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Abstract

The α-lipoic acid (α-LA) is a novel feed additive to improve growth, antioxidant capacity and lipid metabolism in animal husbandry. In this study, isonitrogenous and isoenergetic diets were formulated at seven levels of α-LA (0, 300, 600, 1200, 2400, 4800, or 9600 mg/kg) and fed to the Chinese mitten crab *Eriocheir sinensis* juveniles for eight weeks. According to weight gain and specific grwoth rate, the optimal level of α-LA supplement in the diet is estimated at 1339 -1574 mg/kg for *E. sinensis*. The α-LA supplement significantly increased the activities of SOD, GSH-Px and T-AOC, and the content of GSH in the hepatopancreas except for SOD in the crabs fed 9600 mg/kg α-LA. Excess dietary α-LA supplement increased the content of MDA in the hepatopancreas. The *E. sinensis* fed 2400 mg/kg α-LA in the diet showed the highest mRNA expressions of *es_TGL1*, *es_TGL2*, *es_IL* and *es_DGL* and had higher lipid catabolism when taking 300 mg/kg α-LA than other diets, which coincides with higher mRNA expressions of *es_IL*, *es_DGL* and *CPT-1* in the hepatopancreas. The crab fed 2400 mg/kg α-LA significantly modified 45 metabolites in serum compared with the control. The supplmentation of α-LA significantly influenced fatty acid metabolism, amino acid metabolism, carnitine metabolism and accumulation of plant antioxidants. This study indicates that α-LA is a promising feed additive to regulate growth, antioxidant status and lipid metabolism in *E. sinensis*,

but its inclusion in the diet should be less than 2400 mg/kg α-LA to maximize the benefitial effect and minimize side effects.

1. Introduction

The supplement of functional additives to a diet is an important approach for disease prevention and growth promotion in animal production (Giannenas et al., 2012). Functional additives in diets can be absorbed and utilized more efficiently and cause less environmental pollution than drug splashing such as antibiotics (Ringø et al., 2014). Therefore, application of feed additives in aquaculture has shown a broad development prospect for health management. The α-lipoic acid (α-LA) is considered a "universal antioxidant" or "ideal antioxidant" which widely exists in nature and has received attention in past decades (Packer et al., 2001; Wollin and Jones, 2003). Several studies have indicated that α-LA can be absorbed by oral ingestion and has high bioavailability in a variety of species (Carlson et al., 2007; Somi et al., 2011; Teichert et al., 1998). Thus α-LA derived from conventional diet ingredients or a nutritional supplement can be readily metabolized by the organism (Biewenga et al., 1997; Shay et al., 2009). Furthermore, research evidence has demonstrated that α-LA with a moderate dose in the diet is safe and nontoxic and can be used as a dietary supplement for farmed animals (Cremer et al., 2006a; b).

The α-LA can be rapidly converted into a reduced isoform as dihydrolipoic acid (DHLA) which is a natural antioxidant (Bast and Haenen, 2010, Packer et al., 1995b). Beside scavenging free radicals in

organisms, DHLA can also regenerate a variety of antioxidants such as vitamin C, vitamin E, glutathione, coenzyme Q and ubiquinone (Shay et al., 2009; Trattner et al., 2007). Due to its outstanding antioxidant capacity, α-LA has been thought as a feed additive in animal production. However, nutrition research on α-LA application in aquaculture is still limited, though some studies have been done on abalone *Haliotis discus hannai*, Japanese Flounder *Paralichthys olivaceus*, hybrid tilapia (*Oreochromis niloticus × O. aureus*) and Pacu *Piaractus mesopotamicus* (Wang FQ, 2010; Xiong et al., 2012; Zhang et al., 2010; Terjesen et al., 2004). In our previous research, α-LA supplement was used as a supplement in the diet of Chinese mitten crab *Eriocheir sinensis* with the dosages of 700 and 1400 mg/kg (Xu et al., 2018). Although its addtion has improved growth performance and antioxidant capacity, it is unclear if growth performance of the Chinese mitten crab can be further improved by extending the dosage of α-LA beyond 1400 mg/kg in the diet.

Most previous studies on the effect of dietary α-LA have focussed on the growth performance at the whole organism level, but there is a lack of an approach to study the comprehensive metabolic mechanism in aquatic animals. In this study, we used the approach of metabolomics to explore physiological and biochemical processes in the organism when α-LA was supplemented to the diets. To our best knowledge, this was the first

attempt to use metabolomics approach to study the metabolism of α-LA in aquaculture. This study aims to determine the optimum dosage of α-LA in the diet of juvenile *E. sinensis* and examine its metabolomics mechanisms in economically important crustacean species in aquaculture.

2. Materials and methods

2.1. Experimental diets

The isonitrogenous and isoenergetic practical diets were formulated with seven concentrations of α-LA (0, 300, 600, 1200, 2400, 4800 and 9600 mg/kg). Fish oil, soybean oil, lecithin and cholesterol were the lipid sources (approximately 7% crude lipid). Fish meal, soybean meal and cottonseed meal were the protein sources (approximately 36% crude protein). Lysine and methionine were added to adjust the balance of amino acids in the diets according the optimum requirement of these two amino acids. Butylated hydroxytoluene (BHT) was added at a concentration of 0.05‰ as the antioxidant to reduce lipid oxidation in the diets. Raw materials were ground and sieved through an 80-μm mesh. All dry ingredients were finely ground and mixed throughly before oil was added. The mixture was dissolved by adding deionized water (110 mL/kg diet) and then wet-extruded into 2.5-mm-diameter pellets using a double helix plodder (F-26, SCUT industrial factory, Guangdong, China). The scattered pellets were dried by blowing air at room temperature until

reaching less than 10% moisture. Pellets were sieved to various sizes by 14, 12 and 10 mesh sieves and stored at -20 ºC until use. Ingredient and proximate composition of the seven experimental diets are given in Table 1.

2.2. Experimental crab and management procedure

Juvenile *E. sinensis* were purchased from a local farm near Shanghai, China. After one-week acclimation in tanks (300 L), 840 healthy crabs (1.14 ± 0.06 g) were randomly assigned to 28 tanks (300 L). There were seven treatments with four replicates each, and each replicate had 30 crabs. Five groups of corrugated plastic pipes (12 cm long and 25 mm diameter, six pipes in each group) and five arched tiles were placed in each tank as shelters to reduce attacking behavior. During the trial, crabs were hand-fed thrice daily at 0700, 1600 and 2100 h with the daily ration of 4% body mass for eight weeks. Two hours after feeding, uneaten feed was removed by a siphon. The daily water exchange rate was 50% of the water volume. The incoming fresh water was filtered through a quartz sand filter (Xinyi Water Treatment Equipment Factory, Huzhou, China) and aerated thoroughly before entering the water recirculation system. Dead carbs were removed timely and the weight of each crab was recorded. During the whole cultivation, water temperature ranged from 25.0 to 28.0 ºC and pH ranged from 7.6 to 8.4. Dissolved oxygen was >7.0 mg/L and ammonia-N was <0.05 mg/L.

