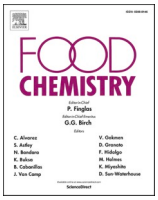
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Dietary L-carnitine supplementation recovers the increased pH and hardness in fillets caused by high-fat diet in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The wide use of high-fat diet (HFD) causes negative effects on flesh quality in farmed fish. L-carnitine, a lipid-lowering additive, enhances mitochondrial fatty acid β-oxidation. However its roles in alleviating the effects of HFD on flesh quality in fish are unknown. We fed Nile tilapia with medium-fat diet (MFD, 6% dietary lipid), high-fat diet (HFD, 12% dietary lipid) and HFCD supplemented with L-carnitine (HFCD + 400 mg/kg L-carnitine) for 10 weeks. The HFD-fed fish had higher fat deposition, pH value, myofiber density and flesh hardness than those fed on MFD. However, feeding the fish with the HFCD improved lipid catabolism, which increased significantly lactic acid content and myofiber diameter in muscle, thus reduced pH and hardness values. HFCD also reduced endoplasmic reticulum stress and myofiber apoptosis caused by HFD in the fish. Our study suggests that dietary L-carnitine supplementation alleviates the negative effects of HFD on flesh quality of farmed fish.

1. Introduction

Globally, the cost of protein feed materials has increased significantly, due to rapid aquaculture development and shortage of wild fisheries resources. Accordingly, high-fat diet (HFD) is commonly used in aquaculture to save protein due to its "protein sparing effect" (Li, Jiang, Liu, & Ge, 2012). However, feeding farmed fish with HFD for long-term causes excessive lipid deposition in the body and induces metabolic diseases (Du, Clouet, Huang, Degrace, Zheng, He, et al., 2008). Consequently, feeding tilapia (*Oreochromis niloticus*) with HFD for extended period reduced antioxidant defense system, induced cell apoptosis and elevated inflammation (Jia et al., 2020). Similarly, HFD significantly increased fat content in muscle and whole-body in triploid brown trout (*Salmo trutta*) (Regost, Arzel, Cardinal, Laroche, & Kaushik, 2001). Recently, we have also shown that, feeding Nile tilapia with HFD for long period affected flesh quality (Lv, Ma, Hu, Lin, Chen, Zhang, et al., 2020) by decreasing moisture and increasing lipid contents in muscle, which changed flesh quality and flavor. These studies suggest that, feeding farmed fish with HFD for long time causes adverse effects in the body.

Previous studies in mice also reported significant effects of HFD on growth and development of myofiber in muscle tissue. For instance, feeding mice with HFD caused a remarkable negative correlation between dietary fat content and myofiber diameter (Trovato, Castrogiovanni, Szychlinska, Purrello, & Musumeci, 2018), indicating that HFD is closely related to growth, development and metabolism of myofiber. In another study, feeding mice with HFD for long-term caused skeletal muscle atrophy, which was mainly manifested through reduction of myofiber area, muscle satellite cells and muscle nucleus (Lee et al., 2015). Furthermore, it is known that, muscle atrophy is related to markers of apoptosis such as cysteine-aspartic acid protease 3 (caspase-3) transcription factor (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999). Caspase-3 plays a key role in muscle atrophy immobilization by acting on the apoptosis and inflammation pathways (Zhu et al., 2013). However, whether HFD causes myofiber atrophy in flesh of farmed fish like in mice is currently unknown. Fish flesh quality has become an

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important concern for consumers (Freitas, Vaz-Pires, & Câmara, 2020). Therefore, research is required to explore approaches for improving flesh quality in farmed fish fed on HFD.

L-carnitine is a dietary lipid-lowering additive commonly used in aquaculture to reduce excess fat deposition and promote growth (Li, Limbu, Ma, Chen, Zhang, Du, 2019). L-carnitine binds to acyl-CoA sourced from long-chain fatty acids and acyl-carnitine (Fritz & McEwen, 1959). Therefore, it is transported by carnitine-acyltransferase I and II (CPT-I and -II) from cytoplasm into mitochondrial matrix for fatty acid β-oxidation (Porter et al., 2017). Thus, L-carnitine is biochemically essential in lipid catabolism and is regarded as an efficient stimulator for lipid utilization in farmed fish. Evidently, L-carnitine promoted fatty acids metabolism in largemouth bass (*Micropterus salmoides*) (Chen et al., 2020) and reduced body lipid content in Asian catfish (*Clarias batrachus*) (Desai, Singh, Sapkale, & Patil, 2010) and Rainbow trout (*Oncorhynchus mykiss*) (Sharifzadeh, Yeganeh, Firouzbakhsh, & Oraji, 2017). In addition, dietary L-carnitine supplementation also reduced inflammation and enhanced antioxidant capacity and immune responses in juvenile black sea bream (*Acanthopagrus schlegelii*) fed on HFD (Jin et al., 2019). However, the potential functions of L-carnitine on flesh quality in fish fed on HFD have not been intensively studied. We hypothesized that L-carnitine may also alleviate the adverse effects on flesh quality caused by feeding farmed fish with HFD for long time.

Nile tilapia is the third largest economically-farmed fish around the world, with China currently the largest producer and exporter (Fao The State of World Fisheries and Aquaculture 2020, 2020). According to Chinese consumption habits, tilapia with over 50 g can be sold in markets. Recently, diets with high fat content has been reported during culture of Nile tilapia (Abdel et al., 2021). Previous studies in our laboratory have proved that 12% fat content in the diet for Nile tilapia increase significantly fat deposition (Limbu, Ma, Zhang, & Du, 2019; Lv, Ma, Hu, Lin, Chen, Zhang, et al., 2020). Our present study aimed to further understand the effects of dietary L-carnitine supplementation on flesh quality of fish fed on HFD. To achieve the objective, juvenile Nile tilapia (initial weight 3.20 ± 0.30 g) were fed on medium fat-diet (MFD), HFD and HFD supplemented with L-carnitine diet (HFCD), for 10 weeks. After the feeding trial, growth indexes, nutrient deposition on fish fillets, myofiber tissue structure and expression of flesh quality-related genes were analyzed. This research provides new insights into the use of L-carnitine as a safe dietary additive on flesh quality in farmed fish fed on HFD.

2. Materials and methods

Ethical statement

All experiments were carried out by following the Guidance of the Care and Use of Laboratory Animals in China. This study was approved by the Committee on the Ethics of Animal Experiments of East China Normal University (F20190101).

2.1. Experimental fish and diet

About 700 all male Nile tilapia were purchased from a fish farm in Guangzhou. Before the formal trial, the Nile tilapia were acclimated for two weeks in eight 250-L tanks, and fed a commercial diet (Chengdu, China) containing 33% dietary protein and 5% dietary fat. Three experimental feeds, named as medium-fat diet (MFD, 6% dietary lipid), high-fat diet (HFD, 12% dietary lipid) and HFD supplemented with L-carnitine (HFCD, 12 % dietary lipid + 400 mg/kg L-carnitine), were formulated as shown in Supplementary Table 1. The diets were prepared separately and extruded into 2 mm pellets as performed regularly in our laboratory (Li et al., 2019; Liu et al., 2020). After drying in an oven at 40 °C for 24 h, the pellets were stored in a refrigerator at −30 °C until needed for the feeding trial.

