCAGCCGC	60
atgl	CTTCCTCTCCGCAACAAGTC	TGGTGCTGTCTGGAGTGTTC	60
drp1	CTCGCCAACAGAAACGGAAC	TGGCACTTTGGTCTTCGACA	60
mfn1b	GTCAACGCTATGCTGAGGGA	TCATCAGAGCCCTCCGTCTT	60
mfn2	TTCCAACGACCCAACACCAA	GTAGGCCCCCAACTGTTCAA	60
mul1	GCTGCCGTGATACGAGTCAT	ACGTTGGACAAGGACTGGAC	60
atg5	TCAGTCGCTGCCATTAGAGC	TCTCGTCACCTGCGAAAACT	60
pgc-1α	AACCCGACTCTTATCCCTCC	CGTATCAACGCCACAGCAC	60
pgc-1β	GTTCCTCCGAACTCCCAGTG	GCAACACCCCTCCAACTACA	60
fis1	GTCCCGGGAGTCATCCTTTG	ACAATGAGCTGGTGAAGGGAG	60
β-actin	CAACTGGGATGACATGGAGAAG	TTGGCTTTGGGGTTCAGG	60

Note: *g6pd*, glucose-6-phosphate dehydrogenase; *hk*, hexokinase; *pfk-1*,phosphofructokinase-1; *pk*, pyruvate kinase; *fbp*, fructose 1, 6-bisphosptase; *g6pase*, glucose-6-phosphatase G-6-pase; *fas*, fatty acid synthase; *srebp-1c*, Sterol regulatory element-binding protein 1c; *ppaα*,peroxisome proliferators-activated receptors; *cpt-1α*,carnitine palmitoyl transferase 1A;*hsl*,hormone-sensitive triglyceride lipase; *atgl*, Adipose Triglyceride;*drp1*, Dynamin-Related Protein 1; *mfn1b*, mitofusin1b; *mfn2*, mitofusin2; *mull*, mitochondrial E3 ubiquitin protein ligase 1; *atg5*, autophagy related 5; *pgc-1α*, proliferator-activated receptor gamma co-activator 1α; *pgc-1β*, proliferator-activated receptor gamma co-activator 1β; *fis1*, mitochondrial fission protein 1; *β-actin*, beta-actin.

2.3. Data Analysis

Variance analysis was performed using SPSS version 23.0. Statistical significance was assessed through one-way ANOVA or t-test. Differences among the experimental groups were evaluated using Dunnett's Test for multiple comparisons. Results are presented as the mean ± standard deviation (SD), with statistical significance set at $P < 0.05$.

3. Results

3.1. Cellular Biochemistry

The addition of BCAAs during cell culture effectively alleviated lipid droplet formation and reduced glycogen accumulation in hepatocytes induced by high-glucose or high-fat exposure.

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Furthermore, ALT and AST activities in the culture medium were significantly lower in the BCAAs-treated group compared to the HG and HF groups (P <0.05; Figure 1).

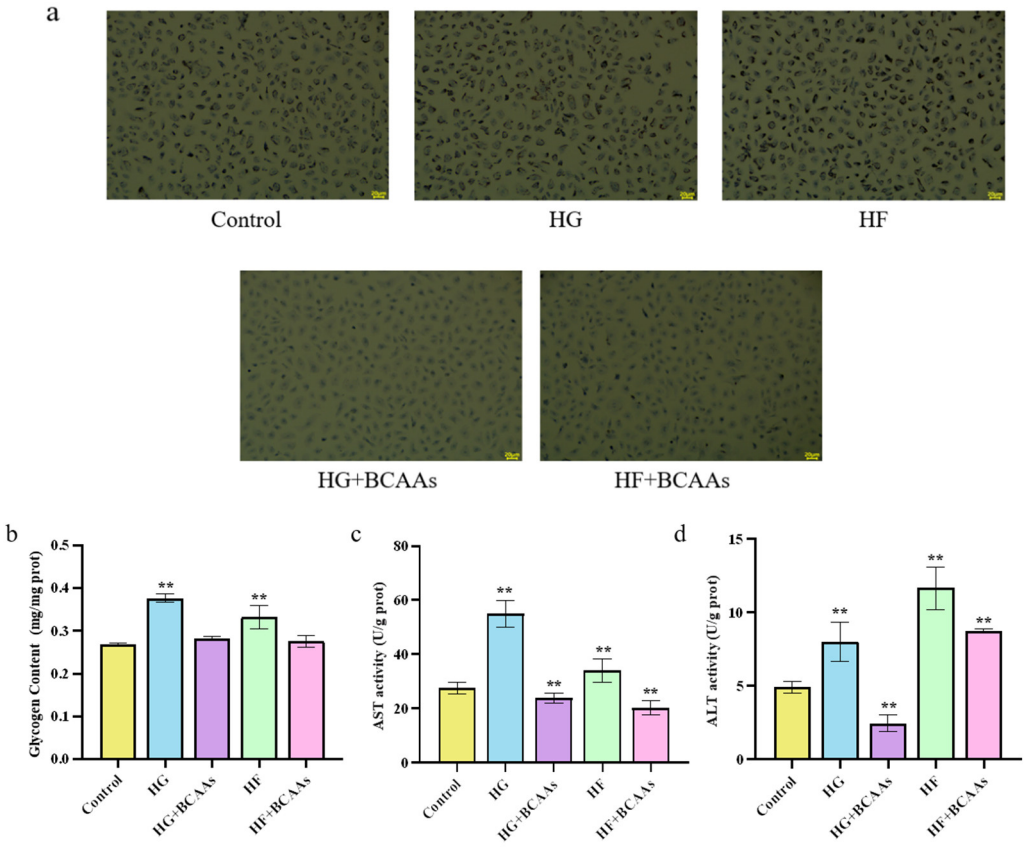

Figure 1. Effects of BCAAs supplementation on hepatocyte biochemistry in spotted seabass (*Lateolabrax maculatus*): (a) Lipid deposition, (b) Glycogen content, (c) ALT activity, (d) AST activity. **Indicates extremely significant differences between groups ($P < 0.01$).

