Functional differences between L- and D-carnitine in metabolic regulation evaluated using a low-carnitine Nile tilapiamodel

Jia-Min Li[1], Ling-Yu Li[1], Yu-Xue Zhang[1], Zhe-Yue Jiang[1], Samwel M. Limbu[1,2], Fang Qiao[1], Pascal Degrace[3], Li-Qiao Chen[1], Mei-Ling Zhang[1], Zhen-Yu Du[1]*

[1]LANEH, School of Life Sciences, East China Normal University, Shanghai200241, China

[2]Department of Aquatic Sciences and Fisheries Technology, University of Dar es Salaam, Dar es Salaam, Tanzania

[3]Team Pathophysiology of Dyslipidemia, INSERM UMR1231 "Lipides, Nutrition, Cancer," Université de Bourgogne Franche-Comté, Dijon, France

Short title:Metabolic regulation of L- and D-carnitine

*Correspondence to: Prof. Zhen-Yu Du, School of Life Sciences, East China Normal University, No.500, Dongchuan Rd, Shanghai 200241, China. Tel: +86-21-54345488. *E-mail address*: zydu@bio.ecnu.edu.cn

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Abstract

L-carnitine is essential for mitochondrial β-oxidation and has been used as a lipid-lowering feed additive in humans and farmed animals. D-carnitine is an optical isomer of L-carnitine and DL-carnitine has been widely used in animal feeds. However, the functional differences between L- and D-carnitine are difficult to study because of the endogenous L-carnitine background. In the present study, we developed a low-carnitine Nile tilapia model by treating fish with a carnitine synthesis inhibitor, and used this model to investigate the functional differences between L- and D-carnitine in nutrient metabolism in fish. L- or D-carnitine (0.4 g/kg diet) were fed to the low-carnitine tilapia for 6 weeks. L-carnitine feeding increased the acyl-carnitine concentrationfrom 3522 to 10822 ng/g and alleviated the lipid depositionfrom 15.89 to 11.97 %in the liver of low-carnitine tilapia. However, as compared with L-carnitine group, D-carnitine feeding reduced the acyl-carnitine concentrationfrom 10822 to 5482 ng/g, and increased lipid deposition from11.97 to 20.21 % and the mRNA expression of the genes involved in β-oxidation and detoxification in the liver. D-carnitine feeding also induced hepatic inflammation, oxidative stress, and apoptosis. A metabolomic investigation further showed that D-carnitine feeding increased glycolysis, protein metabolism, and activity of the tricarboxylic acid cycle and oxidative phosphorylation.Thus, L-carnitine can be physiologically utilized in fish, whereas D-carnitine is metabolized as a xenobiotic and induces lipotoxicity. D-carnitine-fed fish demonstrate increases inperoxisomalβ-oxidation, glycolysis, and amino acid degradation to maintain energy homeostasis. Therefore, D-carnitine is not recommended for use in farmed animals.

Keywords: low-carnitine tilapia; metabolic regulation; L-carnitine; D-carnitine; nutrient metabolism

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Introduction

Carnitine is a vitamin-like substance that plays a vital role in the β-oxidation of long-chain fatty acids (FAs) to produce energy by transporting them from the cytosol to the mitochondrial matrix,*via* carnitine palmitoyltransferase 1 (CPT1) [1, 2]. Carnitine can be synthesized endogenously from lysine and methionine, mainly in the liver[3-5]. In mammals, a deficiency of carnitine causes dyslipidemia and severe lipid accumulation[6, 7], and carnitine has been used as a functional food additive to promote FA oxidation and treat metabolic diseases, such as diabetes and obesity[8-10]. Analogous to its role in mammals, carnitine is thought to aid the maintenance of energy homeostasis in other land and aquatic animals when administered as a dietary supplement[11, 12]. Therefore, carnitine is industrially produced as a drug or feed additive for clinical and nutritional use. However, industrial production yields both optical isomers of carnitine, L-carnitine and D-carnitine[13-15], and a mixture of the two (DL-carnitine) is widely sold. Many mammalian studies have shown that only L-carnitine has beneficial effects[16-18], while D-carnitine can interfere with the uptake and transport of L-carnitine[13, 19], and therefore inhibit the β-oxidation of FAs[20, 21], which has a hepatotoxic effect[22]. However, studies of the functions of L- and D-carnitine in aquatic animals have yielded contradictory results to date[23, 24].

High energy diets are now widely fed to farmed fish, which has led to a number of metabolic diseases, especially severe fat accumulation, developing in various fish species, leading to adverse effects on their growth and health[25, 26]. Therefore, there is a great deal of interest in identifying means of increasing lipolysis to supply energy in the form of FAs and to alleviate excess lipid accumulation in fish. Carnitine has been used as a lipid-lowering feed additive in farmed fish since the 1980s. A number of studies have shown that L-carnitine can efficiently reduce tissue lipid deposition in some fish species; however, it does not affect body lipid content or growth performance in other species, and actually increases hepatic content in a few[27]. In fact, in practical aquaculture, DL-carnitine is more commonly used in feeds. However, the nutritional effects of D-carnitine have only been evaluated in sea bass, and the results showed that D-carnitine reduces growth performance and increases tissue lipid content (in liver and muscle), in addition, the regulatory mechanism of D-carnitine on nutritional metabolism in fishwas not yet known now[23, 28]. Interestingly, a number of studies have shown that DL-carnitine has a

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similar effect in many fish species to L-carnitine to reduce whole-body lipid content, when used at the same dose[24, 29]. These contradictory results have discouraged the use of carnitine in aquafeed, and therefore it would be of great interest to precisely distinguish the effects of L- and D-carnitine on nutrient metabolism in fish.

In addition, it has been difficult to compare the effects of L- and D-carnitine supplementation, because it has not been possible to eliminate the background of endogenous L-carnitine in vivo. Under most physiologic conditions, endogenous carnitine biosynthesis may be sufficient to maintain adequate tissue concentrations[30], and therefore dietary supplementation with D-carnitine might only be partially able to affect L-carnitine concentration in particular tissues. For example, in the rat, dietary D-carnitine supplementation reduces endogenous L-carnitine levels in heart and skeletal muscle by >50%, in kidney by <30%, but not at all in the liver, brain, or plasma[19]. Furthermore, previous work in mammals and zebrafish has shown that mitochondrial β-oxidation of FAs is not efficiently inhibited when the endogenous carnitine concentration is reduced by ≤50%[31, 32]. Therefore, the effects of D-carnitine on metabolism are difficult to investigate using standard animal models, meaning that the individual metabolic effects of L- and D-carnitine supplementation in animals have not been fully determined to date.

Therefore, we aimed to establish a low-carnitine model to investigate the effects of feeding dietary L- or D-carnitine on metabolism. Mildronate[3-(2,2,2-trimethylhydrazinium) propionate; MD] is a structural analogue of a precursor in L-carnitine synthesis (γ-butyrobetaine), which has been used to establish mammalian models of mitochondrial dysfunction, in which endogenous carnitine biosynthesis is efficiently inhibited[33]. Recently, we have established MD-induced low-carnitine models in zebrafish and Nile tilapia, in which endogenous carnitine concentrations were only ~10% of control levels[32, 34].

In order to precisely distinguish the effects of L- and D-carnitine on systemic nutrient metabolism, we first established a low-carnitine Nile tilapia model, which is a useful model for the study of nutrient metabolism and for which there is a great deal of available genomic information[35].Diets containing 0.4 g/kg L- or D-carnitine were fed to low-carnitine fish for 6 weeks, after which, growth, dietary use, and organ biochemistry were assayed, and gene expression analysis and liquid chromatography-mass

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spectrometry (LC-MS)-based metabolomics were performed. The data shows that compared with L-carnitine, D-carnitine inhibits lipid catabolism and causes lipotoxicity. In addition, metabolomic and gene expression analyses together showed the induction of systemic metabolic changes by D-carnitine. To the best of our knowledge, this is the first study to illustrating the different effects of L- and D-carnitine on nutrient metabolism in an animal with a relatively low-carnitine background.

Experimental Methods

Animal ethics

All experiments were conducted under 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.

Fish, diets and sampling

More than 600 juvenile Nile tilapia were purchased from Shanghai Ocean University (Shanghai, China). Before the formal experiment, the fish were acclimated for two weeks, during this period they were fed on a commercial diet (protein 37.1%, lipid 10.9%) (Shenyang, China). To establish the low-carnitine tilapia model and perform the experimental design, two experimental stages were followed as shown in Fig. 1. Accordingly, four diets were prepared: basal diet, basal diet adding mildronate propionate (MD, 25 g/kg diet), basal diet adding MD (25g/kg diet) and L-carnitne (0.4g/kg diet), and basal diet adding MD (25g/kg diet) and D-carnitine (0.4g/kg diet). To prepare the diets, MD, L-carnitine and D-carnitine were firstly dissolved in water, then mixed with the ingredients of the basal diet. The formulations of the diets are listed in SupplementaryTable S1.