2.2. Experimental design

After two weeks of acclimatization, 18 fish (six per treatment) were randomly sampled for body composition determination for calculation of nutrients retention. Afterwards, 270 visually healthy Nile tilapia with an average weight of 3.20 ± 0.30 g were randomly sampled and distributed into three treatments (three tanks per treatment, 30 fish per tank) and fed the three experimental diets for 10 weeks. The Nile tilapia in each tank were fed twice a day (8:00 and 18:00 h) at a feeding rate of 6% average body weight per day. The fish in each tank were bulk weighed every two weeks, and the feeding amount was adjusted accordingly. During the 10-week feeding trial, dissolved oxygen and pH were maintained at ranges from 4.80 to 6.40 mg/L and 7.60 to 8.00, respectively while water temperature was 28.00 ± 2.00 °C. The fish were reared under natural light of 14 h light/10 h dark cycle.

2.3. Determination of growth performance, feed efficiency and nutrients retention

After the feeding trial, the fish in the three treatments were starved for 12 h, then weighed individually to obtain final body weight. Afterwards, fish were individual measured for their body length by using a ruler. Six fish from each treatment were randomly sampled for body composition determination after the feeding trial (n = 6). Specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), lipid efficiency ratio (LER), protein retention ratio (PRR) and lipid retention ratio (LRR) were calculated by using the following formulae:

SGR = 100 × [ln (Final fish weight) – ln (Initial fish weight)]/Time of feeding trial (days)

FCR = Feed intake amount/(Final fish weight- Initial fish weight)

PER = (Final fish weight- Initial fish weight)/total dietary protein intake

LER = (Final fish weight- Initial fish weight)/total dietary lipid intake

PRR = (Final fish protein weight- Initial fish protein weight)/total dietary protein intake

LRR = (Final fish lipid weight- Initial fish lipid weight)/total dietary lipid intake

2.4. Body composition and biochemical parameters analyses

Six fish from each dietary treatment (two fish per tank) were randomly sampled and anesthetized by using eugenol for whole body composition assays (n = 6). Another six fish from each treatment (two fish tank) were randomly selected and anesthetized as above. Each individual fish was dissected to obtain the liver and muscle tissue for composition analysis (n = 6). The moisture content in the whole fish and muscle tissue was determined by drying the samples in an oven at 105 °C for 15 h. The total protein in the whole body and muscle samples was determined by the Kjeldahl method after sulfuric acid digestion. The lipid content in the whole-body, muscle and liver was determined by the chloroform/methanol (2/1, v/v) method (Bligh and Dyer (1959) as done previously in our laboratory (Lv et al., 2020).

The lactic acid and glycogen contents of muscle samples were determined by using specific commercial kits according to the manufacturer's instructions (Jiancheng Biotech Co., China). The remaining muscle samples were used for amino acid and fatty acid composition analyses and isolation of total RNA for quantitative real-time PCR (qRT-PCR).

2.5. Amino acids and fatty acids composition analysis

The remaining muscles from six samples per dietary treatment after proximate composition were used for amino acids and fatty acids composition analyses. Two muscle samples from each treatment were

mixed and used to determine the amino acids and fatty acids composition (n = 3). The amino acids composition was determined as described previously in our laboratory (Wang, Qian, Gu, Zhang, Wang, Lv, et al., 2021). The quantification of amino acids was conducted by using an amino acid automatic analyzer (Hitachi L-8900, Japan). The fatty acid composition of the muscles was analyzed by using a Gas chromatography (GC). The details on the method has been described in our recent publication (Zhang, Jiang, Lv, Jin, Chen, Zhang et al., 2021).

2.6. Texture profile analysis (TPA)

Six fish were randomly sampled from each treatment (2 fish/tank), anesthetized and dissected to obtain white muscle samples for analysis of flesh texture parameters (n = 6). The texture was determined by using a QTS-25 surface analyzer (CNS Farnell Ltd., Hertfordshire, Britain) according to the manufacturer's instructions. The flesh texture characteristics were determined by using a cylindrical steel probe with a diameter of one cm. The speed of probe was set at 30 mm/min with a target value of 4 mm for compression. During texture determination, the sample resistance was automatically recorded by the machine every 0.01 s, and then the sample force-time graph was plotted as described previously (De Huidobro, Miguel, Blázquez, & Onega, 2005). The TPA parameters of the samples were obtained by using the TexturePro Adapt 2 Computer program (CNS Farnell Ltd., Hertfordshire, Britain). For each parameter assay, every fish sample was repeated five times.

2.7. pH measurement and centrifugal loss as markers of flesh quality traits

Six individual muscle samples from each treatment (2 fish/tank) were evaluated for pH value (n = 6). The pH measurement was performed by a pH meter in samples homogenized with distilled water (ratio 1:9) according to the method described previously (Lv et al., 2020). To calculate the centrifugal loss of flesh sample, six fresh muscle samples each weighing one gram (n = 6) were placed in a plastic tube and centrifuged at 1000× g for 30 min. Afterwards, the moisture on the sample surface was dried by using filter papers and the samples were weighed again. The difference of the sample weight before and after centrifugation was recorded as the centrifugal loss (Lv et al., 2020).

2.8. Isolation of RNA, synthesis of cDNA and quantitative real-time PCR (qRT-PCR)

The remaining muscles from six fish samples per dietary treatment after proximate composition were used for isolation of total RNA (n = 6) by using Tri Pure Reagen (Aidlab, China). The NanoDrop 2000 Spectrophotometer (Thermo, USA) was used to verify the quality and quantity of the isolated total RNA. The quality of the samples ranged from 1.90 to 2.0 based on the 260/280 nm absorbance ratio. The cDNAs were synthesized by using a Prime ScriptTM RT reagent Kit (Takara, Japan) based on manufacturer's instructions. The reaction system for quantitative real-time polymerase chain reaction (qRT-PCR) contained 1.0 μL PCR primers, 1.0 μL cDNA template, 3.0 μL nuclease-free water and 5.0 μL SYBR Premix Ex Taq. The reaction was run in a Real-Time System (Bio- Rad CFX Connect, USA) according to the manufacturer's protocol. The procedure and efficiency of qPCR were the same as our previous study (Zhang et al., 2021). The primers sequences used in this study were designed in National Center for Biotechnology Information (NCBI) and are listed in Supplementary Table 2. Elongation factor 1 alpha (EF1a) and β-actin were used as reference genes due to their stability. The relative mRNA expression was evaluated by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.9. Histological analysis

Six individual dorsal muscle samples from each treatment were fixed with 4% paraformaldehyde and then embedded in paraffin as described before (Wang et al., 2021). The sections were stained by using hematoxylin and eosin (H & E), and then observed by using a light microscope (Nikon, Tokyo, Japan). Moreover, three fields of view were randomly selected in each slice (three view per slice, one slice per fish, 6 fish per treatment) for myofiber morphological characteristics (n = 6). The diameter and number of myofiber in a 0.35 mm^2 visual area were calculated by using an Image J software as described previously (Zhang et al., 2021).