3.2. Cellular Antioxidant Defense

As shown in Figure 2, BCAAs supplementation under high-glucose or high-fat conditions significantly enhanced SOD activity ($P < 0.05$), and T-AOC ($P < 0.05$) in hepatocytes. Additionally, it markedly reduced MDA content ($P < 0.05$) and decreased the ROS production.

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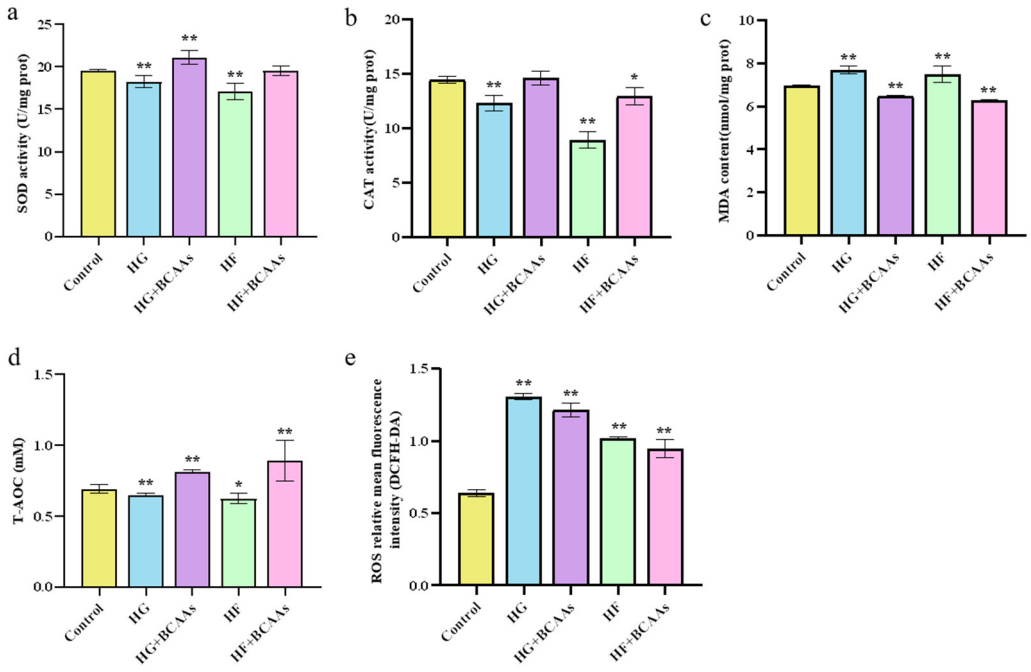

Figure 2. Effects of BCAAs supplementation on antioxidant capacity in hepatocytes of spotted seabass (*Lateolabrax maculatus*): (a) SOD activity, (b) CAT activity, (c) MDA content, (d) T-AOC, (e) ROS concentration. *Indicates significant differences between groups ($P < 0.05$), ** indicates extremely significant differences between groups ($P < 0.01$).

3.3. Cellular Metabolism

The activity of key metabolic enzymes involved in the citric acid cycle is presented in Figure 3. Under high-glucose or high-fat conditions, BCAAs supplementation significantly increased the activity of CS, α-KGDHC, SDH, PEPCK, and LPS ($P < 0.05$) while reducing FAS activity ($P < 0.05$). This was accompanied by a significant increase in ATP content ($P < 0.05$).