2.3. Sample collection and calculations

At the end of eight-week cultivation, all crabs were fasted for 24 h and were counted and weighed for calcualting survival, weight gain and specific growth rate (SGR). Then six crabs at the stage of intermoult from each tank were randomly collected and kept at -20 ºC for the analysis of whole-body composition. Subsequently, crabs were anesthetized in slurry ice and hemolymph was sampled with a 1-mL disposable syringe for metabolomics analysis and then crabs were dissected for assessing hepatosomatic index (HSI) and other subsequent analysis. Hepatopancreas samples were stored at -80 ºC until use. This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

Weight gain, specific growth rate, survival and hepatosomatic index were evaluated using the following formulae:

1) Weight gain (WG, %) = [final weight − initial weight]/initial weight × 100

2) Specific growth rate (SGR, % day^{-1}) = [ln $_{(final\ weight)}$ − ln $_{(initial\ weight)}$] ×100/days

3) Survival (%) = (final crab number/initial crab number) × 100

4) Hepatosomatic index (HSI, %) = (wet hepatopancreatic weight/wet body weight) × 100

2.4. Whole-body composition analysis

The proximate composition of diets and the whole body of all crabs were analyzed following the standard methods (AOAC, 1995). The moisture of whole body and diets was analyzed by drying to a constant weight at 105 ℃. The crude protein content was measured by the Kjeldahl method (8200, Kjeltec, Foss, Sweden). The crude lipid contents of whole body and hepatopancreas were extracted with a chloroform/methanol mixture and a 0.37 mol/L KCl solution following the method of Bligh and Dyer and quantified by gravimetry in the vacuum drying oven (DZF-6050, Jinghong, Ltd., Shanghai, China) (Folch et al., 1951). Kibbling samples were carbonized completely on a heating plate (TR-30A, SuDa, China) and then incinerated in a muffle furnace (PCD-E3000 Serials, Peaks, Japan) at 550 ℃ for 6 h to measure the ash content.

2.5. *Hepatopancreas biochemical analysis and gene expressions*

The contents of total protein, malondialdehyde (MDA) and glutathione peroxidase (GSH) and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and total antioxidant capacity (T-AOC) were measured by using the colorimetric method with the commericial assay kits (A045-2, A003-1, A006-1, A001-1-1, A005 and A015-1, Jiancheng, Bioengineering Institute, Nanjing, China). Hepatopancreases were weighed and homogenized in 10 volumes (v/w) of the pre-chilled saline solution and then were centrifuged at 1500 *g*

(5415R, Eppendorf, Germany) at 4 ºC for 30 min and the supernatant was collected. The supernatant of hepatopancreas homogenates was diluted with a 0.85% saline solution according to the manufacturer's instruction. The measurements of MDA, SOD, GSH-Px, T-AOC, GSH and total protein were based on the methods of Buege and Aust (1978), Nebot et al. (1993), Reiners et al. (1991), Benzie and Strain (1996), Chaney and Spector (1984) and Bradford (1976), respectively.

2.6. Gene expressions of lipid catabolism in hepatopancreas

Total RNA in the six replicaed hepatopancreas was extracted with the Trizol reagent (RN0101, Aidlab, China). RNA quantity and quality were estimated using a Nano Drop 2000 spectrophotometer (Thermo, Wilmington, USA). For each sample, 1 μg of total RNA was reversely transcribed using the PrimeScrip^{TM} RT Master Mix (RR047A, Takara, Japan) at 42 ºC for 2 min to remove genomic DNA and at 37 ºC for 15 min plus 85 ºC for 5 s to complete reverse transcription. cDNA was unified according to the measured concentration and the standard curve before conducting the real-time quantitative PCR (RT-PCR). Each gene in one treatment was run in six copies with β-actin (GenBank accession No. KM24725.1) as the internal control. Primers of *es_TGL1* (triglyceride lipase 1), *es_TGL2* (triglyceride lipase 2), *es_IL* (intracellular triglyceride lipase 2), *es_DGL* (diglyceride lipase) and *CPT-1* (carnitine palmitoyltransferase-1) were designed and validated by Primer Premier

6.0 according to our existing database (Table 5).

RT- PCR was performed in a final volume of 20 μL containing 10 μL of 2× Ultra SYBR mixture (CW0957, KangWei, China), 0.5 μL of 10 mM gene-specific forward and reverse primers, 2.5 μL of diluted first-standard cDNA template and 6.5 μL of H_2O with the following cycling condition: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 94 °C for 15 s, 58 °C for 20 s, 72 °C for 20 s and a 0.5 °C per 5 s increment from 60 °C to 95 °C. The RT- PCR was conducted in the CFX96 Real-Time PCR system (Bio-rad, Richmond, CA). The cycle time (Ct) values of different treatments were compared to their corresponding internal control and then converted to fold change values by comparing to the control group through the quantififed method of $2^{-\Delta\Delta Ct}$ (KJ and TD, 2001). CFX Manager^{TM} Software (version 1.0) was used for data visualization and relative quantification analysis.

2.7. Metabolomic analysis

2.7.1. Preparation of serum for metabolomics

Five serum samples were prepared for the crabs fed 0 and 2400 mg/kg α-LA, respectively. Samples were thawed at room temperature and 200 μL serum of each samples was mixed with an internal quantitative standard (30 μL L-2-chlorophenylalanine add 350 μL 75% methanol, 0.3 mg/mL) and vortexed for 30 s. Then, 150 μL mixed solution with

methanol and acetonitrile (v:v = 2:1) was added to precipitate protein completely. After stewing for 10 min at -20 °C, an ultrasonic extraction in an icy water bath was conducted for 5 min, followed by centrifugation at 4 °C, 1400 rpm for 10 min. The 400-μL supernatant was transferred into a fresh 2 mL LC-MS glass through a 0.22 μm filter membrane.

2.7.2. LC-MS analysis

In this study, LC-MS was performed in an Ultimate 3000-Velos Pro system equipped with a binary solvent delivery manager and a sample manager, coupled with a LTQ Orbitrap mass spectrometer equipped with an electrospray interface (Thermo Fisher Scientific, USA).

LC conditions: The column filler used in this study was the acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm, Waters, Milford, USA). Separation was achieved using the following procedure: 5% B-25% B for 0-1.5 min; 25% B-100% B for 1.5-10 min; 100% B for 10-13 min; 100% B-5% B for 13-13.5 min and 5% B for 13.5-14.5 min at a flow rate of 0.4 mL/min, where B is acetonitrile (0.1% (v/v) formic acid) and A is aqueous formic acid (0.1% (v/v) formic acid). The injected sample volume was 3 μL and the column temperature was set at 45 °C.