After the acclimation, the first stage of the experiment was conducted to establish the low carnitine tilapia model as we recently reported[32, 34]. In this stage, 480 fish were randomly divided into 2 groups (control group and MD group) and fed with the basal diet and basal diet adding MD (25g/kg diet) at 4% body weight (BW), respectively. The control group contained 3 tanks, while the MD group had 9 tanks, each tank contained 40 fish. After 4 weeks, only 10 fish from each tank were anesthetized

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using the MS-222 at 20 mg/L, sampling for the liver, muscle, visceral adipose tissue and plasma to calculate the hepatosomatic index (HSI), intraperitioneal fat index (IPI) and tissue concentration of carnitine. During this stage, the photoperiod was 12h/12h, and the temperature was kept at 26 ± 2 °C. The weight of the fish in each tank was recorded once a week, and the feeding amount was adjusted correspondingly.

The second stage of the experiment was to evaluate the different functions between L- and D-carnitine in nutritional metabolism. The MD-treated fish from the first stage were randomly divided into 3 groups: MD group (MD), MD + L-carnitine group (MD/L-carnitine) and MD + D-carnitine group (MD/D-carnitine). Fish from each dietary group were distributed in 3 tanks (n= 30 fish per tank) and fed 4% BW for 6 weeks. The experimental conditions were similar as in the first stage. Thereafter, fish were fasted for 12-hour and anesthetized (MS-222 at 20 mg/L) for sample collection of plasma, liver, muscle and visceral adipose tissue (VAT). All samples were frozen with liquid nitrogen and then stored at -80°C until use for molecular and biochemical assays.

Carnitine concentration assay

After the whole feeding trial, parts of the liver and muscle of 6 fish were collected from each group, accurately weighted and homogenized by distilled water (1:10, w:v). The tissue homogenate was centrifuged at room temperature, 2000 rpm for 15 min. A 100 μL aliquot of tissue homogenate supernatant was first incubated by using 50 μL KOH (1 mol/L) at 37 °C for 30 min to fully hydrolyze the combined carnitine, and 10 μLHCl (1 mol/L) was then added to neutralize the solution (sample A). A total of 60 μL distilled water was added directly into another 100 μL aliquot of tissue homogenate (sample B). Protein in sample A, B was removed by precipitating with 200 μL of cold acetonitrile (ACN) (containing 1 μg/mL carbachol, the internal standard, IS) and centrifuged at 16, 900 ×g for 20 min. The 1 μL of the supernatant was injected for LC-MS/MS analysis. Total carnitine was defined as the concentration of carnitine in sample A, free carnitine was defined as the concentration of carnitine in sample B, acyl-carnitine was defined as total carnitine minus free carnitine (A minus B). The details of LC-MS measurements were reported in our recent publication[27].The measurement of serum

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carnitine was similar to the testing of the tissue carnitine.

Biochemical composition of whole fish and tissues

The crude lipid of the liver and muscle was extracted and measured using methanol and chloroform (1:2, v:v) as previously described[36, 37]. The glycogen content of liver was assessed by the commercial kit (Jiancheng Biotech Co., China). The crude protein of the liver, muscle and whole fish was measured by KjeltecTM 8200 (FOSS, Sweden). The serum triglyceride (TG) content, malondialdehyde (MDA), superoxide dismutase (SOD), glucose and total amino acid (TAA) were measured by using specific commercial kits (Jiancheng Biotech Co., China).

Histological analysis

Pieces of liver (5×5mm) were fixed in 4% paraformalclehyde and embedded in paraffin as describedpreviously[38]. Sections of 5 μm thickness were stained with hematoxylin and eosin (H & E). The paraffin sectioning and hematoxylin and eosin staining of visceral adipose tissue were performed as described[39]. The two tissues histology were observed and photographed by Olympus BX53.

Quantitative real-time PCR

Total RNA was isolated by using a Tri Pure Reagent (Aidlab, China). The quality and quantity of total RNA were tested by NANODROP 2000 Spectrophotometer (Thermo, USA). The cDNAs of tissues total RNA were synthesized using a PrimerScriptTM RT reagent Kit with a gDNA Eraser (Perfect Real Time) (Takara, Japan) by S1000TM Thermal Cycler (Bio-Rad, USA). Elongation factor 1 alpha (ef1α) and β-actin were used as the reference genes. The primers of all testing genes for Quantitative PCR (qPCR) were listed inSupplementary Table S2. The qPCR assay was carried out as described previously[40].

Metabolomics assay of liver samples

The accurately weighed liver samples (20 mg/sample) from D- or L-carnitine-treated fish were

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prepared to grinde in the cooled tube with internal standard and 80% methanol, and ultrasonicated after vortexed. Samples were then centrifuged at 4 °C, 14000 rpm for 10 min. Two hundreds microliter of the supernatants from each tube was collected by using crystal syringes, filtered through 0.22 μmmicrofilters and transferred to LC vials.

The following LC-MS analysis (ACQUITY UHPLC system Ultimate 3000 coupled with LTQ Orbitrap MS (Thermo Fisher Scientific, USA)), data preprocessing, and statistical analysis, identification of differential metabolites, were performed with some modification from the methods of the previous study[41]. The detailed procedures of liver metabolomics assay are described in the SupplementaryExperimental procedures.

Statistical analyses

All results are presented as mean ± SEM (n = 4-6). Independent-samples t-test was performed to evaluate the significant difference (P<0.05) of variables between control and MD group in the establishment of low-carnitine tilapia. In the data of evaluation of the different functions of L- and D-carnitine, significant differences (P<0.05) of each variable were firstly detected using the one-way ANOVA test, followed by Duncan's multiple range to estimate the differences. All analyses were conducted using the SPSS Statistics 19.0 software (IBM, USA).

Results

Establishment of low-carnitine tilapia

After 4 weeks of feeding with MD (1,000 mg/kg BW/d), a low-carnitine fish model had been successfully established, evidenced by the large reductions in serum, liver, and muscle concentrations of free carnitine, and the greater mass of adipose tissue and the liver (Fig. 2(a)). Throughout the 4-week feeding period, both control and MD-treated fish remained visually in good health and there was no significant growth difference between the two groups (Fig. 2(b)). These low-carnitine tilapias were then used in a subsequent 6-week experiment to investigate the metabolic effects of L- and D-carnitine, which were individually added to the MD diet.

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D-carnitine is associated with lower acyl-carnitine synthesis than L-carnitine and is metabolized by detoxification pathways

After 6 weeks, neither the MD/L-carnitine, nor the MD/D-carnitine diet, affected growth, when compared with MD fish (data not shown). In serum, MD fish had the lowest free carnitine concentration (40 ng/ml) (Fig. 3(a)). Both L- and D-carnitine supplementation significantly increasedthe free carnitine content in serum of MDfishto 108 and 98 ng/ml, respectively, which were similar as the free carnitine concentration in serum of control fish (109 ng/ml) (Fig. 3(a)). However, only L-carnitine supplementation could increase the serum concentration of acyl-carnitine, which is the physiological form of carnitine used in the acyl-carnitine shuttle across the mitochondrial membranes, to the normal level as that in control tilapia (123 vs 141 ng/ml, Control vs MD/L-carnitine), whereas the serum concentration of acyl-carnitine in MD and MD/D-carnitine groups were low and comparable (40 vs 39 ng/ml, MD vs MD/D-carnitine) (Fig 3(a)). Similarly, in liver, both L- and D-carnitine supplementation increased the free carnitine concentration in the low-carnitine tilapia more than three-fold(1795 vs 9018 vs 8797 ng/g, MD vs MD/L-carnitine vs MD/D-carnitine) (Fig. 3(a)), but only L-carnitine increased the hepatic concentration of acyl-carnitine with around two times more being present than in the MD or MD/D-carnitine-fed fish(10822 vs 3522 vs 5482 ng/g, MD/L-carnitine vs MD vs MD/D-carnitine) (Fig. 3(a)). In contrast tothe sensitivity to isomer-specific effects of carnitine in the liver, there were no significant differences in free carnitine or acyl-carnitine concentrations in muscle among all the groups of fish (Fig. 3(a)).

Markers of xenobiotic detoxification were also assayed (Fig. 3(b)). In the liver, the MD/L-carnitine group had significantly lower expression of cytochrome P450 family 1 subfamily A (*CYP1A*) than the MD or MD/D-carnitine groups. A similar trend was identified with respect to liver heat shock protein 70 (*HSP70*), but this did not reach significance. In muscle, the expression of *CYP1A* was comparable among the groups, whereas the L- and D-carnitine groups showed significantly lower expression of *HSP70*.