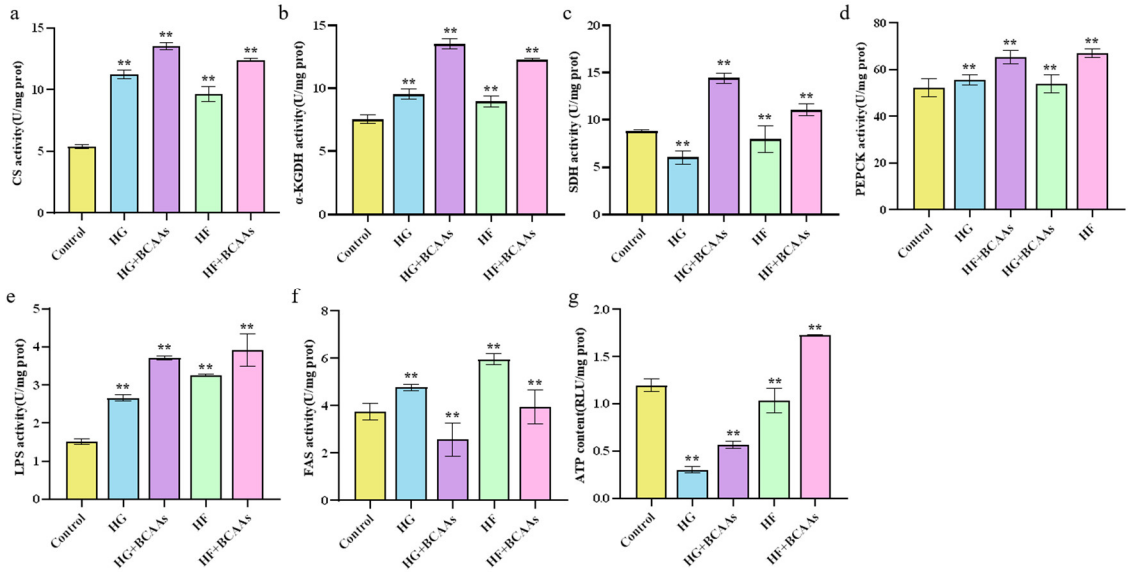

Figure 3. Effects of BCAAs supplementation on hepatocyte metabolism in spotted seabass (*Lateolabrax maculatus*): (a) CS activity, (b) CAT activity, (c) SDH content, (d) PEPCK activity, (e) LPS activity, (f) FAS activity. ** Indicates extremely significant differences between groups ($P < 0.01$).

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The expression of genes related to glucose and lipid metabolism in hepatocytes is shown in Figure 4. Compared to the control group, BCAAs supplementation significantly downregulated the expression of lipogenic genes *fas* and *srebp-1c* (*P<0.05*), while significantly upregulating the expression of lipolytic genes *ppaα* and *atgl* (*P < 0.05*). Furthermore, genes involved in glucose metabolism, including *g6pd*, *hk*, *pfk*, *pk*, *fbp*, and *g6pase*, were significantly upregulated (*P < 0.05*).

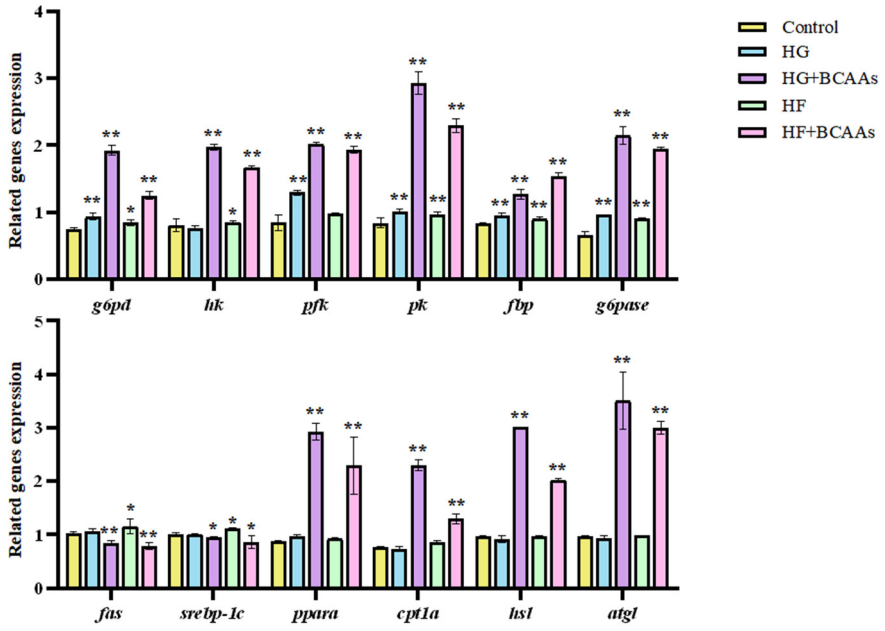
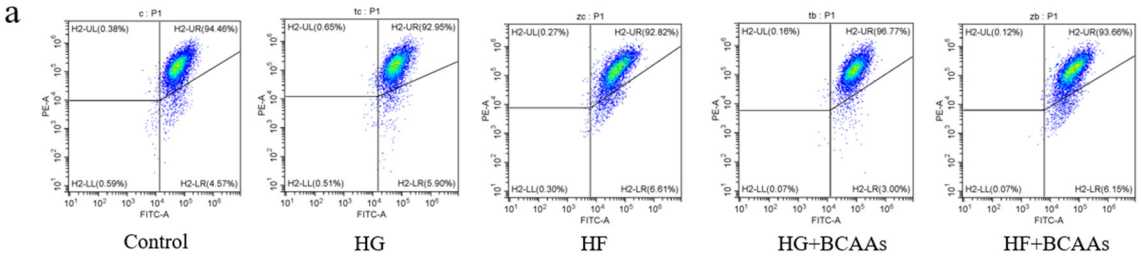


Figure 4. Effects of BCAAs supplementation on the expression of genes related to glucose and lipid metabolism in hepatocytes of spotted seabass (*Lateolabrax maculatus*) under high-glucose or high-fat load. *Indicates significant differences between groups (*P < 0.05*), ** indicates extremely significant differences between groups (*P < 0.01*).

3.4. Mitochondrial Function

The effects of BCAAs supplementation on the mitochondrial membrane potential of *L. maculatus* are shown in Figure 5. BCAAs incorporation alleviated the reduction in membrane potential induced by high-glucose or high-fat conditions, enhanced mitochondrial activity, and reduced DNA damage in hepatocytes.