MS data was collected using a LTQ Orbitrap mass spectrometer equipped with an electrospray ionization (ESI) source operation in either positive or negative ion mode. The capillary and source temperature was set at 350 °C with a desolvation gas flow of 45 L/h. Centroid data were

collected form 50 to 1000 m/z with a 30 000 resolution.

The quality control (QC) sample was prepared by pooling the aliquots into a mixed sample, and then was analyzed using the same method with the analytic samples. The QCs were injected at regular intervals (every 5 samples) throughout the analytical run to provide a set of data from which repeatability was assessed.

2.8. Statistical analyses

The program XCMS (http://masspec.scripps.edu/xcms/scms.php) was used for the extraction of the peak intensities. The variables presented in the results counted for at least 80% of all varialbes extracted, and the variables with a retention time <0.5 min (near the dead time) were excluded owing to the high degree of ion suppression. Variables with <30% relative standard deviation (RSD) in the QC samples were then retained for further multivariate data analysis. The result was in a three-dimensional matrix, including retention time and m/z pairs, sample names and normalized ion intensities. The positive and negative data were combined to get an complete dataset for analysis in the SIMCA-P$^+$ 14.0 software package (Umetrics, Umea, Sweden). Principal component analysis (PCA) and (orthogonal) partial least-squares-discriminant analysis (O) PLS-DA were carried out to visualize the metabolic alterations among experimental groups, after the prodecures of mean centering and unit variance scaling. Variable importance in the projection

(VIP) ranked the overall contribution of each variable to the (O) PLS-DA model, and those variables with VIP >1.0 were considered relevant for group discrimination. The heatmap was produced by the method of H cluster. Furthermore, the KEGG pathway analysis of different metabolities was performed on an online databases, MBRole (http://scbg.cnb.csic.cs/mbrole).

Statistical analysis was carried out with the SPSS statistics 20 (IBM, Armonk, NY, USA). All data were presented as mean ± standard errors (SE). One-way analysis of variance (ANOVA) was used to determine the significance between α-LA dosages in the diet. Post hoc test was used for pairwise comparisons among seven treatments by Duncan's multiple range test. Differences were considered to be statisticaly significant at $P <$ 0.05 and extremely significant at $P < 0.01$.

3. Results

3.1. Growth performance

No significant difference was found in survival after eight-week cultivation. The HSI was lowest in crabs fed 300 mg/kg α-LA among all treatments (Table 2). WG and SGR were higher in crabs fed 1200 and 2400 mg/kg α-LA than in other treatments ($P < 0.05$). WG and SGR of crabs significantly decreased when the supplement of α-LA exceeded 2400 mg/kg in the diets. Based on the results of WG and SGR, the

optimum supplement of α-LA in the diet for *E. sinensis* was estimated as 1339.13 and 1574.09 mg/kg by the broken-line regression method (Figure 1-A, B).

3.2. Whole-body proximate composition and hepatopancreas lipid, TG contents

There was no sinificant difference in moisture, crude protein, crude lipid and ash among all treatments ($P < 0.05$, Table 3). Although a slight increase of hepatopancrease lipid contents in the supplments of 300, 600 and 1200 mg/kg and a slight decrease in 2400 and 4800 mg/kg α-LA supplement were observed in comparison to the control, no significant difference was found among all treatments ($P > 0.05$) (Figure 2-A). Triacylglycerol (TG) contents in hepatopancreas increased gradually with the increase of α-LA supplement in the diet ($P < 0.05$) (Figure 2-B).

3.3. mRNA expressions of genes related to lipid catabolism

The *es_TGL1* and *es_TGL2* showed the highest mRNA expressions in the hepatopancreas of *E. sinensis* fed 2400 mg/kg α-LA ($P < 0.05$) (Figure 3-A, B). The *es_IL* also had the highest mRNA expression in *E. sinensis* fed 2400 mg/kg α-LA despite no significant difference from those fed 300, 1200 and 9600 mg/kg α-LA (Figure 3-C). There was highest mRNA expression of *es_DGL* in the hepatopancreas of *E. sinensis* fed 1200 mg/kg α-LA, but without significant difference from

those fed 300 and 2400 mg/kg α-LA (Figure 3-D). In the hepatopancreas of *E. sinensis* fed 300 mg/kg α-LA, CPT-1 showed significantly higher mRNA expression than that in other treatments ($P < 0.05$, Figure 3-E).

3.4. Antioxidantive paramaters in hepatopancreas

Activities of SOD and GSH-Px in the hepatopancreas increased at the initial stage and then decreased with the increasing dosage of α-LA in the diet and peaked in *E. sinensis* fed 600 mg/kg α-LA ($P < 0.05$, Table 4). T-AOC increased with the increasing α-LA contents in the diet and showed the highest value in *E. sinensis* fed 4800 mg/kg α-LA ($P < 0.05$). The MDA content in the hepatopancreas of *E. sinensis* fed α-LA >4800 mg/kg significantly increased, compared with other treatments ($P < 0.05$). Although no significant change was found, the GSH content in the hepatopancreas increased when α-LA supplement exceeded 600 mg/kg in the diet ($P > 0.05$).

3.5. Identification of metabolites by metabolomics

In this study, a total of 45 significantly changed metabolities were identified from five biological replicates of the serum of crabs fed 0 and 2400 mg/kg α-LA. Among which, 22 metabolities significantly increased and 23 metabolities significantly decreased in comparion to the control (Table 6). These metabolities were categorized into two clusters according to their change patterns (Figure 5).

Three patterns of top19 pathways with significantly changed serum metabolities are showed in Figure 4. Pattern 1: Pathways with three significantly changed metabolities were aminoacyl-tRNA-biosynthesis, linolenic acid metabolism, arachidonic acid metabolism and glycerophospholipid metabolism. Pattern 2: Pathways with two significantly changed metabolities were neuroactive ligand-receptor interation, autophagy-animal, autophagy-other, ABC transporters, cysteine and methionine metabolism and glycosylphosphatidylinositol (GPI)-anchor biosynthesis. Pattern 3: Pathways with one significantly changed metabolities were beta-alanine metabolism, alanine, aspartate and glutamate metabolism, tryptophan metabolism, taurine and hypotaurine metabolism, sulfur relay system, alpha-linolenic cid metabolism, selenocompound metabolism, histidine metabolism and the biosynthesis of phenylalanine, tyrosine and tryptophan.