2.10. Statistical analyses

The data obtained were tested for normality and homogeneity of variances by using Shapiro -Wilk and Levene's tests, respectively.We used One-way analysis of variance (ANOVA) to test for the statistical differences on the mean of the measured parameters among the three treatments. When ANOVA showed significant differences, the specific comparisons among treatments were determined by using Duncan's multiple range test. Results with $P < 0.05$ were considered statistically different. The results obtained are presented as mean ± standard error of the mean (SEM). All the statistical analyses were carried out by using SPSS Statistics 22.0 software (IBM, Michigan Avenue, Chicago, IL, USA).

3. Results

3.1. The effects of L-carnitine on growth performance and whole fish composition

Feeding the fish with the three experimental diets did not cause significant difference in body length (Fig. 1a). The fish fed on HFD had higher final body weight (Fig. 1b), SGR (Fig. 1d) and PER (Fig. 1e) than those fed on MFD and HFCD. However, no significant differences in growth and PER were obtained between fish fed on MFD and HFCD. The fish fed on MFD had significantly higher FCR than the HFD- and HFCD-fed fish (Fig. 1c), while the fish fed on HFD and HFCD had comparable FCR values (Fig. 1c). The fish fed on HFD and HFCD had statistically similar LER (Fig. 1f) and LRR (Fig. 1h) values, but significantly lower than those fed on the MFD. However, no significant difference in PRR was obtained among the three experimental diets (Fig. 1g). The HFD-fed Nile tilapia had significantly lower moisture (Fig. 1i) and total protein (Fig. 1j), but had significantly higher total lipid in whole fish (Fig. 1k) and liver Fig. 1l) than the MFD-fed fish. The HFCD-fed Nile tilapia had significantly higher moisture (Fig. 1i), but lower total lipid in whole fish than the fish fed on HFD (Fig. 1k). However, the fish fed on the HFD and HFCD had comparable total protein in whole fish (Fig. 1j) and total lipid in the liver (Fig. 1l). In short, these data suggest that L-carnitine supplementation mainly alleviated the HFD-caused excessive fat deposition in whole body and liver, and accordingly reduced the final body weight.

3.2. The effects of L-carnitine on the biochemical composition and pH value in muscle tissue

The Nile tilapia fed on HFCD significantly reduced the moisture content (Fig. 2a), while the fish fed on MFD and HFD had statistically similar moisture content in the muscle. Feeding the three diets did not affect total protein (Fig. 2b), total lipid (Fig. 2c) and glycogen contents (Fig. d). Feeding the Nile tilapia with HFD increased significantly the pH value (Fig. 2e), but decreased the lactic acid content (Fig. 2f). Interestingly, feeding the Nile tilapia with HFCD significantly alleviated the HFD-induced increase in pH value and increased lactic acid content to similar levels as those fed on the MFD. The HFCD-fed fish upregulated significantly the expression of hypoxia inducible factor 1 (HIF1) and HIF3 genes than those fed on HFD (Fig. 2g). However, feeding the Nile tilapia with MFD and HFD did not affect the expression of HIF1, HIF2 and HIF3 genes. Feeding the Nile tilapia with HFD down-regulated significantly the expression of fatty acid synthase (FAS), Acetyl-CoA

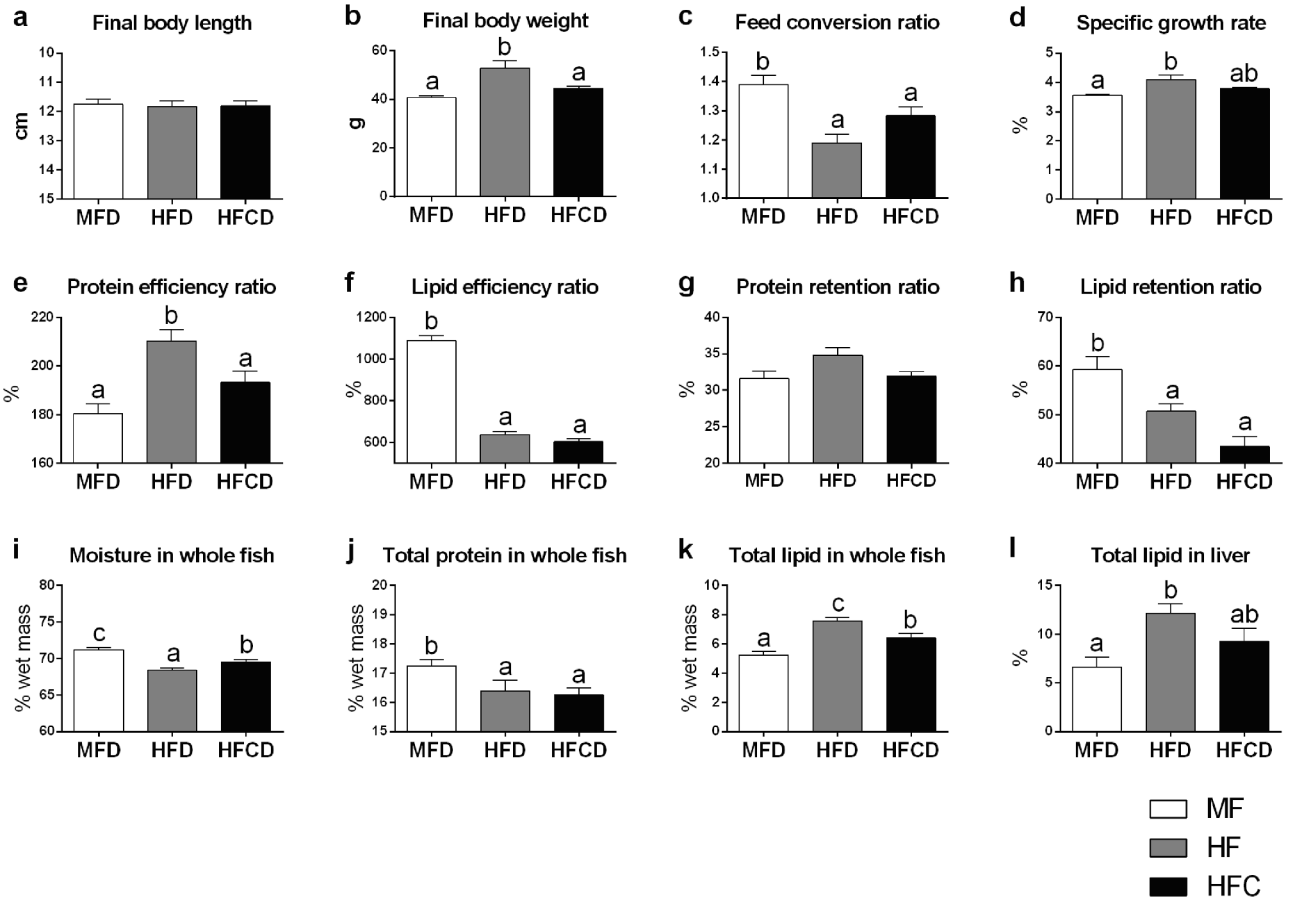


Fig. 1. The effect of L-carnitine on growth performance and Nile tilapia whole body composition. (a) Final body length; (b) Final body weight; (c) Feed conversion ratio; (d) Specific growth rate; (e) Protein efficiency ratio; (f) Lipid efficiency ratio; (g) Protein retention ratio; (h) Lipid retention ratio; (i) Moisture in whole fish; (j) Total protein in whole fish; (k) Total lipid in whole fish; (l) Total lipid in liver. Results are shown as means ± SEM (a-h: n = 3; i-l: n = 6). Significant differences were determined at $P < 0.05$.