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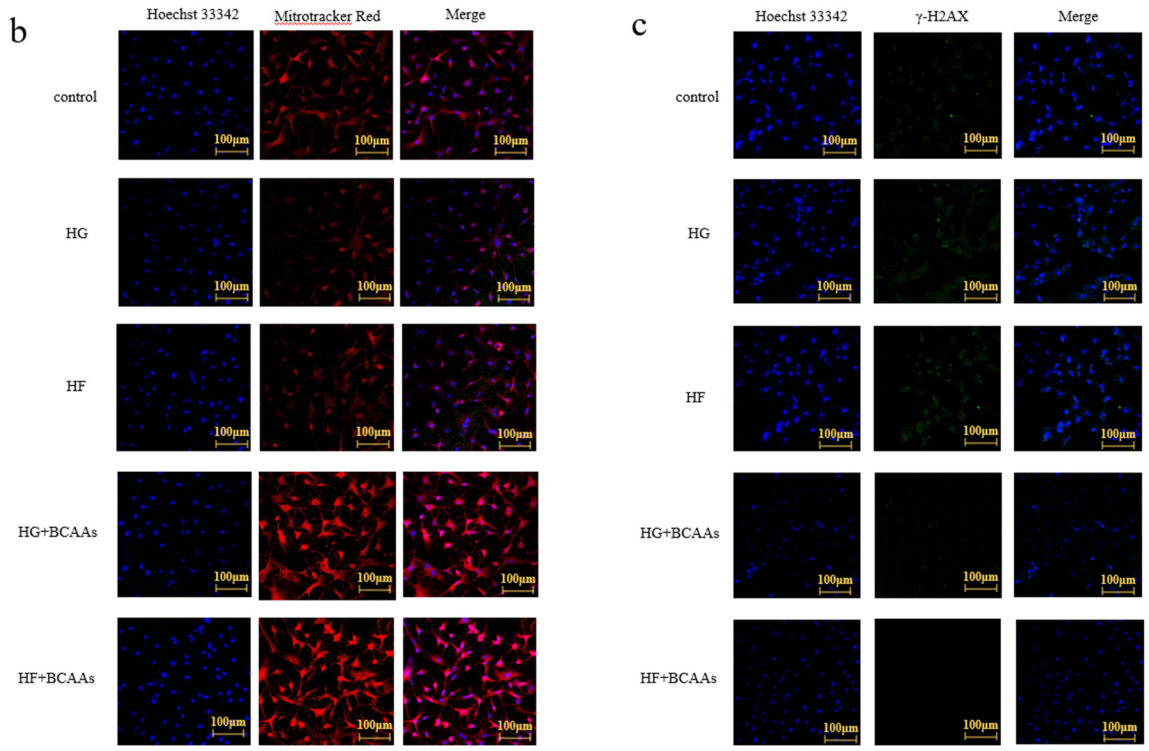

Figure 5. Effects of BCAAs supplementation on mitigating hepatocyte mitochondrial damage induced by high-glucose or high-fat conditions: (a) Cell Membrane Potential, (b) Mitochondrial Activity, (c) Mitochondrial DNA Damage.

The expression of genes associated with hepatocyte mitochondrial fusion, fission, biogenesis, and autophagy is displayed in Figure 6. BCAAs supplementation significantly promoted the expression of mitochondrial fusion genes *pgc1α* and *pgc1b*, as well as biogenesis genes *mfn1b* and *mfn2* (*P<0.05*). Conversely, it suppressed the expression of fission-related genes *fis1* and *drp1*, along with autophagy-related genes *mul1* and *atg5* (*P<0.05*).

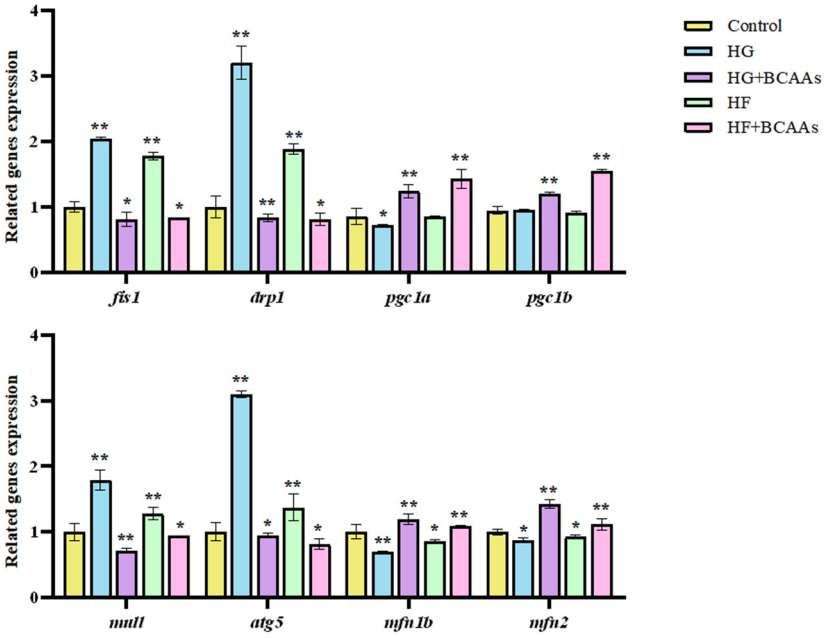

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Figure 6. Effects of BCAAs supplementation on the expression of genes related to mitochondrial fusion and fission under high-glucose or high-fat conditions. *Indicates significant differences between groups ($P < 0.05$), ** indicates extremely significant differences between groups ($P < 0.01$).