4. Discussion

In the present study, the best growth performance was found in the *E. sinensis* fed 1200 or 2400 mg/kg α-LA, but excessive α-LA supplement in the diet decreased weight gain and grwoth. Similarly, in abalone *Haliotis discus hannai*, Japanese flounder *Paralichthys olivaceus* and hybrid tilapia *Oreochromis niloticus × Oreochromis aureus*, excess use of dietary α-LA reduced weight gain (Wang FQ, 2010; Xiong et al., 2012; Zhang et al., 2010). In the present study, appropriate supplement of

α-LA in the diet improved antioxidant capacity of *E. sinensis*. The SOD and GSH-Px in the hepatopancreas showed higher activities in the crab fed 600, 1200 or 2400 mg/kg α-LA and the contents of GSH and T-AOC also increased by α-LA supplement. DHLA can promote the conversion of cystine to cysteine which is a limiting factor for GSH biosynthesis (Han et al., 1997; Suh et al., 2004). These results indicate that appropriate α-LA addition to the diet can activate the enzymatic and non-enzymatic antioxidant systems and improve antioxidant capacity in *E. sinensis*. The significant decrease of T-AOC and increase of MDA contents by supplementing 9600 mg/kg α-LA indicate that excess α-LA in the diet can negatively impact antioxidant capacity in *E. sinensis* in a similar way reported in *H. discus hannai* (Zhang et al., 2010).

In the present study, the amount of plant antioxidants, including pueraria glycoside 3, glycitein, chicoric acid, dihydrozeatin and asparagoside, significantly increased in the serum of *E. sinensis* fed 2400 mg/kg α-LA compared to the control. Among these antioxidants, the increase of glycitein and dihydrozeatin in serum indicates that α-LA can improve the utilization and absorption efficiency of other dietary ingredients in *E. sinensis* (Dan and Birac, 2010; Martin et al., 1989). Chicoric acid and asparagoside A can decrease mRNA expressions of interleukin (IL) and tumor necrosis factor-α (TNF-α) and reduce inflammation and improve antioxidant capacity in mice (Liu et al., 2009;

Xiao et al., 2013). Similarly, pueraria glycoside can improve the activity of SOD in rats (Chen et al., 2012; Chen et al., 2018). Therefore, the increased absorption and accumulation of plant antioxidants in the serum suggest that supplementation of α-LA in the diet is a possible approach to enhance the level of antioxidative capacity in *E. sinensis*.

Dietary α-LA did not significantly influence crude lipid and hepatopancreas lipid, but the TG content in the hepatopancreas significantly increased with the increase of dietary α-LA supplement. However, in our previous study, α-LA in the diet can promote the accumulation of crude lipid and hepatopancreas lipid regardless of dietary lipid percentage (Xu et al., 2018), which is different from the present study as the previous study did not include a large rage of α-LA dosage. In mammals, α-LA intake by oral administration or muscular injection can reduce body weight through reducing appetite via inhibiting the phosphorylation of AMP-activated protein kinase-α (AMPK-α) in the hypothalamus (Kim et al., 2004). Similarly, lipolysis can be accelerated and lipogenesis can be suppressed by dietary α-LA through accelerating the phosphorylation of AMPK-α in the liver and the muscle which can decrease the lipid content in rats (Butler et al., 2009; Kumar et al., 2006; Seo et al., 2012). In our study, the higher mRNA expressions of *es_TGL1* and *es_TGL2* in *E. sinensis* fed 2400 mg/kg α-LA indicate a better ability of triglyceride digestion in the hepatopancreas (Cherif et al., 2007; Miled

et al., 2000). Relative to the crabs fed 1200 and 2400 mg/kg of α-LA, crabs fed 300 mg/kg α-LA showed higher active lipid catabolism, as indicated by higher mRNA expressions of *es_IL*, *es_DGL* and *CPT-1* in the hepatopancreas, which coincides with the significant reduction of HSI (Rivera-Pérez and García-Carreño, 2011). It indicates that α-LA shows a distinguished function in lipid metabolism due to the change of α-LA dosage in the diet of *E. sinensis*.

Active lipid metabolism was also observed in the serum of crabs fed 2400 mg/kg α-LA by metabolomics analysis. Fatty acid metabolism and carnitine metabolism were significantly influenced by the dosage of α-LA in the diet and especially reflected in the change of glycerophospholipid such as PC (18:1/20:5), PE (16:1/22:6) and PE (20:5/22:6). The change of carnitine is a main physiological response to fatty acid metabolism (Fritz, 1963). In rats, α-LA can regulate the metabolism of arachidonic acid and linoleic acid as an efficient drug to diabetes cure (Doss et al., 1997; Packer et al., 1995a). Similarly, in this study, the increased metabolism of polyunsaturated fatty acid induced a significant increase in the contents of 13-L-Hydroperoxylinoleic acid and 13-OxoODE that both can activate peroxisome proliferator-activated receptor γ (PPARγ) (Schopfer et al., 2005). It might be the reason why the TG content of hepatopancreas significantly increased in the *E. sinensis* fed an increasing level of α-LA, because other researchers have also demonstrated that PPARγ is critical

for lipid biosynthesis and storage (Brun and Spiegelman, 1997; He et al., 2015).

The metabolism of amino acids including cysteine, methionine, histidine, phenylalanine, beta-alanine and tryptophan significantly changed in the *E. sinensis* due to the variation of α-LA intake. Active carnitine metabolism was closely associated with the change of amino acid metabolism because it is the main ramification of amino acid and plays an important role in energy metabolism by acylation of hydroxyl groups (Fritz, 2010). Methyl-malonyl-carnitine can be produced by metabolizing amino acids such as valine, methionine and isoleucine (Verbrugghe et al., 2012). In previous research, α-LA was used to treat Alzheimer's syndrome due to its critical role in protein biodegradation and modification amino acid residues (Bilska and Włodek, 2005; Butterfield et al., 1994). Furthermore, DHLA can be rapidly released to the intercellular space and transfer cystine to cysteine to promote biosynthesis of GSH by neutral amino acid transporters (Han et al., 1997). Meanwhile, cysteine can provide the reduced form of sulfur to multiple biological molecules such as methionine, LA and coenzyme A (Kredich, 2008). Methionine is also a precursor of GSH biosynthesis which can reduce oxidative damage and promote detoxification in the cell (Caylak and Aytekinb, 2008; Reed and Orrenius, 1977). Therefore, amino acids such as cysteine, histidine and phenylalanine were significantly

influenced by dietary supplement of α-LA in *E. sinensis*.

5. Conclusion

The α-LA can be supplemented to the diet of *E. sinensis* to improve growth performance and antioxidant capacity. The recommended optimal supplement dosage of α-LA is 1339 -1574 mg/kg. The appropriate supplement of α-LA can promote digestion and absorption of plant antioxidants and lipids in the diet. However, the dosage of α-LA in the diets determines the level of influence on the lipid metabolic pattern. The recommended maximum dosage should not exceed 2400 mg/kg in the diet and excessive α-LA supplement can reduce growth performance and antioxidant capacity in *E. sinensis*.