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D-carnitine causes greater lipid deposition and expression of genes involved in the β-oxidation of fatty acids than L-carnitine

As shown in Fig.4, among the four groups, the control andMD/L-carnitine groupsdisplayed lower serum triglyceride (TG) concentration (Fig. 4(a)) and lipid content in liver and muscle (Fig. 4(b)) than the MD and MD/D-carnitine groups (serum TG: 2.60 vs 1.99 vs 3.30 vs 4.29 mmol/L,liver lipid: 8.33 vs 11.97 vs 15.89 vs 20.21%,muscle lipid: 1.72 vs 1.44 vs 1.98 vs 1.86%,Control vs MD/L-carnitine vs MD vs MD/D-carnitine). The MD/D-carnitine group had the significantly higher serum TG concentrations and liver lipid contentthan those in the MD/L-carnitine group, and also had significantly higher liver lipid content than that in the MD group. As a consequence, the nearlymasses of the liver and adipose tissue in the control andMD/L-carnitine group were lower than the other two groups, but the MD/D-carnitine group had the highest liver mass(Fig. 4(c)). These trends were also reflected in histological images of the liver and adipose tissue, which showed that the control andL-carnitine group had thelower, and D-carnitine the highest, degree of lipid accumulation in the liver and adipose tissue (Fig. 4(d) and (e)). Thus, L-carnitine feeding reduced the lipid content of the liver, adipose tissue, and serumto the normal level, but D-carnitine did not.

The mRNA expression of genes involved in lipid metabolism in liver, muscle, and adipose are shown in Fig. 5. Interestingly, the MD and MD/L-carnitine groups showed similar expression levels of genes involved in lipid catabolism. In contrast, diacylglycerol O-acyltransferase 2 (*DGAT2*), the gene responsible for FA esterification; and fatty acid synthase (*FAS*) and sterol regulatory element-binding transcription factor 1 (*SREBP1*), which are critical for *de novo* lipogenesis, showed similar levels of expression in the L- and D-carnitine groups. However, MD/D-carnitine treatment was associated with significantly higher mRNA expression of *CPT1α* and acyl-CoA oxidase (*ACO*) in the liver and muscle, and peroxisome proliferator-activated receptor β (*PPARβ*) in muscle, than L-carnitine or MD. These genes all encode proteins that are involved in mitochondrial or peroxisomal β-oxidation.

D-carnitine stimulates higher glucose utilization than L-carnitine

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The effects of the two carnitine isomers on glucose metabolism are shown in Fig. 6. The MD/L-carnitine group showed the highest concentrations of serum glucose and liver glycogen of the three groups, but serum glucose was not significantly different in the MD/L-carnitine and MD/D-carnitine groups (Fig. 6(a) and (b)). Consistent with this, there were also differences in the expression of genes involved in glucose metabolism. In the liver, MD/L-carnitine significantly increased the mRNA expression of the gluconeogenic enzymes fructose-1,6-bisphosphatase 1 (*FBP1*)and glucose-6-phosphataseα (*G6Pα*), while higher expression of the glycolytic enzymes PFK and PK was found in the MD/D-carnitine group (Fig. 6(c)). In muscle, higher expression of *FBP1* and *G6Pα*was also found in the MD/L-carnitine group.

L- and D-carnitine do not significantly affect protein metabolism

The effects of the two carnitine isomers on protein metabolism are presented in Fig. 7. Only MD/D-carnitine significantly increased TAA (Fig. 7(a)), but the total protein content of liver, muscle, and whole fish were comparable among the three groups (Fig. 7(b)). The mRNA expression of genes involved in protein metabolism, were unaffected by the type of carnitine fed (genes involved in protein catabolism: initiation factor 2 kinase (*GCN2*), activating transcription factor 4 (*ATF4*), and glutamate dehydrogenase 1 (*GDH1*); genes involved in synthesis: mechanistic target of rapamycin (*mTOR*)) in liver. In muscle, the MD/D-carnitine group demonstrated lower expression of the protein catabolism genes *ATF4* and *GDH1* than the MD group. However, considering the comparable protein content of the whole body and tissues, it is clear thatL- and D-carnitine do not differentially affect protein metabolism.

D-carnitine has the potential to cause more severe inflammation, oxidative stress, and apoptosis than L-carnitine

The mRNA expression of the genes involved in inflammation, oxidative stress, and apoptosis is shown in Fig. 8. MD/D-carnitine induced higher expression of pro-inflammatory factors, particularly interleukin 1β (*IL-1β*) and transforming growth factor β1 (*TGF-β1*) in liver and muscle, and tumor

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necrosis factor α (*TNF-α*) in muscle, than MD/L-carnitine and MD (Fig.8(a)). However, MD/L-carnitine feeding only caused higher expression of *TNF-α* in muscle and *IL-1β* in adipose tissue than MD. Similarly, MD/D-carnitine feeding resulted in higher serum concentrations of peroxidation products (MDA) and tended to increase the mRNA expression of the antioxidant enzymes glutathione S-transferase (*GST*) and *SOD* in the liver. However, MD/L-carnitine and MD feeding was associated with similar levels of these oxidative stress markers (Fig.8(b)). D-carnitine also induced higher hepatic mRNA expression of caspase 8 (*Casp8*), a marker of apoptosis, and lower muscle mRNA expression of B-cell lymphoma 2 (*Bcl2*), an anti-apoptosis protein, than MD and MD/L-carnitine feeding (Fig.8(c)). Although MD/D-carnitine also reduced the mRNA expression of caspase 3(*Casp3*) and BCL2 associated X (*Bax*) in muscle (Fig. 8(c)), the marked upregulation of Casp8 implies a greater potential for apoptosis in the MD/D-carnitine group.

A metabolomic analysis reveals distinct metabolic responses to the feeding of D- and L-carnitine

A metabolomic analysis was performed to further aid understanding of the contrasting effects of L- and D-carnitine in the liver of low-carnitine tilapia. The score plots of the OPLS-DA model show that all the samples from the D-carnitine and L-carnitine-treated groups were within the 95% Hotelling T^2 ellipse, and that the metabolomic profiles of L- and D-carnitine groups were clearly separated (Fig. 9(a)), implying differing metabolomic profiles. When the D-carnitine and L-carnitine groups were compared, with the L-carnitine group being used as the control, a total of 85 metabolites with differing concentrations between the groups were identified (VIP>1 and P<0.05) (Supplementary Table S3). Of these 84metabolites, two acyl-carnitines and 11 lysophospholipids were present at lower concentrations, and 23 phospholipids and glycerophosphoric acid (the substrate of diglyceride (DG)) were present at higher concentrations in the MD/D-carnitine group than the MD/L-carnitine group.

D-carnitine also increased the anaerobic production of lactic acid and D-citramalic acid from pyruvate, but was associated with reductions in the concentrations of five essential amino acids in tilapia (Leu, Val, Met, Trp, and Phe) and higher concentrations of His and (+)-threo-2-Amino-3,4-dihydroxybutanoic acid. Moreover, D-carnitine increased the concentrations of

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fumaric acid, 2,3-methylenesuccinic acid, carbonic acid, adenosine 5'-monophosphate (AMP), and phosphoric acid, which are by-products of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. All the metabolites showing marked differences between the D-carnitine and L-carnitine groups are displayed in a comprehensive metabolic map as Fig. 9(b). In summary, the hepatic metabolomic analysis shows that D-carnitine feeding is associated with greater lipid deposition, glycolysis, and amino acid catabolism, to maintain energy homeostasis.

Discussion

The contrasting effects of L-carnitine and D-carnitine on lipid metabolism

Although carnitine has been used as a feed additive for more than 20 years, the specific effects of L- and D-carnitine in many aquatic species have yet to be properlydetermined[42-47]. Contradictory findings generated by previous studies have been attributed to inter-species differences or variations in dietary components[12, 30]. However, the previous studies could not avoid the confounding effects of the endogenous carnitine background when attempting to evaluate the effects of administering exogenous L- or D-carnitine. We were able to inhibit the synthesis of endogenous carnitine using MD and thereby establish a low-carnitine model in the zebrafish and tilapia in our previous studies[32, 34]. The present study was the first to evaluate the specific effects of L- and D-carnitine in fish with a low endogenous carnitine background.

Dietary L-carnitine efficiently increased the free carnitine and acyl-carnitine concentrations in the serum and liver of the low-carnitine tilapia, and also reduced lipid accumulation in tissues of the low-carnitine tilapia. Of note, these values of L-carnitine group were similar as those in normal fish, showing that the dietary supplementation of L-carnitine was sufficient to recover the carnitine shortage caused by MD.Because acyl-carnitine is the physiologically active type of carnitine, this is consistent with L-carnitine being the physiological carnitine isomer in tilapia and also confirms that exogenous L-carnitine can be utilized in fish. Of note, dietary supplementation with L-carnitine did not significantly increase the concentrations of free carnitine or acyl-carnitine in muscle in the low-carnitine tilapia. The tissue-specific effect of L-carnitine were found in many experiments in

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mammals or fish, in which the carnitine content of muscle was relatively stable, whereas the liver was more sensitive to carnitine supplementation or deficiency[32, 48, 49]. However, some fish studies also reported that dietary L-carnitine could increase the muscle carnitine content in African catfish and zebrafish[27; 50; 51]. Therefore, the tissue-specific effects of L-carnitine are likely to be different among species, and thus more low-carnitine animal models should be established to intensively evaluate the possible different functions of L-carnitine among species.