4. Discussion

4.1. BCAAs Supplementation Mitigated Metabolic Disorders in Hepatocytes Induced by High-Glucose or High-Fat Load

The liver is a critical metabolic organ in fish and other animals, making it a frequent focus of metabolism-related studies. Previous research has demonstrated that high-glucose or high-fat diets can lead to glycogen and lipid deposition, resulting in liver damage [27,28]. In the present study, ALT and AST activities in the supernatant of hepatocytes significantly increased under high-glucose or high-fat conditions, indicating liver damage. However, the addition of BCAAs alleviated lipid deposition and glycogen accumulation in hepatocytes subjected to these conditions, while also reducing ALT and AST activities. This finding aligns with studies in mice fed high-fructose or high-fat diets, suggesting that isolated hepatocytes exhibit metabolic behavior similar to *in-vivo* conditions.

Enzymes in the liver provide the catalytic foundation for physiological metabolism in fish [29]. To better understand the effects of BCAAs on glucose and lipid metabolism in hepatocytes, the activities of key enzyme involved in the citric acid cycle, gluconeogenesis, and lipid metabolism were evaluated. BCAAs supplementation significantly increased the activities of CS, α-KGDHC, SDH, PEPCK, and LPS while reducing FAS activity. Enhanced activities of CS, α-KGDHC, and SDH indicate increasedentry of glucose and lipids into the citric acid cycle. PEPCK, a rate-limiting enzyme in gluconeogenesis [30,31], also showed higher active in the BCAA-supplemented groups, reflecting accelerated gluconeogenesis and reduced glycogen accumulation.

HK, G6pd, PFK, and PK are pivotal glycolytic enzymes essential for energy production. FBP and G6Pase, key regulators of gluconeogenesis, play critical roles in glycogen breakdown and maintaining blood glucose homeostasis [32–34]. The study demonstrated that in the BCAAs-supplemented groups, gene expression of glycolysis-promoting enzymes (*g6pd, hk, pfk, pk*) and gluconeogenesis-related enzymes (*fbp, g6pase*) were significantly upregulated. These findings suggest that BCAAs supplementation enhances both glycolysis and gluconeogenesis in hepatocytes, thereby strengthening energy metabolism and reducing glycogen accumulation.

Sterol regulatory element-binding protein (SREBP) family members are key regulators of cholesterol, fatty acids, triglycerides, and glycerophospholipids synthesis, with SREBP-1c specifically controlling the transcription of lipogenic genes such as *fas* and *acc*. FAS and acetyl-CoA carboxylase (ACC) are major enzymes involved in fatty acid biosynthesis [35–37]. ATGL plays a critical role in lipolysis, initiating triglyceride catabolism. Reduced ATGL expression can lead to triglyceride accumulation, contributing to obesity and other metabolic disorders [38–41]. This study found that BCAAs supplementation significantly downregulated the expression of lipogenic genes *fas* and *srebp-1c*, while upregulating lipolytic genes *ppaα* and *atgl*. These findings suggest that BCAAs promote lipid breakdown and inhibit lipid synthesis, thereby alleviating fat deposition in the liver of *L. maculatus*. BCAAs likely modulate the expression of SREBP precursors, reducing *srebp-1c* expression and its downstream targets *fas* and *acc*. Simultaneously, BCAAs enhanced the expression of lipolysis-related genes such as *atgl*, thereby improving lipid homeostasis by reducing triglyceride accumulation.

Antioxidant markers provide valuable insights into organismal health. AOC reflects the cumulative antioxidant potential from substances and enzymes, with SOD and CAT serving as key enzymes for neutralizing free radicals [42,43]. MDA, a product of lipid peroxidation, serves as a marker of oxidative stress [44,45]. In this study, high-glucose and high-fat conditions caused a significant decline in T-AOC, and activities of SOD, and CAT, along with increased MDA levels, indicating oxidative stress in *L. maculatus* hepatocytes. However, BCAAs supplementation effectively alleviated oxidative stress, restoring antioxidant capacity and enzyme activity.

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4.2. Adding BCAAs Alleviated Mitochondrial Damage Caused by High-Glucose or High-Fat Loads

The structure and function of mitochondria are intricately linked to liver metabolism [46]. The mitochondrial membrane acts as a critical barrier, protecting the organelle from damage. However, its permeability increases significantly when mitochondria are compromised [47,48]. In this study, high-glucose or high-fat exposure caused a decrease in mitochondrial membrane potential and a decline in mitochondrial activity in hepatocytes. Conversely, BCAAs supplementation helped maintain normal mitochondrial membrane potential and activity, suggesting that BCAAs stabilize the mitochondrial membrane and preserve mitochondrial function.

In cells, the mitochondrial respiratory chain is the primary site of ROS production. Disruption of this chain can lead to electron leakage, which combines with oxygen and other molecules to generate ROS. This study found that high-glucose or high-fat conditions inhibited ATP production and increased ROS levels. However, BCAAs supplementation significantly alleviated these adverse effects.

Mitochondrial DNA (mt DNA), a circular DNA located within mitochondria, encodes proteins essential for energy metabolism. Unlike nuclear DNA, mt DNA is highly susceptible to oxidative damage caused by ROS, which can impair mitochondrial function and overall cellular health. This study demonstrated that BCAAs supplementation significantly mitigated mt DNA damage induced by high-glucose or high-fat conditions, reducing oxidative stress and preserving mitochondrial integrity in hepatocytes.

Mitochondria are highly dynamic organelles that undergo constant renewal under normal physiological conditions. Two key processes—mitochondrial biogenesis (the generation of new mitochondria) and mitochondrial autophagy (the clearance of damaged or aged mitochondria)—work together to maintain mitochondrial homeostasis [49,50]. PGC-1α and PGC-1β, are crucial transcriptional regulators of mitochondrial biogenesis. They coordinate the activation of downstream transcription factors, enhancing mt DNA transcription and the synthesis of key mitochondrial enzymes, thereby promoting mitochondrial generation [51]. Notably, PGC-1β has been found to be more effective than PGC-1α in driving mitochondrial biogenesis in fish.