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Table 1

Ingredient formulation (g/kg dry basis) and proximate composition (%) of the seven experimental diets fed to *E. sinensis*

Ingredients	Content (g/kg dry basis)						
	0	300	600	1200	2400	4800	9600
Fish meal	250	250	250	250	250	250	250
Soybean meal	190	190	190	190	190	190	190
Cottonseed meal	190	190	190	190	190	190	190
Corn starch	150	150	150	150	150	150	150
Fish oil : Soybean oil (1:1)	35	35	35	35	35	35	35
Lecithin	5	5	5	5	5	5	5
Cholesterol	5	5	5	5	5	5	5
Lysine[a]	4	4	4	4	4	4	4
Methionine[a]	6	6	6	6	6	6	6
Vitamin premix[b]	30	30	30	30	30	30	30
Mineral premix[c]	20	20	20	20	20	20	20
Choline chloride	5	5	5	5	5	5	5
Carboxymethyl cellulose	20	20	20	20	20	20	20
Butylated hydroxytoluene	0.05	0.05	0.05	0.05	0.05	0.05	0.05
α-lipoic acid (mg/kg diet)[d]	0	300	600	1200	2400	4800	9600
Cellulose	90	89.7	89.4	88.8	87.6	85.2	80.4
Total	1000	1000	1000	1000	1000	1000	1000
Analysed proximate composition							
Moisture	9.90	10.01	10.45	10.49	10.45	10.40	10.65
Crude protein	36.56	36.80	36.66	36.53	36.42	36.38	36.40
Crude lipid	7.08	7.31	7.49	7.67	7.03	7.43	7.34
Ash	10.37	10.47	10.64	10.70	10.43	10.80	10.95

[a] Hainachuan pharmaceutical, Ltd., Guangdong, China

[b] Vitamin premix (per 100 g premix): retinol acetate, 0.043 g; thiamin hydrochloride, 0.15 g; riboflavin, 0.0625 g; Ca pantothenate, 0.3 g; niacin, 0.3 g; pyridoxine hydrochloride, 0.225 g; para-aminobenzoic acid, 0.1 g; ascorbic acid, 0.5 g; biotin, 0.005 g; folic acid, 0.025 g; cholecalciferol, 0.0075 g; α-tocopherol acetate, 0.5 g; menadione, 0.05 g; inositol, 1 g. All ingredients are filled with α-cellulose to 100 g.

[c] Mineral premix (per 100 g premix): KH_2PO_4, 21.5 g; NaH_2PO_4, 10.0 g; $Ca(H_2PO_4)_2$, 26.5 g; $CaCO_3$, 10.5 g; KCl, 2.8 g; $MgSO_4 \cdot 7H_2O$, 10.0 g; $AlCl_3 \cdot 6H_2O$, 0.024 g; $ZnSO_4 \cdot 7H_2O$, 0.476 g; $MnSO_4 \cdot 6H_2O$, 0.143 g; KI, 0.023 g; $CuCl_2 \cdot 2H_2O$, 0.015 g; $CoCl_2 \cdot 6H_2O$, 0.14 g; Calcium lactate, 16.50 g; Fe- citrate, 1 g. All ingredients are diluted with α-cellulose to 100 g.

[d] Sangon Biotech, Ltd., Shanghai, China.

Table 2

Growth performance and survival of *E. sinensis* fed diets of different α-lipoic acid contents (mean ± SE)

α-lipoic acid levels (mg/kg diet)	WG (%)	SGR (%)	HSI (%)	Survival (%)
0	130.40 ± 6.53[a]	1.49 ± 0.05[a]	8.64 ± 0.25[b]	82.50 ± 4.38
300	139.15 ± 4.20[ab]	1.56 ± 0.03[ab]	7.40 ± 0.39[a]	82.50 ± 3.15
600	150.47 ± 5.65[b]	1.64 ± 0.08[b]	8.70 ± 0.33[b]	89.17 ± 6.58
1200	170.64 ± 1.96[cd]	1.78 ± 0.03[cd]	8.29 ± 0.39[ab]	81.67 ± 3.97
2400	177.46 ± 4.10[d]	1.82 ± 0.05[d]	8.62 ± 0.29[b]	88.33 ± 3.97
4800	157.51 ± 8.80[bc]	1.69 ± 0.06[bc]	8.57 ± 0.30[b]	79.17 ± 6.29
9600	154.13 ± 7.40[b]	1.66 ± 0.05[bc]	8.39 ± 0.33[b]	90.00 ± 1.36

WG, weight gain; SGR, specific growth rate; HSI, hepatosomatic index.

[a, b, c, d] Values within a column without a common superscript letter are different (b indicated the higher value). $P < 0.05$ means significant difference; $P < 0.01$ means extreme significant difference. Values of WG, SGR and Survival are mean of four. Values of HSI are mean of 20.

Table 3

Proximate composition of *E. sinensis* (% wet weight) fed diets of different α-lipoic acid levels
(mean ± SE)

Diets α-lipoic acid level (mg/kg diet)	Moisture (%)	Crude protein (%)	Crude lipid (%)
0	67.28 ± 0.77	12.27 ± 0.39	3.86 ± 0.08
300	67.14 ± 0.71	11.74 ± 0.11	4.40 ± 0.15
600	68.73 ± 0.91	11.83 ± 0.25	3.85 ± 0.12
1200	66.18 ± 0.91	12.50 ± 0.35	4.32 ± 0.15
2400	68.64 ± 1.51	11.83 ± 0.68	3.97 ± 0.20
4800	67.82 ± 1.22	12.13 ± 0.56	3.75 ± 0.13
9600	68.17 ± 1.91	11.84 ± 0.94	3.89 ± 0.39

Values of moisture, crude protein, crude lipid and ash are mean of four.

Table 4

Contents of protein, MDA, GSH and the activities of SOD, GSH-Px and T-AOC in the hepatopancreas of *E. sinensis* fed diets of different α-lipoic acid levels (mean ± SE)

Diets α-lipoic acid level (mg/kg diet)	Protein (mg/mL)	MDA (nmol/mgprot)	SOD (U/mgprot)	GSH-Px (U/mgprot)
0	3.38 ± 0.15	2.84 ± 0.22[a]	7.05 ± 0.35[ab]	44.77 ± 7.27[a]
300	3.11 ± 0.18	3.06 ± 0.44[a]	7.69 ± 0.31[bc]	58.55 ± 6.17[ab]
600	2.82 ± 0.26	3.70 ± 0.38[abc]	8.30 ± 0.41[c]	70.04 ± 6.94[b]
1200	3.15 ± 0.17	3.17 ± 0.33[ab]	8.25 ± 0.19[c]	66.22 ± 6.44[b]
2400	2.96 ± 0.15	3.50 ± 0.31[ab]	8.00 ± 0.40[bc]	67.68 ± 5.82[b]
4800	3.39 ± 0.20	4.68 ± 0.52[c]	7.21 ± 0.30[abc]	62.84 ± 4.97[ab]
9600	3.41 ± 0.07	4.30 ± 0.33[bc]	6.44 ± 0.30[a]	52.04 ± 4.82[ab]

MDA, malondialdehyde. SOD, superoxide dismutase. GSH-Px, glutathione peroxidase. GSH, reduced glutathione. T-AOC, total antioxidant capacity.