The supply of additional carnitine would be expected to promote the transport of FAs into mitochondria, β-oxidation to produce energy, and a reduction in lipid storage.As strong evidence for this, in the present study, L-carnitine supplementation significantly ameliorated the lipid deposition caused by the MD-induced carnitine deficiency. In our recent study of normal zebrafish, dietary L-carnitine supplementation also increased hepatic carnitine concentration, mitochondrial activity, and the mRNA expression of *CPT1*[27], which was associated with lower body lipid content. Interestingly, L-carnitine supplementation in low-carnitine tilapia did not cause an upregulation of the expression of genes involved in β-oxidation. This is likely explained by our previous finding that, compared to normal tilapia, *CPT1* is overexpressed in MD-treated tilapia as a compensatory response to carnitine deficiency and the associated low β-oxidation capacity[34]. This could explain why when exogenous L-carnitine was supplied, the already highly up-regulated mitochondrial β-oxidation could be effective at reducing the accumulated lipid.

In contrast to the effects of L-carnitine, dietary supplementation with D-carnitine did not elevate acyl-carnitine concentrations in the low-carnitine tilapia, and in fact tended to reduce it in muscle.It also further aggravated the lipid deposition in the low-carnitine tilapia. Consistent with these effects, the mRNA expression of genes involved in β-oxidation was compensatorilyup-regulated in the D-carnitine group. These effects were similar to those previously observed in MD-induced low-carnitine tilapia, zebrafish, and rodents[32, 34, 52], and indicate abnormal lipid metabolism as part of a metabolic syndrome. This confirms that D-carnitine should not be given to fish, and also suggests that some of the findings of previous studies, which showed no differences between the inclusion of L-, D-, and DL-carnitine in the diet[24, 29], were confounded by the presence of endogenous L-carnitine.

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As further evidence, D-carnitine supplementation caused a greater detoxification response than L-carnitine, suggesting that D-carnitine is more likely to be treated as a xenobiotic substrate than L-carnitine in tilapia.Moreover, the feeding of D-carnitine demonstrated more potential for inflammation, oxidative stress, and apoptosis than L-carnitine. This could be caused by D-carnitine itself, or indirectly by the lipotoxicity it induced, which is the result of excess fat deposition. It is known that excess lipid accumulation in non-adipose tissues is associated with higher concentrations of free FAs and TG in the circulation, and that this is often accompanied by inflammation, oxidative stress, and cellular dysfunction or death[53-55]. In summary, D-carnitine cannot be physiologically used, such that the high serum FA levels cannot be lowered, because they cannot enter mitochondria for oxidation, resulting in lipotoxicity.

However, in the present study, the difference in the effects of the two carnitine isomers on muscle metabolism was less marked. A possible reason for this is that there is a higher background concentration of carnitine in muscle than in the liver, or that the degree of supplementation with L- and D-carnitine was insufficient to significantly alter the concentration of acyl-carnitine, such that any change in lipid metabolism in muscle was not obvious. However, D-carnitine did induce adverse effects in muscle, indicated by upregulation of inflammatory and apoptotic markers.

Thus, the present study provides clear evidence that, at least in Nile tilapia, dietary L-carnitine is physiologically utilized in the transport of acyl-carnitine into mitochondriaand improves FA utilization, while dietary D-carnitine appears to be treated as a xenobiotic and disrupts metabolism.

Metabolic adaptation of tilapia in response to D-carnitine ingestion

In addition to facilitating a comparison of the effects of L- and D-carnitine on lipid metabolism, another aim of the present study was to investigate the metabolic adaptation of tilapia in response to D-carnitine.

Cooperation between mitochondria and peroxisomes

Previous studies in mammals and zebrafish have shown that whenmitochondrial β-oxidation is inhibited and fat accumulates in the liver or heart, *CPT1* gene expression or enzyme activity

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compensatorily increases to enhance FA transport into the mitochondrial matrix[32, 52, 56]. In the present study, *CPT1* gene expression was also higher in the liver of D-carnitine-treated fish. However, because D-carnitine significantly reduced the acyl-carnitine concentration, acyl-CoA would not be able to efficiently enter the mitochondrial matrix for β-oxidation. Nevertheless, in addition to increasing the expression of genes involved in mitochondrial β-oxidation, cooperation can also occur between mitochondria and peroxisomes as an adaptive mechanism to facilitate β-oxidation in mammals[57, 58]. Some previous studies in mammals have shown that gene expression or enzyme activities associated with peroxisomal β-oxidation can be enhanced as an adaptation to the inhibition of mitochondrial β-oxidation[56, 59-62]. In the present study, D-carnitine-treated tilapia exhibited higher expression of *ACO*, which encodes a key enzyme of peroxisomal β-oxidation, suggesting that peroxisomal β-oxidation may be up-regulated after D-carnitine treatment. However, a number of studies have indicated that peroxisomal β-oxidation has a significantly lower capacity than that of mitochondrial β-oxidation, particularly in muscle[63-65]. Therefore, even though peroxisomal β-oxidation may have been up-regulated in the D-carnitine group, this would have been insufficient to compensate for the depressed mitochondrial β-oxidation of FAs.

Energy homeostasis regulation

In sports medicine,L-carnitine is used to inhibit glycolysis by suppressing pyruvate dehydrogenase[66], or phosphofructokinase activity[67], which reduces exercise-associated lactate accumulation[68-70], and delays the development of fatigue[71, 72]. This indicates that carnitine is involved in the partition between lipid and carbohydrate use. In the present study, L-carnitine supplementation induced glucose sparing by normalizing FA utilization, while D-carnitine-fed tilapia used more glucose and protein in response to a lack of energy derived from lipids. Compared with mammals, fish have a lower ability to use carbohydrates as energy sources[73], but interactions between lipid and carbohydrate metabolism have been demonstrated in fish[74, 75]. In the present study, D-carnitine not only stimulated the expression of genes involved in glycolysis and reduced the expression of genes involved in gluconeogenesis, but also reduced liver glycogen stores and enhanced the production of lactic acid and D-citramalic acid from pyruvate, as demonstrated by the metabolomics data. This indicates that

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D-carnitine-fed fish use glucose more efficiently. Greater use of glucose by the glycolytic pathway has also been reported in mice in which mitochondrial β-oxidation is impaired[62, 76].

Energy homeostasis involves the balanced use of lipid, glucose, and protein substrates[27, 77, 78]. In the present study, we found that essential amino acid concentrations in the liver were much lower and the total concentration of amino acids (TAA) in the plasma was higher. This suggests that there is greater protein catabolism in D-carnitine-treated fish and that free amino acids, including essential amino acids, are metabolized to produce additional energy. Because the total protein content of tissues was not affected by the D-carnitine treatment, it is also possible that ingested protein is digested more efficiently in these fish, such that amino acid absorption from the gut can be increased. However, this possibility requires further investigation. Interestingly, D-carnitine-treated fish also produced higher concentrations of intermediate metabolites and end-products of the TCA circle, and end-products of oxidative phosphorylation. This supports the assertion that although mitochondrial β-oxidation was impairedto induce greater lipid deposition, these tilapias could still obtain energy from catabolism ofglucose and amino acids to maintain homeostasis, which was associated with greater activity of the TCA cycle and oxidative phosphorylation. This differential regulation of energy substrate use in the D-carnitine-treated fish is summarized in Fig. 10.

In conclusion, using a low-carnitine tilapia model, the present study has shown that dietary L-carnitine can be physiologically utilized in fish, and it has the effect of reducing lipid accumulation in the liver. In contrast, dietary D-carnitine intake reduced the acyl-carnitine concentration in the body and induced lipid accumulation in the liver, which was accompanied by lipotoxicity. Moreover, dietary D-carnitine stimulated glycolysis and amino acid degradation to maintain energy homeostasis. Therefore, D-carnitine is not recommended for use as feed additives in animals.

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Authors contribution were as follows: Z. D. and J. L. designed the research; J. L., L. L.,Y. Z., F. Q. conducted the research; J. L., Z. J.,S. L., L.C. and M. Z. analyzed data; S. L., and M. Z.. contributed to the final writing of the paper; J. L., P. D. and Z. D. wrote the manuscript. All authors read and

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The Authors declare that there are no competing interests associated with the manuscript.