This study demonstrated that high-glucose or high-fat exposure significantly downregulated the expression of *pgc-1α* and *pgc-1β*, while BCAAs supplementation restored their expression levels. These findings indicate that BCAAs can reverse the inhibition of mitochondrial biogenesis caused by high-glucose or high-fat conditions in the liver of *L. maculatus*, thereby promoting mitochondrial generation.

Mitochondrial autophagy, regulated by PINK1/Parkin pathway, is another critical process for maintaining mitochondrial health, with genes such as *mul1* and *atg5* playing essential roles in this pathway [52]. This study revealed that high-glucose or high-fat exposure significantly suppressed the expression of *mul1* and *atg5*. However, BCAAs supplementation upregulated these genes, thereby activating mitochondrial autophagy in the liver of *L. maculatus*. A balanced relationship between mitochondrial autophagy and biogenesis is vital for maintaining mitochondrial function. Disruption of this balance can impair mitochondrial homeostasis and biogenesis. The findings of this study underscore the protective effects of BCAAs in maintaining this balance, reducing mitochondrial damage, and promoting overall mitochondrial health in hepatocytes under high-glucose or high-fat stress.

5. Conclusion

In summary, the isolated hepatocytes of *L. maculatus* exhibited physiological responses comparable to those observed *in-vivo* under high-glucose or high-fat conditions, establishing them as a reliable model for studying fish metabolism. BCAAs supplementation effectively alleviated mitochondrial damage in hepatocytes exposed to high-glucose or high-fat loads, promoted mitochondrial biogenesis, and preserved oxidative phosphorylation processes. These effects mitigated oxidative stress-induced damage to both hepatocytes and mitochondria, thereby

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supporting the maintenance of normal cellular metabolic homeostasis. In the future, aquaculture production can benefit from optimizing feed formulations by incorporating appropriate amounts of BCAAs. This strategy has the potential to improve feed utilization, reduce the incidence of metabolic diseases during the breeding process, and promote the healthy growth and production efficiency in fish.

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Institutional Review Board Statement: Ethical approval is not necessary, as this study only uses the liver cell line of the spotted sea bass.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Tamanna,N.; Mahmood,N. Emerging Roles of Branched-Chain Amino Acid Supplementation in Human Diseases. *Int. Math. Res. Not*.2014, 235619.
2. De Bandt,J.P.; Coumoul,X.; Barouki,R. Branched-Chain Amino Acids and Insulin Resistance, from Protein Supply to Diet-Induced Obesity. Nutrients.2022, 15,68.
3. Bonvini,A.; Rogero,M.M.; Coqueiro,A.Y.; Raizel,R.; Bella,L.M.; Fock, R. A.; Borelli, P.; Tirapegui, J. Effects of different branched-chain amino acids supplementation protocols on the inflammatory response of LPS-stimulated RAW 264.7 macrophages. AminoAcids. 2021, 53,597–607.
4. Valerio,A.D.; Antona,G.; Nisoli,E. Branched-chain amino acids, mitochon-drial biogenesis, and healthspan: an evolutionary perspective. Aging.2011,3,464–478.
5. Blair,M.C.; Neinast,M.D.; Arany,Z. Whole-body metabolic fate of branched-chain amino acids. Biochemical.J. 2021, 478,765–776.
6. Ra,S.G.; Miyazaki,T.; Ishikura.K.; Nagayama,H.; Komine,S.; Nakata,Y.; et al. Combined effect of branched-chain amino acids and taurine supple-mentation on delayed onset muscle soreness and muscle damage inhigh-intensity eccentric exercise.J. Int. Soc. Sports Nutr.2013,10,51.
7. Fouré,A.; Bendahan,D. Is branched-chain amino acids supplementation an efficient nutritional strategy to alleviate skeletal muscle damage? A systematic review. Nutrients. 2017,9,E1047.
8. Holeček,M. Branched-chain amino acid supplementation in treatment of liver cirrhosis: updated views on how to attenuate their harmful effects on cataplerosis and ammonia formation. Nutrition. 2017,41,80–85.
9. Weber,M.G.; Dias,S.S.; de Angelis,T.R.; Fernandes,E.V.; Bernardes,A.G.; Milanez,V.F.; et al. The use of BCAA to decrease delayed-onset muscle soreness after a single bout of exercise:a systematic review and meta-analysis. Amino Acids.2021,53,1663–1678.
10. Doma,K.; Singh,U.; Boullosa,D.; Connor,J.D. The effect of branched-chain amino acid on muscle damage markers and performance following strenuous exercise: a systematic review and meta-analysis. Nutr. Metab.2021,46,1303–1313.
11. Hormoznejad,R.; Zare Javid,A.; Mansoori,A. Effect of BCAA supplemen-tation on central fatigue, energy metabolism substrate and muscle damage to the exercise: a systematic review with meta-analysis. Sport Sci Health. 2019,15,265–279.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 February 2025 doi:10.20944/preprints202502.0661.v1