[a, b, c] Values within a column without a common superscript letter are different (d indicated the highest value). *P*< 0.05 means significant difference; *P*< 0.01 means extreme significant difference.

Values of hepatopancreas protein, MDA, SOD, GHS-Px, GSH and T-AOC are the mean of eight replicates.

Table 5

Primer pair sequences and product size of the genes used for real-time PCR (qPCR)

Gene	Position	5'-3' primer sequence	Length	Tm	Product size (bp)
es_TGL1	Forward	CCTCCTCCTTCGGTATCA	18	54.9	109
	Reverse	GTTGTAGGCTATCAGGTCTT	20	53.4	
es_TGL2	Forward	CTACAACTTCCTCTTCCTGAT	21	53.7	118
	Reverse	GTAGTGGTTGACGGTGTG	18	54.9	
es_IL	Forward	CGATCCTACGAGTTCTTCA	19	53	125
	Reverse	GCACTTGGTGTTGTCATC	18	52.6	
es_DGL	Forward	CCAAGCATTACCGAACCT	18	50	93
	Reverse	TGACCACAGACACCATAAC	19	55.6	
CPT-1	Forward	GAATCTGTGACGGAGGAG	18	54.9	97
	Reverse	TGGCTGACGATGGTTATG	18	52.6	
β-actin	Forward	ATCACTGCTCTTGCTCCT	18	53.4	145
	Reverse	ACTCGTCGTATTCCTCCTT	19	53.5	

es_TGL1, digestive triacylglycerol lipase. es_TGL2, digestive triacylglycerol lipase 2. es_IL, intracellular lipase. es_DGL, diacylglycerol lipase. CPT-1, carnitine palmitoyl transferase-1.

Table 6

Significantly changed metabolites in the serum of *E. sinensis* fed 0 and 2400 mg/kg α-lipoic acid.

Metabolites	Compound ID	VIP[a]	FC[b]	*P* value[c]
Pueraria glycoside 3	3.42_447.1269m/z	2.1457	6.254	0.024
Glycitein	3.42_285.0746m/z	1.5419	6.250	0.025
Chicoric acid	2.68_497.0734m/z	1.3280	4.294	0.025
Methylmalonylcarnitine	1.51_284.1094m/z	2.8912	3.888	0.017
Dihydrozeatin	1.48_244.1171m/z	2.0424	3.459	0.003
19-Norandrosterone	4.98_299.1992m/z	1.1788	3.014	0.001
13-OxoODE	4.98_317.2096m/z	1.4780	2.933	0.001
Asparagoside A	13.63_579.3892m/z	1.2545	2.716	0.006
12,13-DiHODE	4.98_335.2201m/z	1.4620	2.694	0.001
9-Hexadecenoylcarnitine	7.47_398.3245m/z	1.2201	2.666	0.046
Prostaglandin F2a	4.91_377.2281m/z	1.1629	2.557	0.006
Prostaglandin E2	4.97_374.2052n	1.7101	2.324	0.016
26,26,26,27,27,27-hexafluoro-1alphaa-hydroxyvitamin D3/ 26,26,26,27,27,27-hexafluoro-1alphaa-hydroxycholecalciferol	8.19_526.3114m/z	2.2884	2.131	0.022
1-Stearoylglycerophosphoserine	7.65_524.2969m/z	1.7493	1.908	0.021
PS(20:3(8Z,11Z,14Z)/0:0)	6.43_570.2803m/z	1.2861	1.797	0.026
PE(20:5(5Z,8Z,11Z,14Z,17Z)/22:6(4Z,7Z,10Z,13Z,	11.89_809.4963	3.112	1.62	0.02

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		7	6	4
16Z,19Z))	n	7	6	4
13-L-Hydroperoxylinoleic acid	7.54_330.2622m/z	1.1496	1.616	0.018
4alpha-Carboxy-5alpha-cholesta-8-en-3beta-ol	12.05_453.3316 m/z	1.5811	1.475	0.040
2,5-Dihydro-2,4-dimethyloxazole	1.50_100.0752m/z	4.2612	1.461	0.043
LysoPE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	6.50_525.2834n	3.5144	1.435	0.012
3-Deoxy-D-glycero-D-galacto-2-nonulosonic acid	0.64_291.0686m/z	1.5135	1.396	0.013
PE(16:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	12.92_784.4882 m/z	1.4086	1.366	0.040
2-Ketohexanoic acid	1.22_131.0712m/z	1.7847	0.305	0.001
5-Pentyltetrahydro-2-oxo-3-furancarboxylic acid	0.64_218.1377m/z	1.9466	0.314	0.010
3-Hydroxyglutaric acid	0.80_171.0257m/z	1.1404	0.343	0.001
9-hydroxyoctadecanoic acid	9.86_339.2315m/z	5.3353	0.375	0.007
Propionylcarnitine	1.22_218.1379m/z	5.1533	0.400	0.026
Isobutyryl-L-carnitine	2.10_232.1535m/z	2.2632	0.401	0.003
Isovalerylcarnitine	3.09_246.1691m/z	1.6254	0.424	0.035
3,4-Methylenesebacic acid	4.28_227.1282m/z	1.1355	0.428	0.008
MG(16:0/0:0/0:0)	8.96_369.2419m/z	1.7651	0.436	0.004
3-Methylglutarylcarnitine	1.62_290.1589m/z	1.0495	0.456	0.004
Octadecanedioic acid	8.07_353.2106m/z	2.6664	0.467	0.049
PG(16:0/20:3(5Z,8Z,11Z))	14.26_795.5142 m/z	1.3372	0.468	0.020
PG(16:1(9Z)/18:1(9Z))	13.91_769.4992	2.889	0.49	0.02

	m/z	2	9	6
2-hydroxy-heptadecanoic acid	9.25_309.2409m/z	2.6403	0.503	0.030
L-Histidine	0.63_77.5366n	1.1975	0.513	0.012
5-Hydroxy-2-furoic acid	0.57_129.0192m/z	2.2011	0.515	0.001
L-Methionine	0.64_132.0239n	2.0163	0.630	0.025
1-(O-alpha-D-glucopyranosyl)-(1,3R,29S,31R)-dotriacontanetetrol	12.88_676.5488n	4.5885	0.653	0.043
L-Acetylcarnitine	0.64_204.1220m/z	2.1206	0.668	0.013
L-Alanine	0.62_90.0544m/z	1.5382	0.711	0.005
2-Aminobenzoic acid	0.62_138.0543m/z	3.3157	0.759	0.005
PC(18:1(11Z)/20:5(5Z,8Z,11Z,14Z,17Z))	13.27_805.5594n	22.891	0.808	0.047
2,3-Methylenesuccinic acid	1.92_180.9892m/z	1.1051	0.820	0.020

[a] Variable importance in the projection (VIP) was acquired from the OPLS-DA model with a threshold of 1.0.