carboxylase α (ACCα) and muscle-type carnitine palmitoyltransferase 1 (CPT1β) compared to those fed on the MFD. Interestingly, these genes were comparatively up-regulated in the HFCD-fed fish, without any significant differences between fish fed on the HFD and HFCD (Fig. 2h). These data reveal that L-carnitine supplementation alleviated the reduction of lactic acid and the corresponding increase in pH value caused by feeding Nile tilapia with HFD, but did not affect composition of macronutrients in muscle.

The HFD-fed Nile tilapia had significantly higher total polyunsaturated fatty acids (∑PUFA) and ∑n-6, but had significantly lower total short chain fatty acids (∑SFA) and ∑n-3/∑n-6 ratio than the MFD-fed fish (Supplementary Table 3). The MFD- and HFD-fed Nile tilapia had statistically similar total monounsaturated fatty acids (∑MUFA), eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) and ∑n-3. The HFCD-fed Nile tilapia had significantly lower EPA + DHA, ∑n-3, ∑n-6 and ∑PUFA values, but had markedly higher ∑SFA compared with those fed on HFD. The fish fed on HFD had significantly higher total delicious amino acids (∑DAA) value, but had significantly lower total flavor amino acids (∑FAA) value than those fed on MFD, whereas those fed on HFD and HFCD had statistically similar total essential amino acids (∑EAA), ∑DAA and ∑FAA values. In summary, L-carnitine supplementation partly changed the fatty acids and amino acids composition in muscle tissue of Nile tilapia fed on HFD.

3.3. The effects of L-carnitine on texture profile of muscle tissue

The Nile tilapia fed on HFCD decreased significantly hardness (Fig. 3a) and adhesiveness (Fig. 3f), but increased significantly springiness (Fig. 3c) of muscle, compared with the fish fed on HFD. Feeding the Nile tilapia with HFD and HFCD did not affect gumminess (Fig. 3b), springiness (Fig. 3c), cohesiveness (Fig. 3e), adhesiveness (Fig. 3f), chewiness (Fig. 3g) and centrifugal loss (Fig. 3h). The Nile tilapia fed on HFD increased significantly viscosity than those fed on MFD (Fig. 3d). However, feeding the Nile tilapia with MFD and HFD did not affect hardness, gumminess, springiness, cohesiveness, adhesiveness, chewiness and centrifugal loss. These results show that, the dietary L-carnitine supplementation reduced hardness and adhesiveness and increased springiness caused by feeding Nile tilapia with HFD.

3.4. Effects of L-carnitine on morphological characteristics of myofiber

The Nile tilapia fed on HFD reduced significantly myofiber diameter than those fed on MFD (Fig. 4a, b and n). The HFCD-fed fish increased obviously myofiber diameter compared to the HFD-fed fish (Fig. 4b, c and n). The distribution of myofiber diameters from the three dietary treatments are shown in Fig. 4d to m. The HFD-fed fish and HFCD-fed fish had much smaller myofibers compared to those fed on MFD (Fig. 4g, i, j, l). The HFCD-fed fish had much bigger myofibers than those fed on HFD (Fig. 4h, k). More specifically, the fish fed on HFD had significantly higher values of small-diameter myofiber (0–40 μm), but lower values of larger-diameter fibers (80–130 μm) than those fed on MFD. The HFCD-fed fish had lower values of small-diameter myofiber (0–40 μm), but higher values of larger-diameter fibers (80–130 μm) than those fed on the HFD (Fig. 4m). Therefore, the average diameter of myofiber in the HFD-fed fish was significantly lower than those fed on MFD, but it was significantly increased in the fish fed on HFCD (Fig. 4n).