12. Khemtong,C.; Kuo,C.H.; Chen,C.Y.; Jaime,S.J.; Condello,G. Does branched-chain amino acids (BCAAs) supplementation attenuate muscle damage markers and soreness after resistance exercise in trained males? A meta-analysis of randomized controlled trials. Nutrients. 2021,13,1880.
13. Rahimi,M.H.; Shab-Bidar,S.; Mollahosseini,M.; Djafarian,K. Branched-chain amino acid supplementation and exercise-induced muscle damage in exercise recovery: A meta-analysis of randomized clinical trials. Nutrition.2017,42,30–36.
14. Rahimlou,M.; Ramezani,A.; Mahdipour,M.; Palimi,E. Moradipoodeh B. Reduction of muscle injuries and improved post-exercise recovery by branched-chain amino acid supplementation: a systematic review and meta-analysis. J Nutr Health Aging.2020,8,1–16.
15. McGarrah,R.W.; White,P.J. Branched-chain amino acids in cardiovascular disease. Nat. Rev. Cardiol,2023,20,77-89.
16. Lauer,S.M.; et al. Recruitment of BAG2 to DNAJ-PKAc scaffolds promotes cell survival and resistance to drug-induced apoptosis in fibrolamellar carcinoma. Cell Rep.2024, 43,113678.
17. Lo,E.K.K.; et al. The Emerging Role of Branched-Chain Amino Acids in Liver Diseases. Biomedicines, 2022,10,1444.
18. Ananieva,E.A.; Van Horn,C.G.; Jones,M.R.; Hutson,S.M. Liver BCATm transgenic mouse model reveals the important role of the liver in maintaining BCAA homeostasis. J. Nutr. Biochem.2017, 40,132–140.
19. Kamalam,B.S.; Medale,F.; Panserat,S. Utilisation of dietary carbohydrates in farmed fishes: New insights on influencing factors, biological limitations and future strategies. Aquaculture. 2017, 467,3-27.
20. Sari,D.N. ; Ekasari,J. ; Nasrullah,H; Suprayudi,M.A. ; Alimuddin,A. High carbohydrate increases amylase, plasma glucose, and gene expression related to glycolysis in giant gourami *Osphronemus goramy*. Fish Physiology and Biochemistry.2022, 48,1495-1505.
21. Lin,S.M.; Shi,C.M.; Mu,M.M.; et al. Effect of high dietary starch levels on growth, hepatic glucose metabolism, oxidative status and immune response of juvenile *largemouth bass*, Micropterus salmoides. Fish Shellfish Immunol.2018, 78,121-126.
22. Lu,K.L.; Wang,L.N.; Zhang,D.D.; et al. Berberine Attenuates Oxidative Stress and Hepatocytes Apoptosis Via Protecting Mitochondria in Blunt Snout Bream Megalobrama amblycephala Fed High- Fat Diets. Fish Physiol. Biochem, 2017, 43,65-76.
23. Xia,T.; Mao,X.; Zhang,J.; Rahimnejad,S.; Lu,K. Effects of quercetin and hydroxytyrosol supplementation in a high-fat diet on growth, lipid metabolism, and mitochondrial function in spotted seabass (*Lateolabrax maculatus*), Aquaculture.2024, 582, 740538.
24. Dong,Y.; Yu,M.; Wu,Y.; Xia,T.; Wang,L.; Song,K.; Zhang,C.; Lu,K.; Rahimnejad,S. Hydroxytyrosol Promotes the Mitochondrial Function through Activating Mitophagy. Antioxidants.2022, 11, 893.
25. Ren,H.J.; Ke,Y.X.; Lu,K.L.; Li,X.S.; Wang,L.; Song,K.; Zhang,C.X. Effects of high-glucose load on energy metabolism and mitochondrial function of hepatocytes from spotted seabass (*Lateolabrax maculatus*). Journal of Fishery Sciences of China,2024,31,513-523.
26. Mao,X.J. Alleviation effect and mechanism of coenzyme Q10 on excessive fat deposition in liver of *Lateolabrax maculatus* induced by high-fat diet. Jimei University, China, Xiamen,2024.
27. Jung,S.M.; Doxsey,W.G.; Le,J.; Haley,J.A.; Mazuecos,L.; Luciano,A.K.; Li,H.; Jang,C.; Guertin,D.A. In vivo isotope tracing reveals the versatility of glucose as a brown adipose tissue substrate. Cell Rep.2021,36,109459.
28. Zhu,J.; Pan,J.; Wang,X.; et al. Alleviation of the adverse effect of dietary carbohydrate by supplementation of myo-inositol to the diet of Nile tilapia (*Oreochromis niloticus*). Animals.2020, 10,2190.
29. Li,S.L.; Li,Z.Q.; Zhang,J.C.; et al. The impacts of dietary carbohydrate levels on growth performance, feed utilization, glycogen accumulation and hepatic glucose metabolism in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus*♂). Aquaculture.2019, 512, 734351
30. Bornstein,M.R.; Neinast,M.D.; Zeng,X.; Chu,Q.; Axsom,J.; Thorsheim,C.; Li,K.; Blair,M.C.; Rabinowitz,J.D.; Arany,Z. Comprehensive quantification of metabolic flux during acute cold stress in mice. Cell Metab.2023, 35:2077–2092.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 February 2025 doi:10.20944/preprints202502.0661.v1