[b] FC: fold change

[c] *P* value was calculated from two-tailed t-test.

Figure legends

Figure 1

A. Effect of α-LA on weight gain (WG) and the optimum supplement of α-LA in the diet for *E. sinensis* is 1339 mg/kg by broken-line regression calculation.

B. Effect of α-LA on specific growth rate (SGR) and the optimal supplement of α-LA in the diet for *E. sinensis* is 1574 mg/kg by broken-line regression calculation.

Figure 2

Lipid contents (A) and TG contents (B) in the hepatopancreas of *E. sinensis* fed diets with different α-LA concentrations. The x-axis represents different supplement dosages (0, 300, 600, 1200, 2400, 4800 and 9600 mg/kg) of α-LA in the diet.

Figure 3

The mRNA expressions of *es_TGL1* (A), *es_TGL2* (B), *es_IL* (C), *es_DGL* (D) and *CPT-1* (E) in the hepatopancreas of *E. sinensis* fed a diet with different α-LA concentrations. The x-axis represents different supplement dosages (0, 300, 600, 1200, 2400, 4800 and 9600 mg/kg) of α-LA in the diet. The y-axis represents the fold change of mRNA expressions.

Figure 4

Three patterns of top 19 pathways of significantly changed metabolites by serum metabolomics analysis in *E. sinensis*.

Figure 5

Hierarchical clustering analysis of the significantly changed metabolites in the serum of *E. sinensis* between the control and 2400 mg/kg α-LA. The rows represent individual metabolites ID. The left column represents the control and the right column represents the 2400 mg/kg α-LA treatment. The metabolites with increased and decreased contents in abundance are indicated in red and green, respectively.
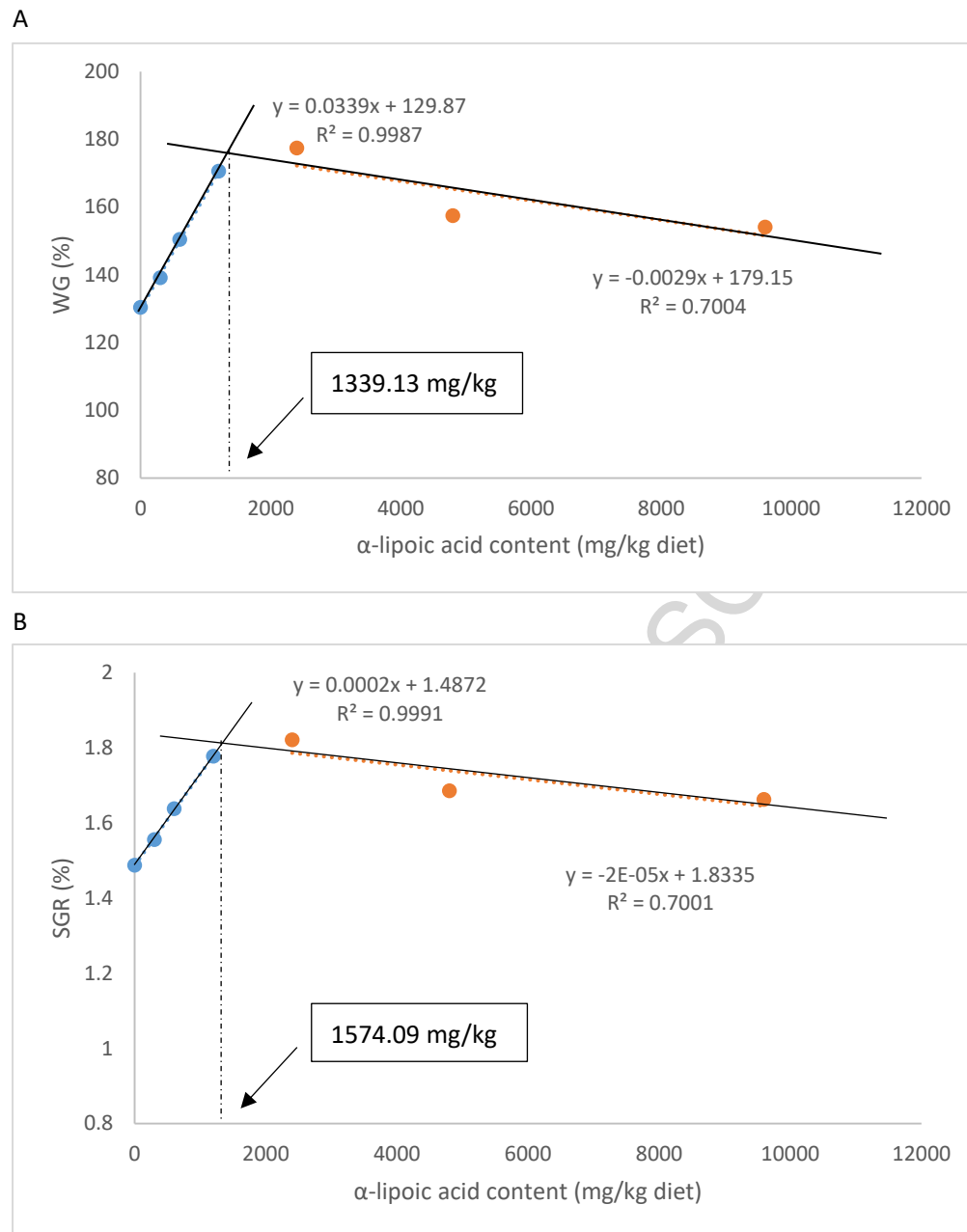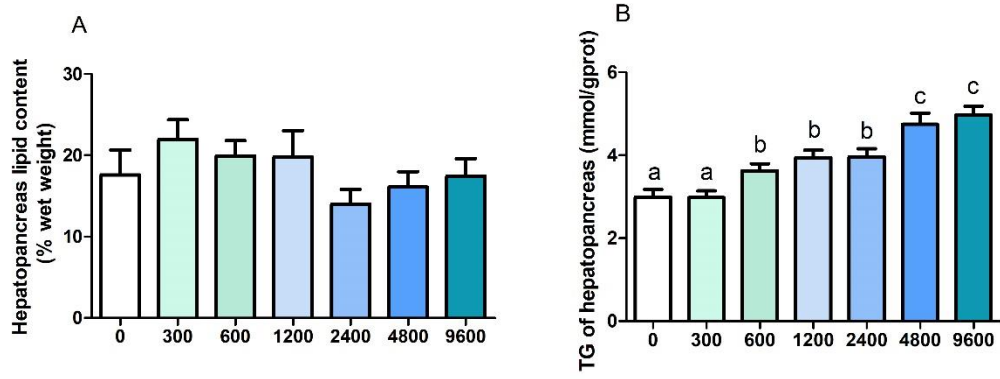
A

y = 0.0339x + 129.87
R² = 0.9987

y = -0.0029x + 179.15
R² = 0.7004

1339.13 mg/kg

WG (%)