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Figure Legends

Fig. 1

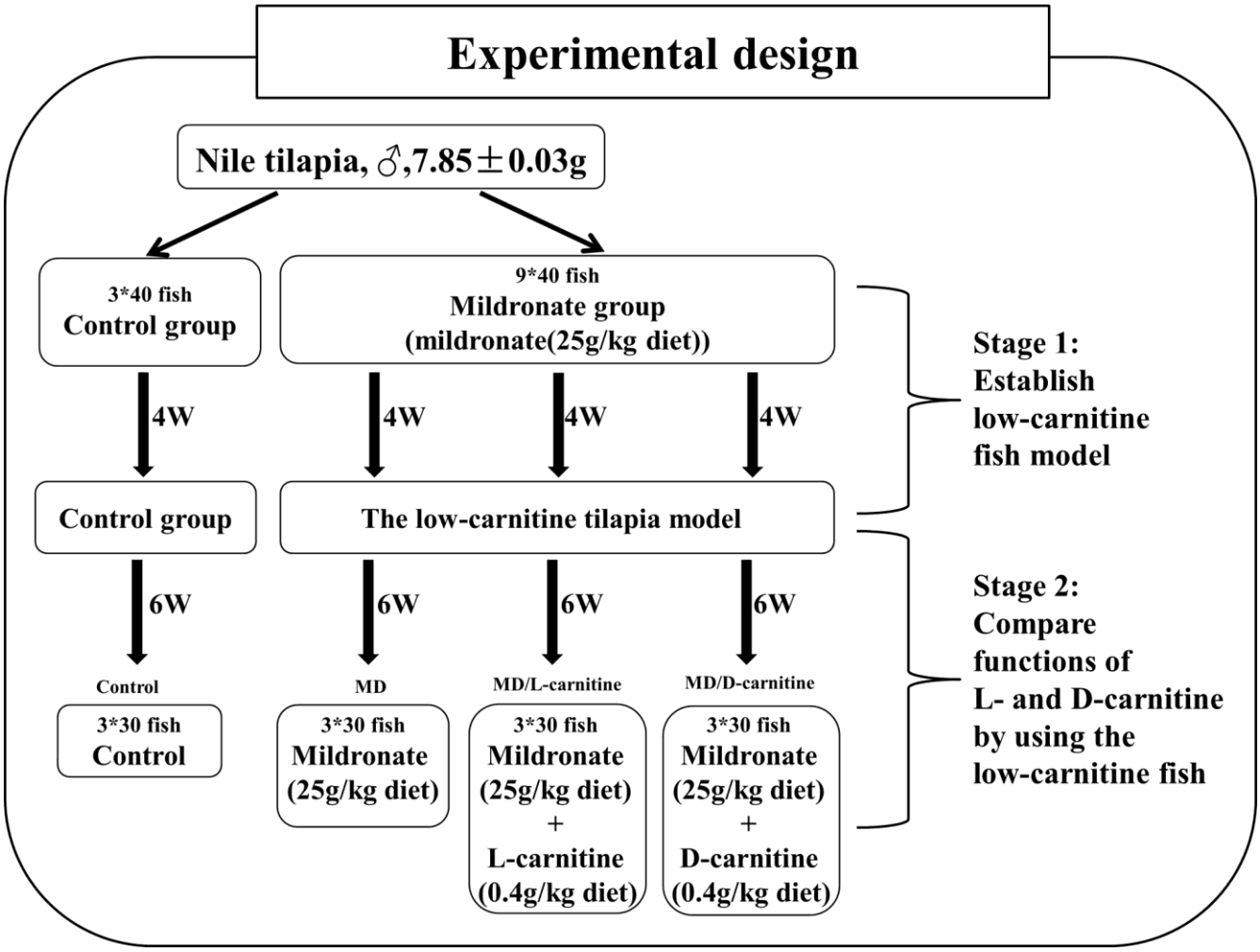


Fig. 1. The design and the protocol of the present experiment.

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Fig. 2

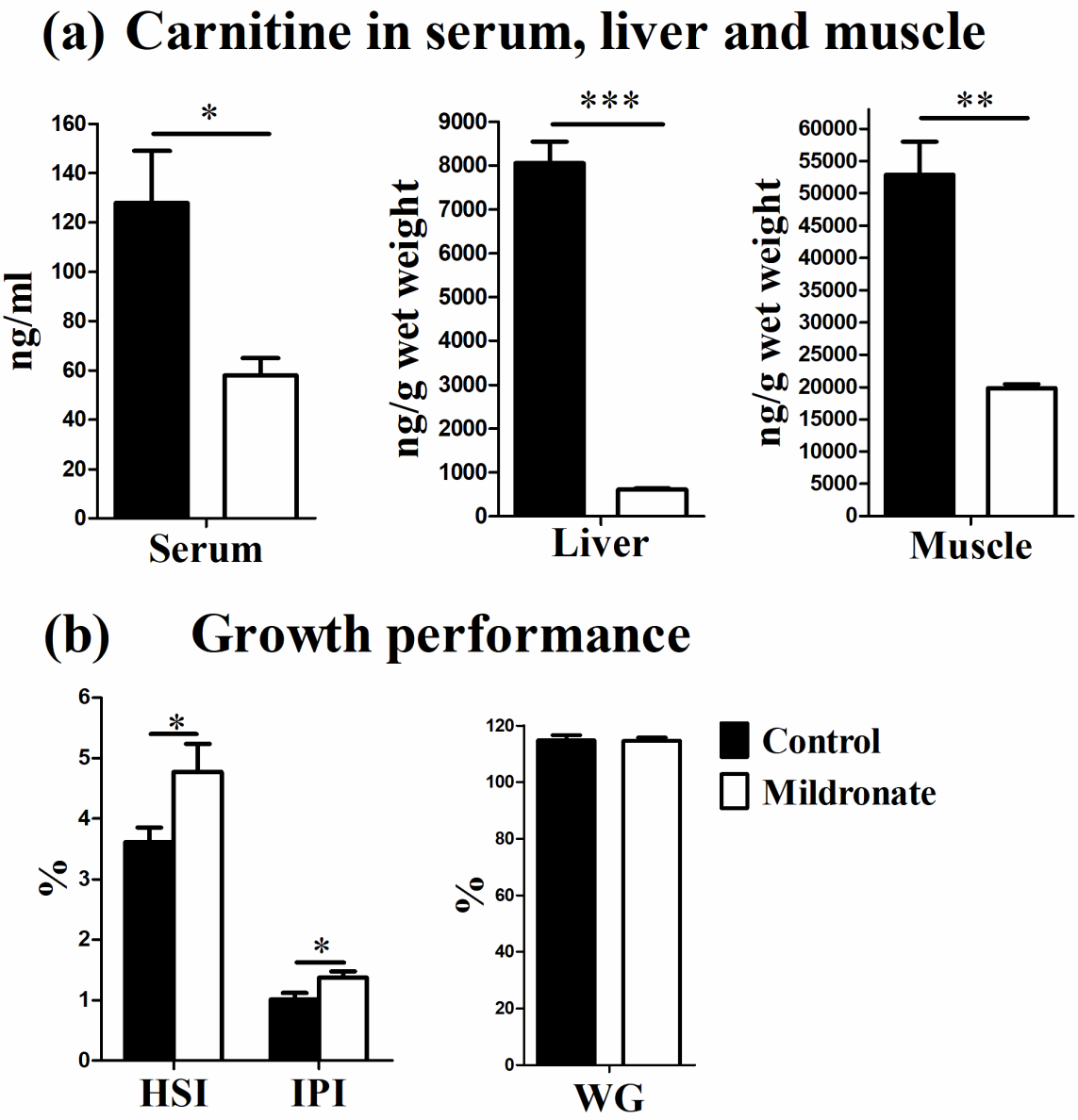


Fig. 2. The establishment of low-carnitine tilapia at the 4th week.(a) The free carnitine concentration in serum, liver and muscle; (b) The growth performance (HSI, IPI, WG). All values are means ± SEM (n = 6). The values with * and ** statistically differ at $P<0.05$ and $P<0.01$, respectively. hepatosomatic index, HSI; intraperitioneal fat index, IPI; weight gain, WG.