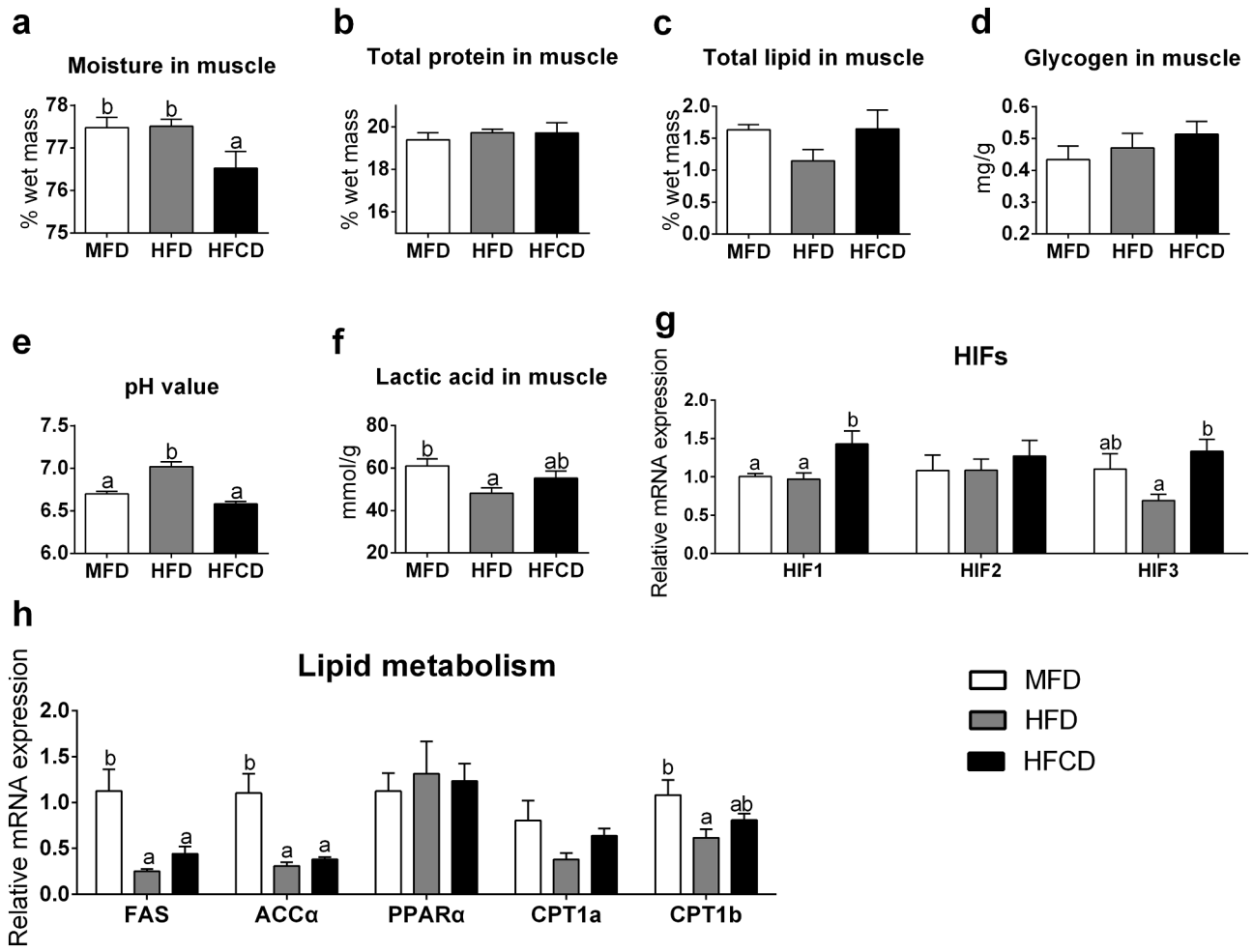


Fig. 2. The effect of L-carnitine on biochemical composition and pH value in muscle tissue. (a) Moisture in muscle; (b) Total protein in muscle; (c) Total lipid in muscle; (d) Glycogen in muscle; (e) pH value; (f) Lactic acid in muscle; (g) HIFs (hypoxia inducible factor): HIF1, HIF2, HIF3; (h) Lipid metabolism: FAS (fatty acid synthase), ACCα (Acetyl-CoA carboxylase α), PPARα (Peroxisome proliferators-activated receptors α), CPT1α (palmitoyltransferase 1α), CPT1β (palmitoyltransferase 1β). Results are shown as means ± SEM (a-h: n = 6). Significant differences were determined at $P < 0.05$.

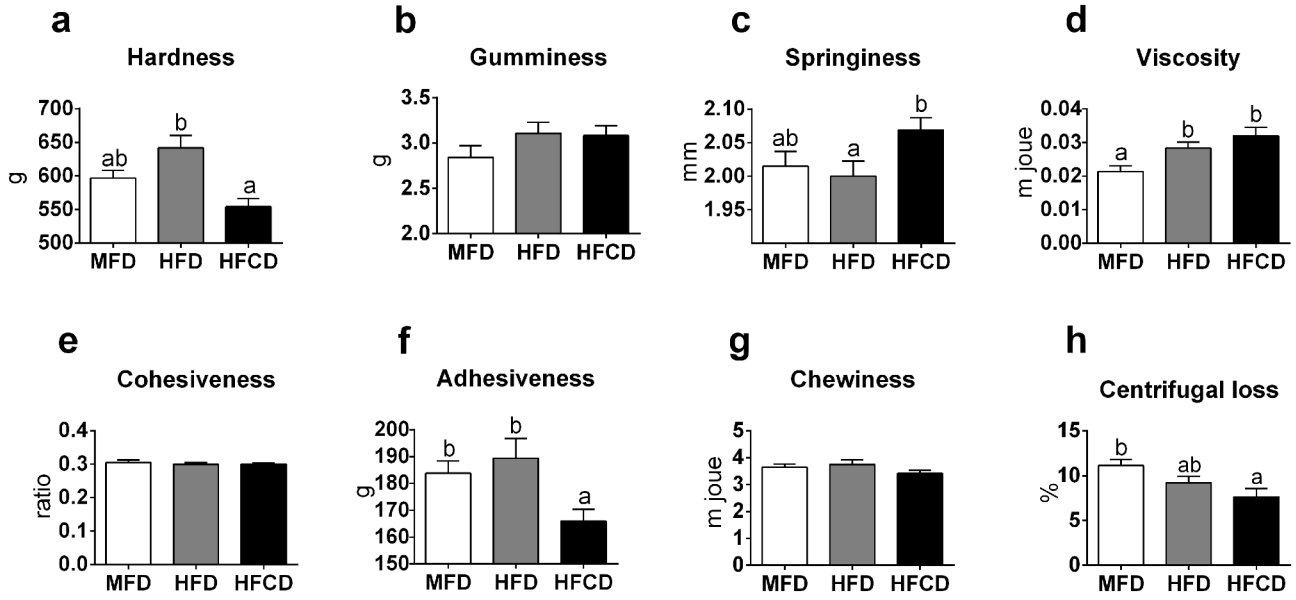


Fig. 3. The effect of L-carnitine on texture profile of muscle tissue. (a) Hardness; (b) Gumminess; (c) Springiness; (d) Viscosity; (e) Cohesiveness; (f) Adhesiveness; (g) Chewiness; (h) Centrifugal loss. Results are shown as means ± SEM (a-h: n = 6). Significant differences were determined at $P < 0.05$.