α-lipoic acid content (mg/kg diet)

B

y = 0.0002x + 1.4872
R² = 0.9991

y = -2E-05x + 1.8335
R² = 0.7001

1574.09 mg/kg

SGR (%)

α-lipoic acid content (mg/kg diet)

Figure 1

Figure 2

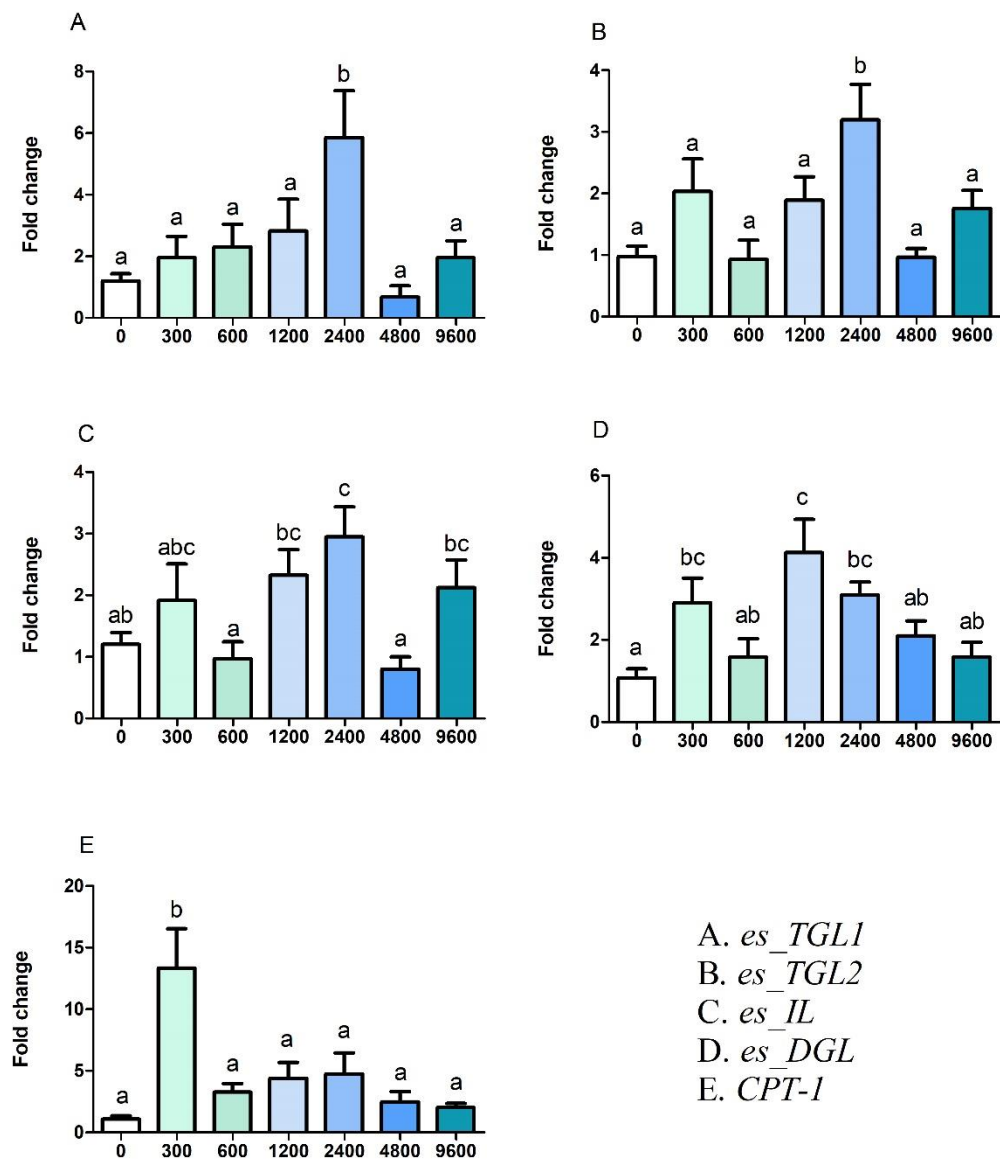A. *es_TGL1*
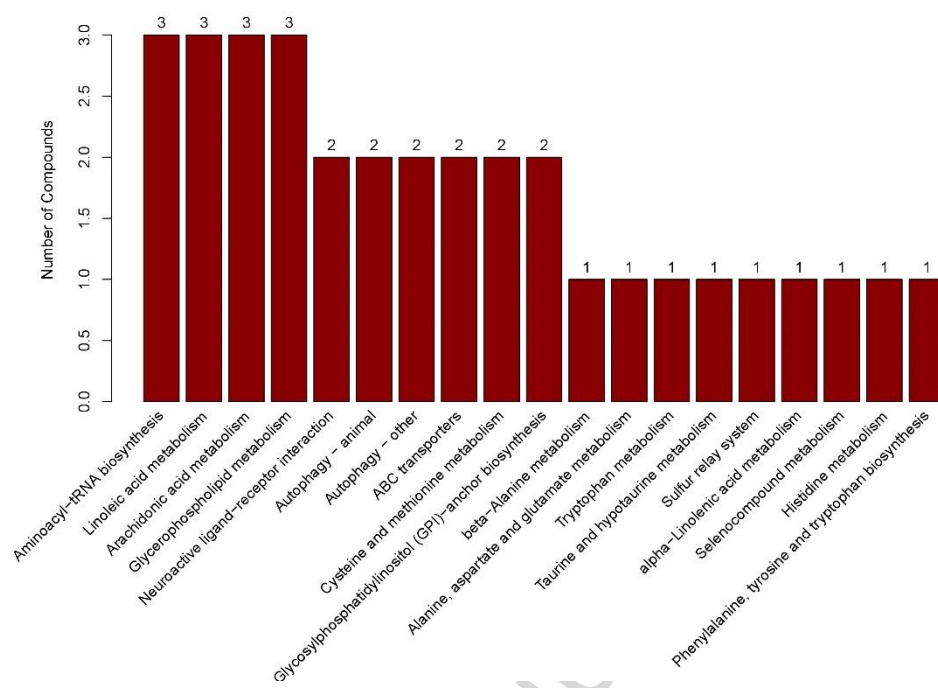B. *es_TGL2*
C. *es_IL*
D. *es_DGL*
E. *CPT-1*

Figure 3

Figure 4

Figure 5

1. The α-LA supplement in diet can significantly improve growth performance and antioxidant capacity of *E. sinensis* and the recommended optimal supplement dosage is 1339-1574 mg/kg.

2. The appropriate supplement of α-LA can promote digestion and absorption of plant antioxidants and lipids in diets by metabonomics analysis.

3. The recommended maximum dosage should not exceed 2400 mg/kg and the excessive α-LA in diet can reduce growth performance and antioxidant capacity in *E. sinensis*.