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Fig. 3

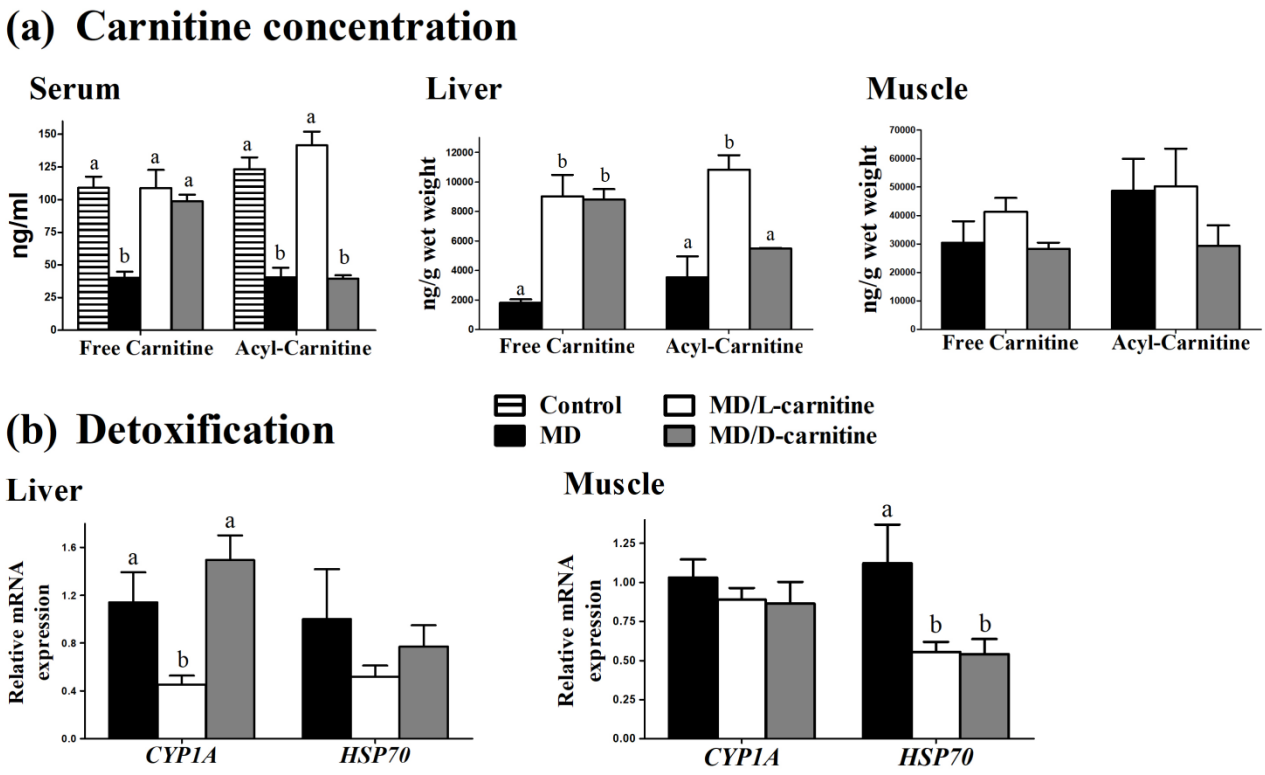


Fig. 3. The effect of L-carnitine and D-carnitine on the carnitine concentration and detoxification of the low-carnitine tilapia. (a) The free carnitine and acyl-carnitine in serum, liver and muscle; (b) The genes related to the detoxification in liver and muscle. All values are means ± SEM (n = 6). The values that are not followed by the same letter are significantly different (P <0.05).Cytochrome P450 1A ,*CYP1A*; heat shock protein 70, *HSP70*.

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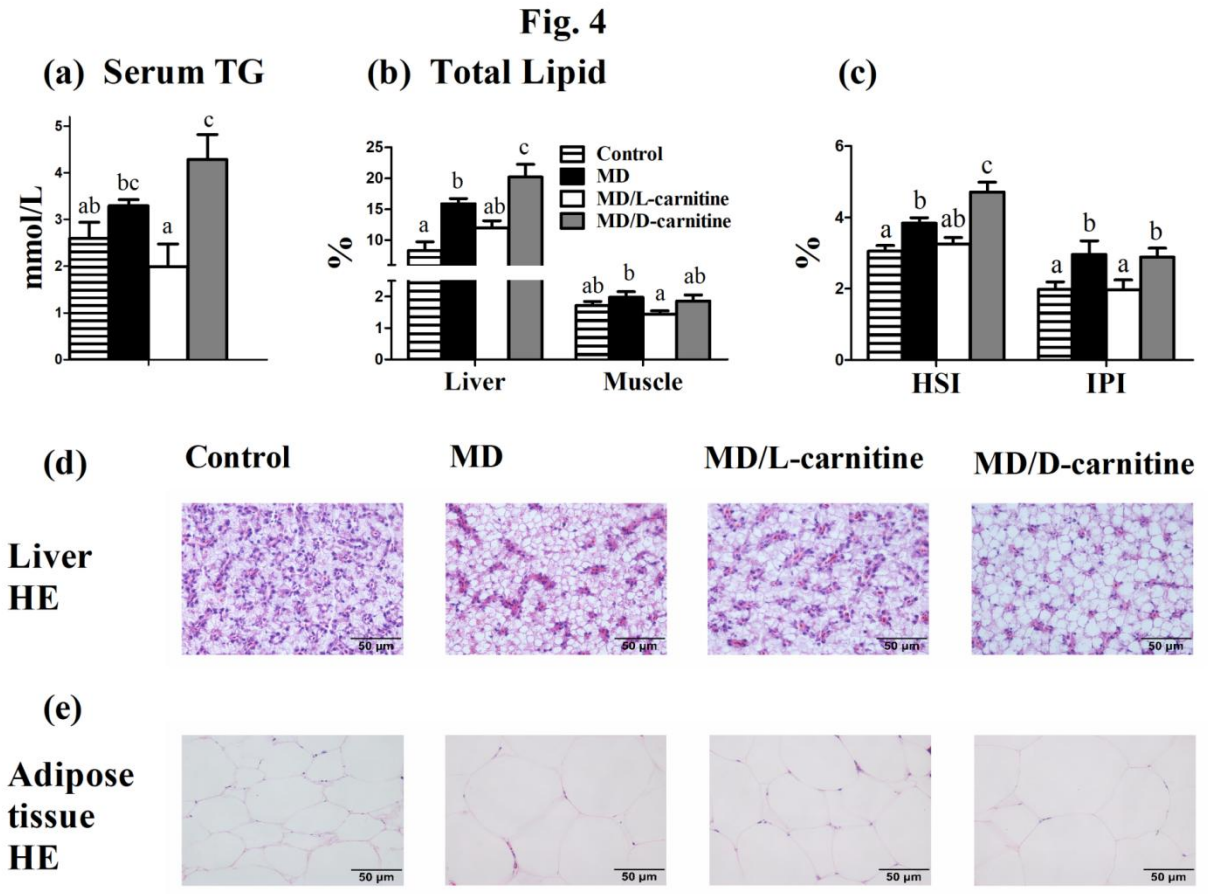


Fig. 4. The effect of L-carnitine and D-carnitine on the tissues lipid deposition.(a) The TG in serum; (b) The total lipid in liver and muscle; (c) The liver and adipose tissue weight; (d) The liver histological characteristics (HE stain); (e) The adipose tissue histological characteristics (HE stain). All values are means ± SEM (n = 6). The values that are not followed by the same letter are significantly different ($P < 0.05$).

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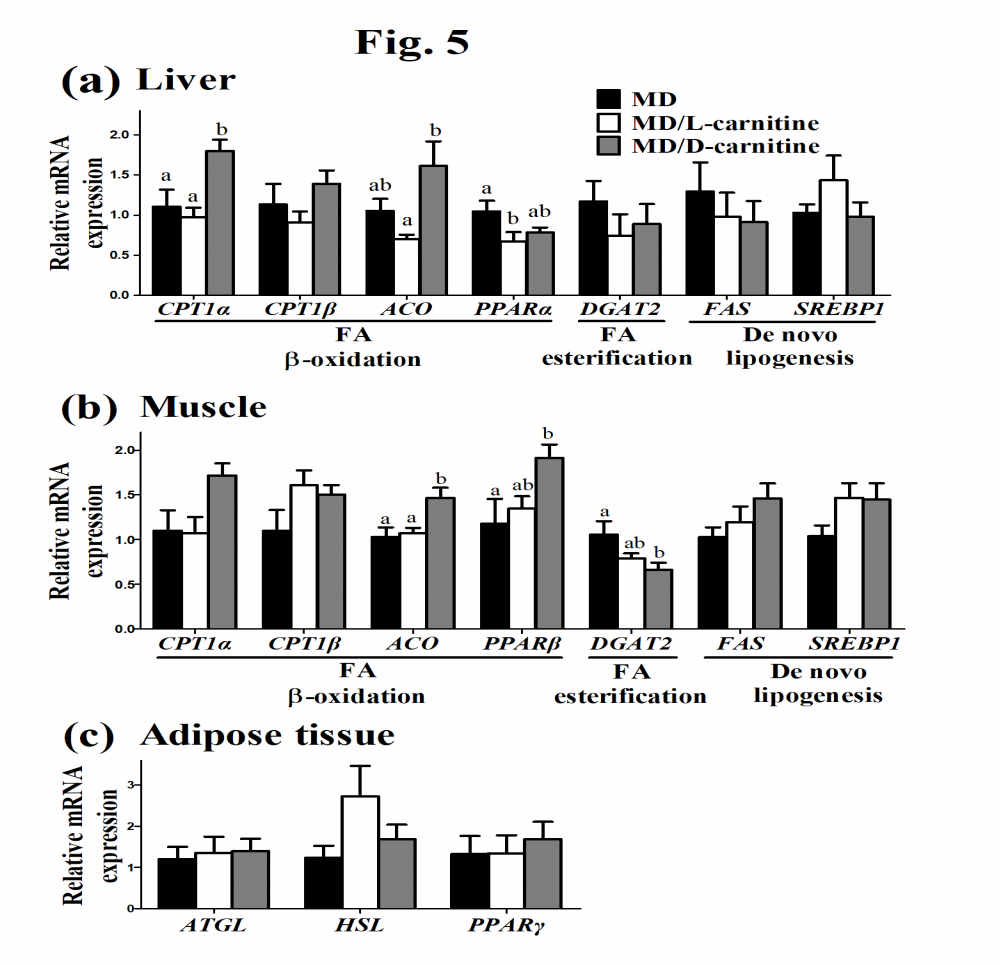


Fig. 5. The effect of L-carnitine and D-carnitine on the mRNA expression of lipid metabolism.(a) Liver lipid metabolism; (b) Muscle lipid metabolism; (c) Adipose tissue lipid metabolism. All values are means ± SEM (n = 6). The values that are not followed by the same letter are significantly different (P <0.05). Carnitine palmitoyltransferase 1, *CPT1*; acyl-CoA oxidase, *ACO*; peroxisome proliferator-activated receptor alpha, *PPARα*; fatty acid synthase, *FAS*; diacylglycerol O-acyltransferase 2, *DGAT2*; sterol regulatory element binding transcription factor 1, *SREBP1*; Adipose triglyceride lipase, *ATGL*; hormone-sensitive lipase, *HSL*; peroxisome proliferator-activated receptor gamma, *PPARγ*.