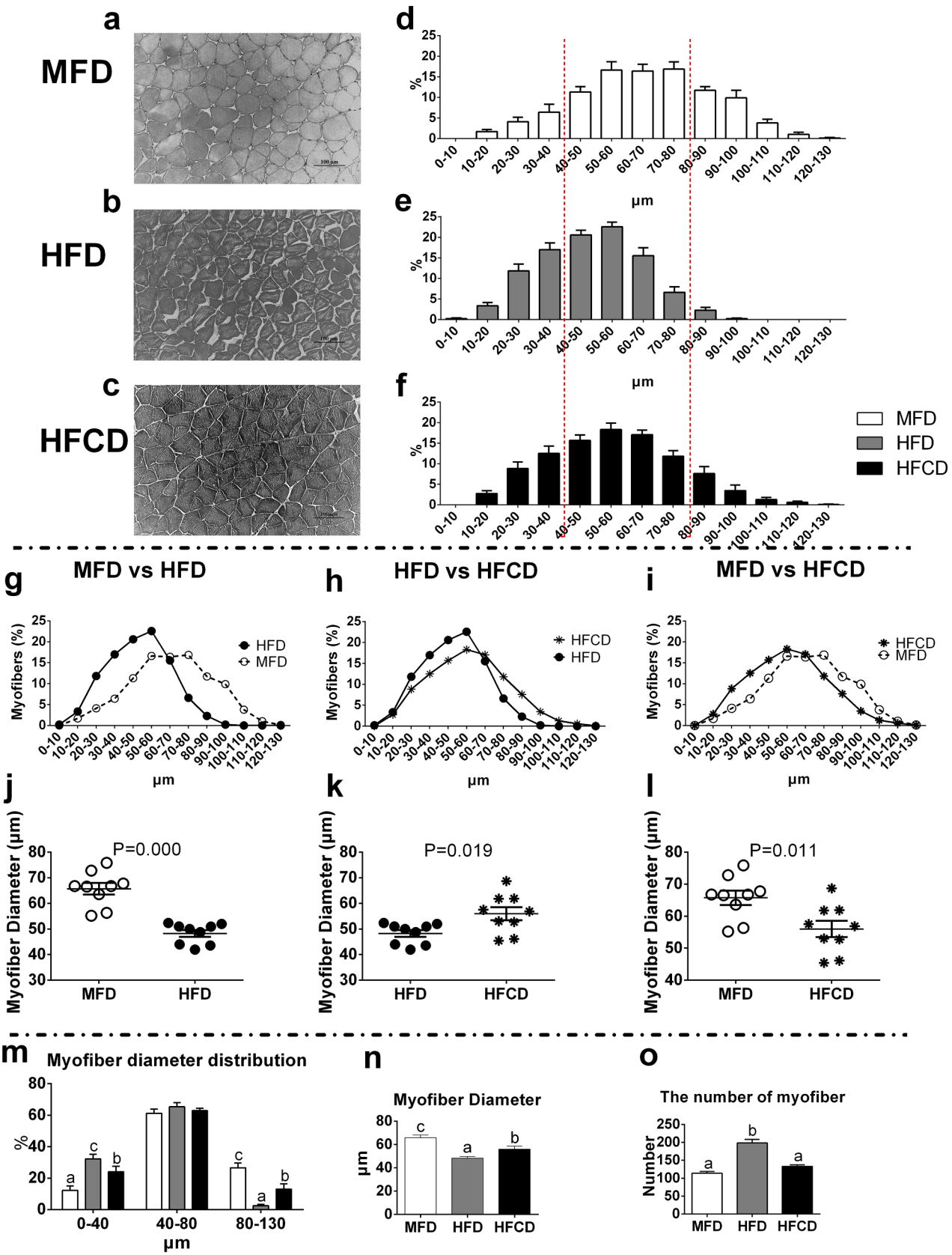


Fig. 4. Effects of L-carnitine on myofiber diameter, number and area in muscle tissues. (a-c) Histological characteristic of myofiber of Nile tilapia fed with MFD, HFD and HFCD under a field of view of 0.35 mm^2 area (200x); (d-f and m) Histograms of myofiber diameter in flesh of Nile tilapia fed with MFD, HFD and HFCD under a field of view of 0.35 mm^2 area (200x); (g-l) Myofiber diameter distribution in MFD vs HFD; HFD vs HFCD; MFD vs HFCD; (n) Numbers of myofiber under a field of view of 0.35 mm^2 area (200x); (o) Myofiber diameter. Results are shown as means ± SEM (a-o: n = 6). Significant differences were determined at $P < 0.05$.

The HFD-fed fish increased the number of myofibers compared to those fed on MFD, while the HFCD-fed fish decreased them to a similar level as those fed on MFD (Fig. 4o). To sum up, dietary L-carnitine alleviated the decrease of myofiber diameter caused by feeding Nile tilapia with HFD.

3.5. The effects of L-carnitine on expression of genes related to myofiber growth and apoptosis

Feeding the Nile tilapia with the three dietary treatments did not affect the expression of myogenin (MyoG; Fig. 5a), myogenic differentiation antigen (MyoD; Fig. 5b), mechanistic target of rapamycin (mTOR; Fig. 5c), protein kinase B (Akt; Fig. 5d) and Caspase-9 (Fig. 5f). The HFD-fed fish significantly upregulated the expression of Caspase-3 gene than those fed on MFD, but feeding the Nile tilapia with HFCD reduced significantly the expression of Caspase-3 (Fig. 5e) gene. The above results pointed that, dietary L-carnitine supplementation alleviated myofiber apoptosis caused by HFD-feeding, but did not affect myofiber growth and development in Nile tilapia.

3.6. The effects of L-carnitine on endoplasmic reticulum stress, autophagy, inflammation and oxidative stress in the muscle tissue

The Nile tilapia fed on HFD increased the expression of genes related to endoplasmic reticulum (ER) stress such as activating transcription factor 4 (ATF4) and inositol-requiring enzyme 1 (IRE1) than those fed on MFD (Fig. 6a), but not binding immunoglobulin protein (BiP). The Nile tilapia fed on HFCD showed a downward trend of ATF4 and IRE1 genes compared with those fed on HFD, without any significant difference (Fig. 6a). The Nile tilapia fed on HFD and HFCD up-regulated the genes related to inflammation, such as interleukin 10 (IL-10), interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) compared with those fed on MFD (Fig. 6c). Feeding the Nile tilapia with the three dietary treatments did not affect autophagy related genes such as perilipin2, microtubule-associated protein light chain 3 (Lc3I) and Lc3II (Fig. 6b) and oxidative stress related genes such as cytochrome P450 1A (CYPIA), glutathione peroxidase 1 (GHS-PX1) and glutathione S-transferase (Fig. 6d). These results suggest that L-carnitine supplementation alleviated the ER stress caused by feeding Nile tilapia with HFD, but did not affect inflammation, autophagy and oxidative stress.

4. Discussion

4.1. Dietary L-carnitine supplementation restored fatty acids β-oxidation and pH value in fillet changed by HFD

The pH value, a key index affecting flesh quality, is negatively linked to the lactic acid content in muscle (Lv et al., 2020). The change of pH influences water holding capacity and myofiber morphology, consequently affects muscle tenderness (Kristoffersen, Tobiassen, Steinsund, & Olsen, 2006). In our present study, the pH value and lactic acid content of flesh in the HFD-fed fish was lower and higher, respectively than in the MFD-fed fish. However, L-carnitine supplementation in the diet increased lactic acid concentration and significantly decreased pH value to similar levels as those fed on the MFD. Previous reports have indicated that feeding HFD impair oxygen-consuming processes such as mitochondrial β-oxidation activities in grass carp (Du et al., 2008), yellow catfish (Cui et al., 2018) and Nile tilapia (Tao et al., 2018). Accordingly, fish fed on HFD reduced HIF3 gene expression, which is a cellular oxygen-sensitive factor in fish (Ma et al., 2020), suggesting oxygen-consumption was inhibited. This means that cellular oxygen content is expected to be higher in fish fed on HFD than MFD. At the same time, it is well known that cellular lactic acid content, as the main product of anaerobic metabolism, is negatively correlated to cellular oxygen level. Evidently, the fish fed on HFD reduced lactic acid, and