31. Qian,L.; Li,N.; Lu,X.C.; Xu,M.; Liu,Y.; Li,K.; Zhang,Y.; Hu,K.; Qi,Y.T.; Yao,J.; Wu,Y.L.;Wen,W.;Huang,S.; Chen,Z.J.; Yin,M.; Lei,Q.Y. Enhanced BCAT1 activity and BCAA metabolism promotes RhoC activity in cancer progression. Nat Metab. 2023,5,1159-1173.
32. Wang,J.; Liu,Y.; Lian,K.; Shentu,X.; Fang,J.; Shao,J.; et al. BCAA catabolic defect alters glucose metabolism in lean mice. Front. Physiol.2019,10,1140.
33. Hui,S.; Cowan,A.J.; Zeng,X.; Yang,L.; TeSlaa,T.; Li,X.; Bartman,C.; Zhang,Z.; Jang,C.; Wang,L.; et al. Quantitative Fluxomics of Circulating Metabolites. Cell Metab.2020, 32,676–688.
34. Yoneshiro,T.; Kataoka,N.; Walejko,J.M.; Ikeda,K.; Brown,Z.; Yoneshiro,M.; Crown,S.B.; Osawa,T.; Sakai,J.; McGarrah,R.W.; et al. Meta-bolic flexibility via mitochondrial BCAA carrier SLC25A44 is required for optimal fever. Elife,2021, 10, 66865.
35. Espenshade,P.J. SREBPs:Sterol-Regulated Transcription Factors. J Cell Sci. 2006, 119,973-976.
36. Brown,M.S.; Goldstein,J.L.; Cholesterol Feedback: from Schoenheimer's Bottle to Scap's MELADL. Lipid Res. 2009, 50 Suppl,S15-S27.
37. Dong,Y.Z.; Wei,Y.; Wang,L.; Song,K.; Zhang,C.X.; Lu,K.L.; Rahimnejad,S. Dietary n-3/n-6 PUFA ratio modulates growth performance in spotted seabass (*Lateolabrax maculatus*) through regulating lipid metabolism, hepatic antioxidant capacity and intestinal health. Anim Nutr. 2023, 20-31.
38. Athanikar,J.N.; Sanchez,H.B.; Osborne,T.F. Promoter Selective Transcriptional Synergy Mediated by Sterol Regulatory Element Binding Protein and Sp1: a Critical Role for the Btd Domain of Sp1.Mol Cell Biol. 1997, 17,5193-5200.
39. Wilentz,R.E.; Witters,L.A.; Pizer,E.S. Lipogenic Enzymes Fatty Acid Synthase and Acetyl-Coenzyme a Carboxylase are Coexpressed with Sterol Regulatory Element Binding Protein and Ki-67 in Fetal Tissues. Pediatr Dev Pathol. 2000, 3,525-531.
40. Zimmermann,R.; Strauss,J.G.; Haemmerle,G.; et al. Fat Mobilization in Adipose Tissue is Promoted by Adipose Triglyceride Lipase. Science.2004, 306,1383-1386.
41. Schreiber,R.; Xie,H.; Schweiger,M. Of Mice and Men: the Physiological Role of Adipose Triglyceride Lipase (ATGL). BBA.2019,1864,880-899.
42. Calabrese,G.; Morgan,B.;Riemer, J. Mitochondrial Glutathione: Regulation and Functions. ARS. 2017, 27,1162–1177.
43. Zhan,Q.Y.; Han,T.; Li,X.Y.; et al. Effects of dietary carbohydrate levels on growth, body composition, and gene expression of key enzymes involved in hepatopancreas metabolism in mud crab Scylla paramamosain. Aquaculture. 2020, 529,735638.
44. Forman,H.J.; Zhang,H.; Rinna,A.; Glutathione: Overview of its protective roles, measurement, and biosynthesis. Mol. Aspects Med.2009, 30, 1–12.
45. Pfanner,N.; Warscheid,B.; Wiedemann,N. Mitochondrial proteins: From biogenesis to functional networks. Nat. Rev. Mol. Cell Biol. 2019, 20,26.
46. Palikaras,K.; Lionaki,E. Taverarakis. Balancing Mitochondrial Biogenesis and Mitophagy to Maintain Energy Metabolism Homeostasis. Cell Death Differ.2015,22,1399-1401.
47. Yu,E.P.K.; Bennett,M.R. The Role of Mitochondrial DNA Damage in the Development of Atherosclerosis. Free Radic. Biol. Med.2016,100: 223-230.
48. Mao,S.; Leone,T.C.; Kelly,D.P.; et al. Mitochondrial Transcription Factor A is Increased but Expression of ATP Synthase Beta Subunit and Medium-Chain Acyl-CoA Dehydrogenase Genes are Decreased in Hearts of Copper-Deficient Rats. J Nutr. 2000, 130,2143-2150.
49. Bremer,K.; Kocha,K.M.; Snider,T.; et al. Sensing and Responding to Energetic Stress:The Role of the AMPK-PGC1alpha-NRF1 Axis in Control of Mitochondrial Biogenesis in Fish. Comp Biochem Physiol B Biochem Mol Biol. 2016, 199,4-12.
50. Lazarou,M.; Sliter,D.A.; Kane,L.A.;et al. The Ubiquitin Kinase PINK1 Recruits Autophagy Receptors to Induce Mitophagy. Nature (London).2015, 524,309-314.
51. Palikaras,K.; Taverarakis,N. Mitochondrial Homeostasis: The Interplay between Mitophagy and Mitochondrial Biogenesis. Exp. Gerontol.2014, 56,182-188.
52. Beaulant,A.; Dia,M.; Pillot,B.; et al. Endoplasmic Reticulum-Mitochondria Miscommunication is an Early and Causal Trigger of Hepatic Insulin Resistance and Steatosis. J. Hepatol.2022,77,710-722.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 February 2025 doi:10.20944/preprints202502.0661.v1

53. Watanabe,T. Strategies for Further Development of Aquatic Feeds. Fisheries Sci.2022, 68,242-252.

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