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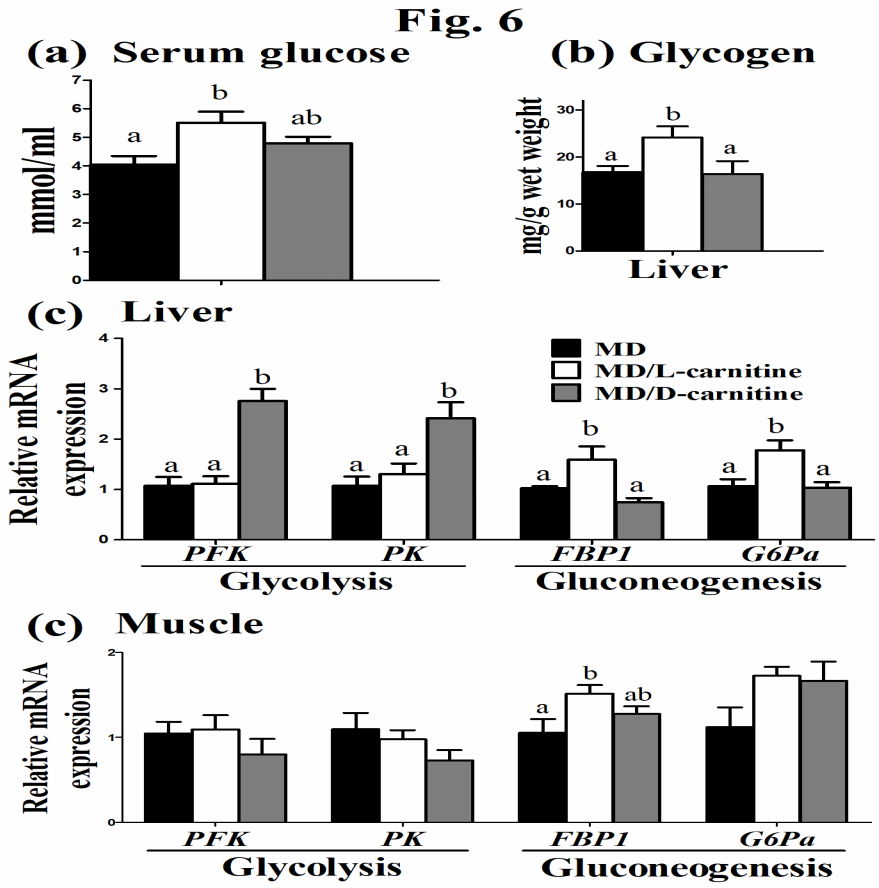


Fig. 6. The effect of L-carnitine and D-carnitine on the glucose metabolism. (a)The serum glucose; (b)The glycogen in liver; (c) Liver glucose metabolism; (d) Muscle glucose metabolism. All values are means ± SEM (n = 6). The values that are not followed by the same letter are significantly different ($P < 0.05$). Phosphofructokinase, *PFK*; pyruvate kinase, *PK*; fructose-1,6-bisphosphatase 1, *FBP1*; glucose-6-phosphatase, *G6P*.

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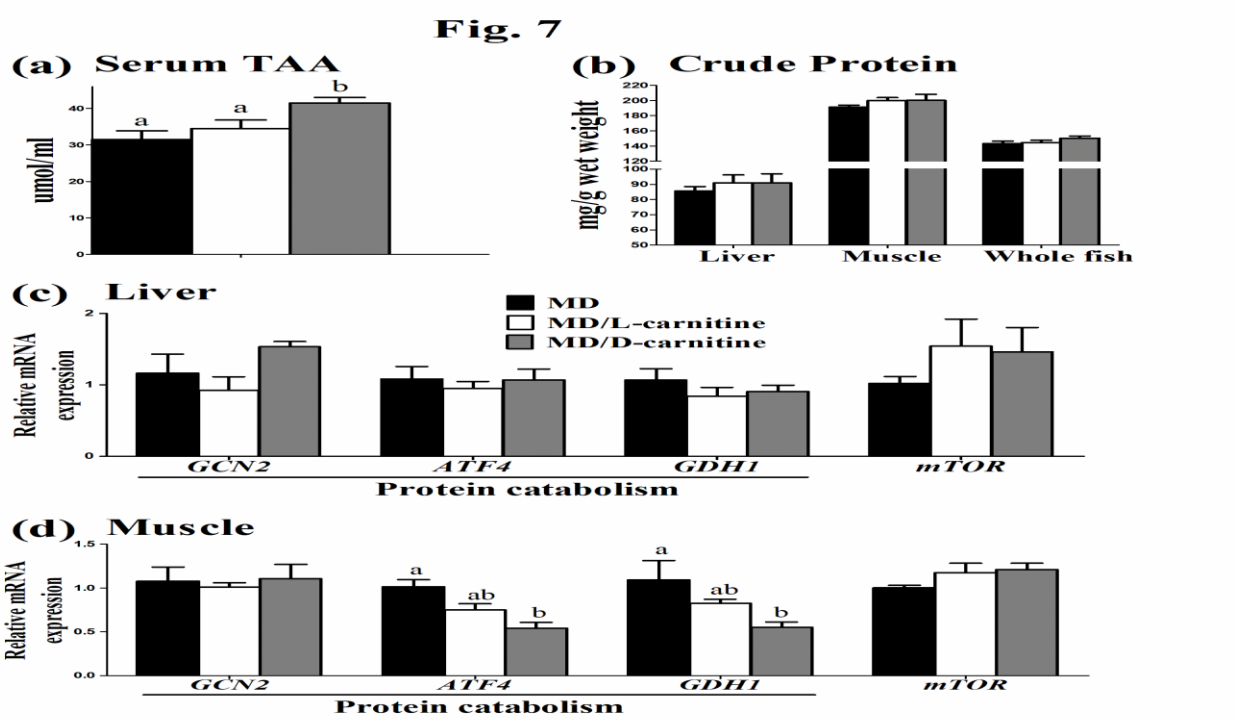


Fig. 7. The effect of L-carnitine and D-carnitine on the protein metabolism. (a)The serum TAA; (b) The crude protein in tissues and whole body; (c) Liver protein metabolism; (d) Muscle protein metabolism. All values are means ± SEM (n = 6). The values that are not followed by the same letter are significantly different (P <0.05). initiation factor 2 kinase, *GCN2*;activating transcription factor 4, *ATF4*;glutamate dehydrogenase 1, *GDH1*; mechanistic target of rapamycin, *mTOR*.