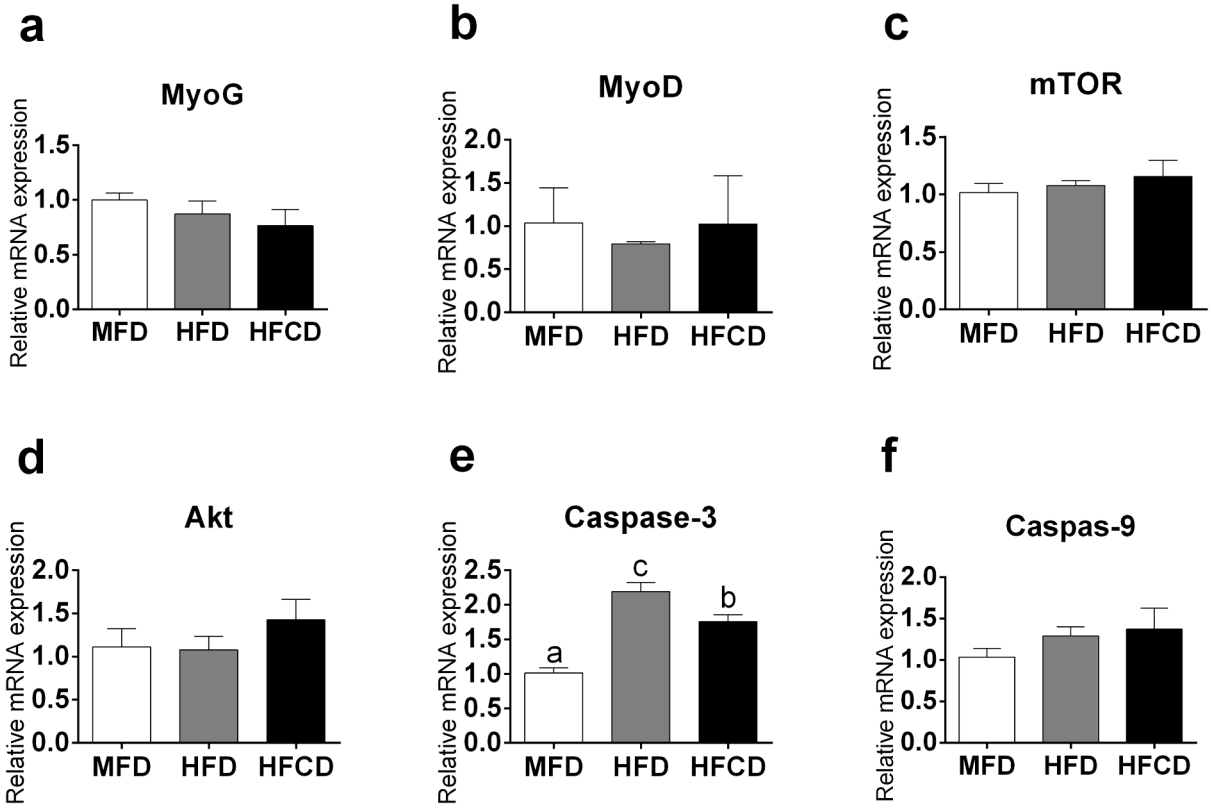


Fig. 5. Effects of L-carnitine on myofiber related genes expression of Nile tilapia. (a) MyoG (Myogenin); (b) MyoD (Myogenic differentiation antigen); (c) mTOR; (d)Akt; (e) Caspase-3 (Cysteine-aspartic acid protease 3); (f) Caspase-9 (Cysteine-aspartic acid protease 9). Results are shown as means ± SEM (a-f: n = 6). Significant differences were determined at $P < 0.05$.

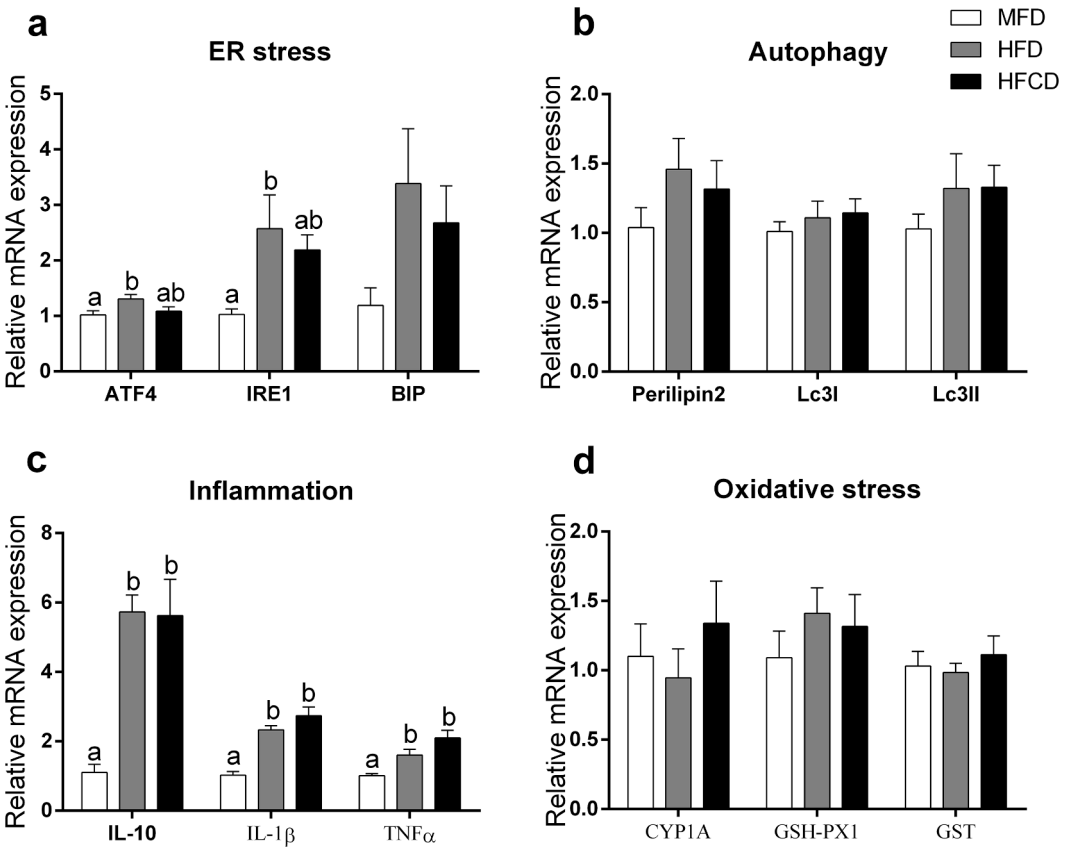


Fig. 6. Effects of L-carnitine on the mRNA expression of endoplasmic reticulum stress, autophagy, inflammation and oxidative stress in muscle tissue. (a) endoplasmic reticulum stress: ATF4 (Activating transcription factor), IRE1 (Inositol requiring enzyme 1), BiP (Binding immunoglobulin protein); (b) Autophagy: Perilipin, Lc3I, Lc3II; (c) Inflammation: IL-10 (Interleukin 10), IL-1β (Interleukin 1β), TNFα (Tumor necrosis factor α); (d) Oxidative: CYPIA (Cytochrome P450 1A), GSH-PX1 (Glutathione peroxidase 1), GST (Glutathione S-transferase). Results are shown as means ± SEM (a-d: n = 6). Significant differences were determined at $P < 0.05$.

therefore resulted in an increase of pH value. The L-carnitine supplementation has been frequently reported to stimulate mitochondrial β-oxidation activity and increase cellular oxygen consumption (Li et al., 2017; Li et al., 2019). Accordingly, the fish fed on HFCD had higher expression of HIF3 and lactic acid, and lower pH value than those fed on HFD, indicating that the impaired oxygen-consumption as a metabolic process and lactic acid production were restored. Consequently, the Nile tilapia fed on HFCD alleviated the abnormal increase of pH value caused by feeding them with HFD. Therefore, our present study indicates that L-carnitine supplementation in the diet restored fatty acids β-oxidation and pH value in fish fillet changed by HFD. This also indicates that muscle pH value might be regulated by L-carnitine in fish.

4.2. Dietary L-carnitine reduced myofiber thinning caused by HFD through decreasing endoplasmic reticulum stress and myofiber apoptosis

Physical performance indicators such as hardness, adhesiveness and chewiness are important indexes in evaluating fillet quality (Periago, Ayala, López-Albors, Abdel, Martinez, García-Alcázar, et al., 2005). Among them, hardness is tightly correlated to other physical indexes, including springiness, adhesiveness and chewiness. Hardness has also been used as an essential fillet quality indicator to reflect shear force, taste and economic value in many fish species (Johnston et al., 2000; Wang et al., 2021; Zhang et al., 2021). In our present study, we revealed that the HFD-fed fish increased muscle hardness, while dietary L-carnitine supplementation decreased hardness and improved springiness of flesh. It has been reported that muscle hardness is negatively correlated with myofiber size in mammals (Hatae, Yoshimatsu, & Matsumoto, 1990). Similarly, some fish studies have also pointed out that muscle hardness is also negatively correlated with the myofiber diameter or size (Periago et al., 2005). Accordingly, in our experiment, the fish fed on HFD had smaller myofibers and their number, while those fed on HFCD had larger myofibers and their number decreased. Likewise, a previous report on mice also reported that dietary fat content had an inverse relationship with myofiber diameter (Trovato et al., 2018). All the previous findings and the present study indicate that feeding HFD in animals induce smaller myofiber and cause higher distribution density of myofiber in muscle, resulting in higher hardness. However, for the first time, our study indicates that these effects can be alleviated by dietary L-carnitine supplementation in farmed fish.

A previous study reported that myofiber growth mainly depends on its hypertrophy and hyperplasia, which cause increase in myofiber diameter and numbers, respectively (White, Baumgarner, Watanabe, Alam, & Kinsey, 2018). In our present study, the expression of genes related to myofiber hyperplasia (MyoG and MyoD) and myofiber hypertrophy (mTOR and Akt) did not change significantly in Nile tilapia fed on the three diets, indicating that feeding HFD or HFCD does not affect myofiber growth and development. Nevertheless, the muscle physical characteristics is also affected by apoptosis or atrophy. In some studies, feeding mice with HFD induced muscle atrophy and reduced myofiber diameter (Sishi et al., 2011), indicating damaged muscle function and myofiber atrophy. Accordingly, in our present study, feeding the Nile tilapia with HFD up-regulated significantly caspase-3, which is an important apoptosis marker gene. Interestingly, feeding the fish with L-carnitine supplementation down-regulated the expression of caspase-3 gene. This suggests that feeding fish with HFD induce muscle atrophy and reduce myofiber diameter, which can be alleviated by L-carnitine supplementation.

Muscle apoptosis is highly correlated to ER stress (Deldicque, 2013). Moreover, feeding mice with HFD induced ER stress, apoptosis, autophagy and oxidative stress in muscle (Koh et al., 2013). In our present study, the fish fed on HFD increased ER stress and inflammation-related genes than those fed on MFD. This suggests that feeding HFD in fish might mediate apoptosis through ER stress and inflammatory process. Of note, the expression of ER stress genes tended to decrease in Nile tilapia fed on HFCD, indicating that dietary L-carnitine supplementation might alleviate cell apoptosis by reducing ER stress. In fact, a mice study also indicated that L-carnitine, as an antioxidant, inhibited significantly ER stress (Unsal, Deveci, Ozmen, & Tumer, 2021), which was consistent with our findings. Therefore, our study shows that dietary L-carnitine supplementation reduces ER stress, consequently decrease myofiber apoptosis caused by feeding fish with HFD.

4.3. Dietary L-carnitine supplementation alleviated fat deposition in whole body and liver caused by HFD

L-carnitine has lipid-lowering effects on whole body and liver in different fish species (Li et al., 2019). Our present study also revealed that dietary L-carnitine reduced excessive fat deposition in the whole body and liver caused by feeding Nile tilapia with HFD. This might also be the reason for the reduced body weight but similar body length in the HFCD-fed fish compared with the HFD-fed fish. It should be noted that some reports also indicated that fish muscle lipid content is reduced after L-carnitine supplementation in diets (Li, Li, Qin, Ning, Lu, Li et al., 2017; Li, et al., 2020). However, the Nile tilapia fed on HFD and HFCD in the present study, had similar total protein, total lipid and glycogen contents in the muscles. Previous studies have suggested that the main target tissue for L-carnitine is liver (Li et al., 2019; Stephens, Constantin-Teodosiu, & Greenhaff, 2007). In addition, as a typical lean fish, Nile tilapia contain low lipid content (1 to 3% fat) in fillet, thus the change of lipid content in muscle is supposed to be marginal. Therefore, the influence of L-carnitine in muscle lipid content in Nile tilapia fed on HFD might not be significant on flesh quality.

Apart from muscle composition, fatty acids and amino acids have a great influence on the nutritional value and flavor of fish fillets (Zhang et al., 2021). It is generally agreed that consuming products with less ∑SFA and more ∑n-3 PUFA in flesh is beneficial for human health (European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition & Allergies (EFSA) Panel on Dietetic Products, Nutrition & Allergies, 2010). Our research indicated that the contents of ∑n-6 PUFA, ∑PUFA and EPA + DHA decreased while that of ∑SFA increased in the HFCD-fed fish than the HFD-fed fish, indicating that L-carnitine supplementation did not improve the nutritional value of fish fillets based on fatty acids composition. On the contrary, our present study indicated that the fish fed on HFD increased DAA ratio but reduced FAA ratio compared with the MFD-fed fish, respectively. Interestingly, the Nile tilapia fed on HFCD reduced and increased DAA and FAA, respectively, to similar levels as the fish fed on MFD. Since the balance of DAA and/or FAA affects greatly the flesh flavor, our study illustrates that dietary L-carnitine supplementation alleviates the flesh flavor caused by feeding Nile tilapia with HFD.

5. Conclusion

To sum up, the present study indicates that feeding HFD in Nile tilapia for 10 weeks caused excessive fat deposition in whole body and liver, higher hardness and pH value, and smaller size of myofiber, as compared with those fed on MFD. However, these abnormal changes were alleviated by dietary L-carnitine supplementation, through reduced ER stress and myofiber apoptosis. For the first time, our study shows that dietary L-carnitine supplementation efficiently recovers the changes induced by HFD on flesh quality of fish. Therefore, using properly L-carnitine as a feed additive might help to maintain or promote flesh quality in fish fed on HFD for long-term.

Declaration of Competing Interest
All authors declare no conflicts of interests.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132367.

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