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Fig. 8

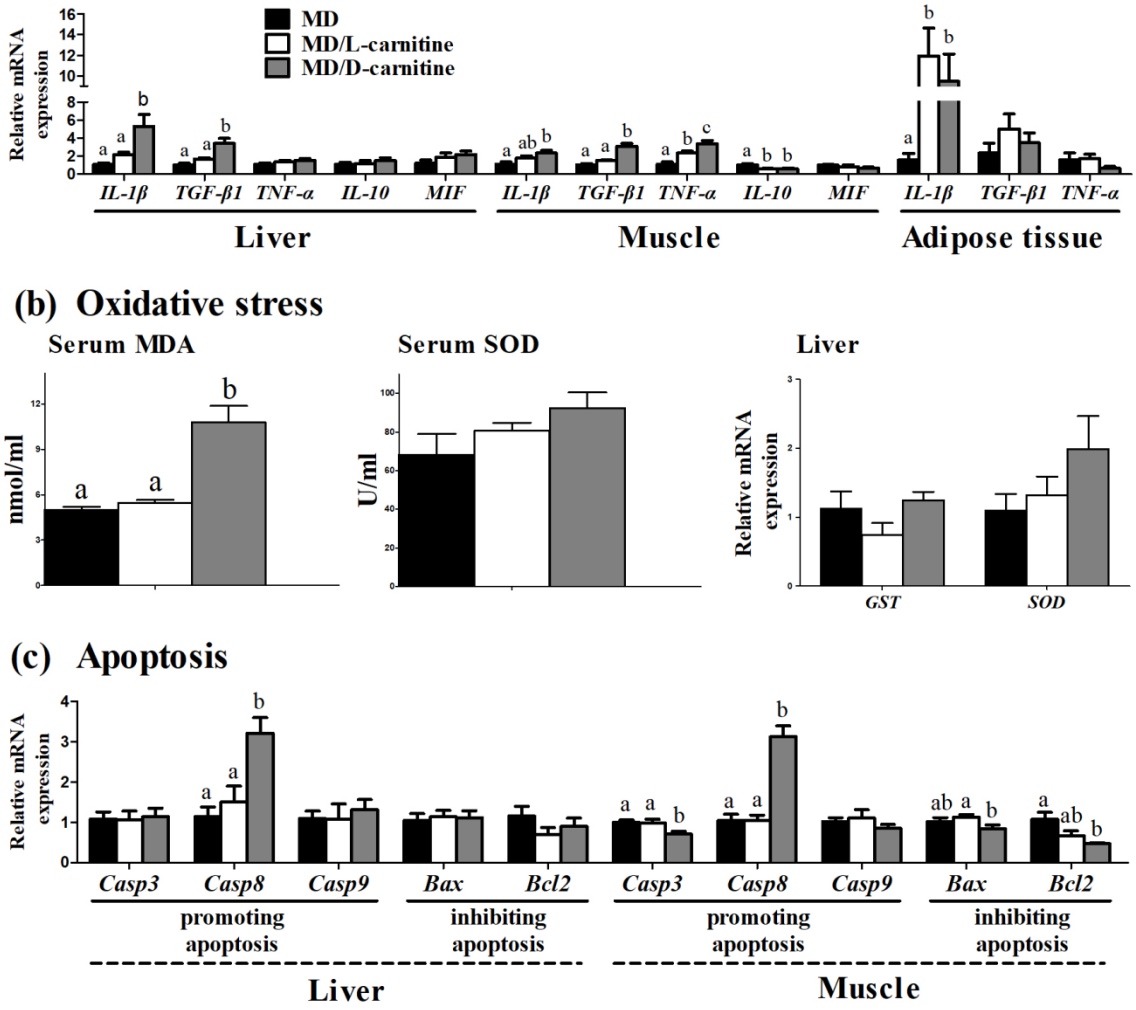


Fig. 8. The effect of L-carnitine and D-carnitine on the inflammation, oxidative stress and apoptosis. (a)The genes related to inflammation; (b) The serum MDA and SOD, the mRNA expression of *GST* and *SOD*; (c)The genes related to apoptosis. All values are means ± SEM (n = 6). The values that are not followed by the same letter are significantly different (P <0.05). interleukin 1 beta, *IL-1β*; transforming growth factor β1, *TGF-β1*; tumor necrosis factor α, *TNFα*; interleukin 10, *IL-10*; macrophage migration inhibitory factor, *MIF*; glutathione S-transferase, *GST*; superoxide dismutase, *SOD*; caspase 3, *Casp3*; caspase 8, *Casp8*; caspase 9, *Casp9*; BCL2 associated X, *Bax*; B-cell lymphoma 2, *Bcl2*.

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Fig. 9

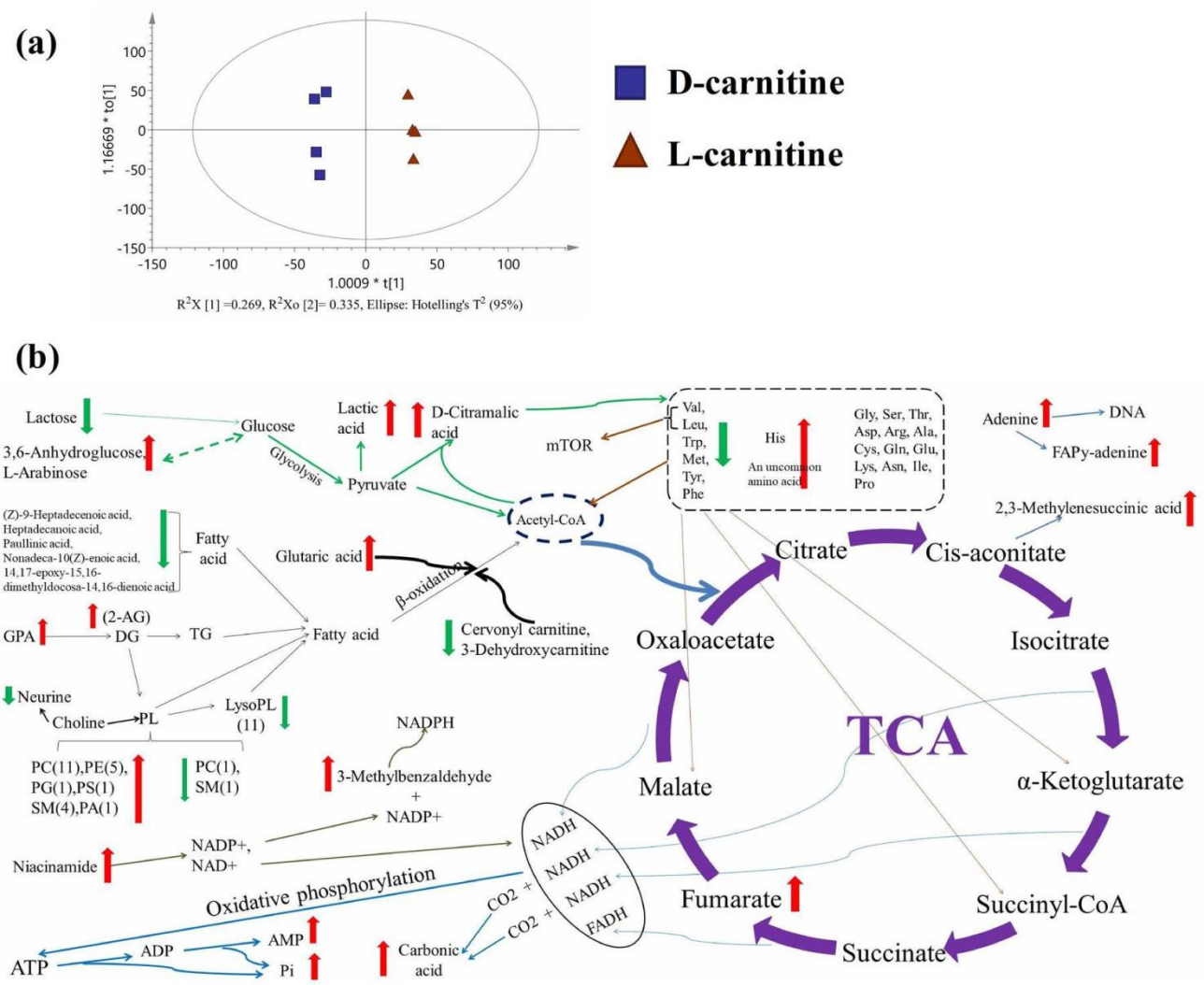


Fig. 9. Evaluation and KEGG pathway of the liver metabolimocs (D-carnitine/L-carnitine).(a) OPLS-DA analysis; (b) KEGG pathway of analysis.

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Fig. 10

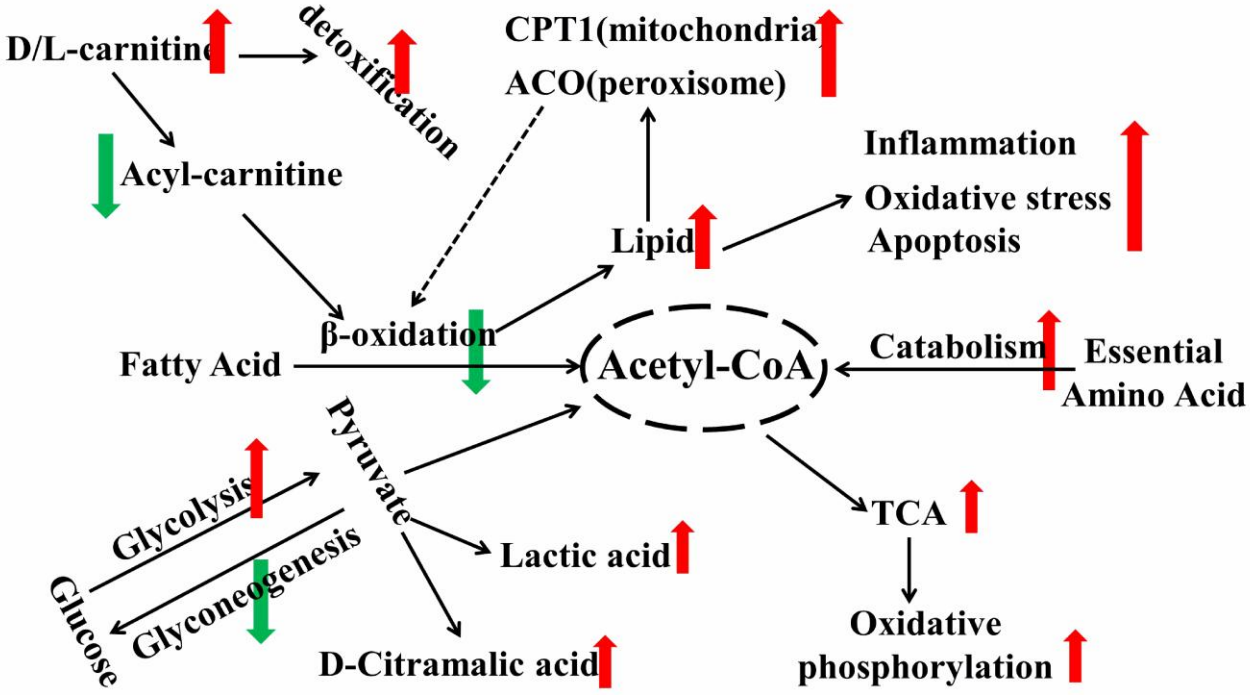


Fig. 10. The different function of L-carnitine and D-carnitine on metabolic regulation of fish.

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