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Original Research Article

Dietary supplementation with succinic acid improves growth performance and flesh quality of adult Nile tilapia (*Oreochromis niloticus*) fed a high-carbohydrate diet

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ABSTRACT

To evaluate the effects of dietary supplementation with succinic acid on growth performance, flesh quality, glucose, and lipid metabolism of Nile tilapia (*Oreochromis niloticus*) fed a high-carbohydrate diet (HCD), five iso-nitrogenous and iso-lipidic diets were prepared as follows: HCD (control group) consisting of 55% corn starch and HCD supplemented with 0.5%, 1.0%, 2.0%, and 4.0% succinic acid, respectively. Tilapia with an initial body weight of 204.90 ± 1.23 g randomly assigned to 15 tanks with 3 replicates per group and 10 fish per tank fed for 8 weeks. Increasing dietary succinic acid supplementation resulted in significant second-order polynomial relationship in the weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency rate (PER), viscerosomatic index, condition factor, and contents of muscular crude lipid and glycogen ($P < 0.05$). The hepatosomatic index, mesenteric fat index, liver glycogen content and crude lipid contents of the whole-body and liver demonstrated significantly linear and second-order polynomial relationship ($P < 0.05$). Quadratic curve model analysis based on WGR, SGR, PER, and FCR demonstrated that optimal supplementation with succinic acid in the HCD of Nile tilapia ranged from 1.83% to 2.43%. Fish fed with 1.0% succinic acid had higher muscular hardness, increased the contents of alkali-soluble hydroxyproline in collagen, docosahexaenoic acid (DHA) and n-3 polyunsaturated fatty acid (n-3PUFA) in muscle, and lower total fatty acid content in muscle ($P < 0.05$) compared with the control group. Compared to the control group, dietary supplementation with 1.0% succinic acid significantly increased the contents of total bounding amino acid (arginine, histidine, isoleucine, lysine, methionine, alanine, proline), total flavor amino acid (free aspartic acid), the catalase (CAT) activity and total antioxidant capacity, and the mRNA relative expression levels of *CAT*, superoxide dismutase (*SOD*), and nuclearfactor erythroidderived 2-like 2 (*Nrf2*) in muscle ($P < 0.05$). Furthermore, succinic acid supplementation significantly up-regulated mRNA relative expression levels of glycolysis genes (hexokinase 2 [*HK2*], phosphofructokinase, muscle-A [*PFKMA*], and phosphofructokinase, muscle-B [*PFKMB*]), a key glycogen synthesis gene (glycogen synthase [*GYS*]), and lipid catabolism genes (carnitine palmitoyltransferase-1B [*CPT1B*], hormone sensitive lipase [*HSL*], and lipoprotein lipase [*LPL*]), while down-regulating the mRNA relative expression level of fatty acid synthase (*FASN*) in muscle ($P < 0.05$). In conclusion, dietary supplementation with 1.83% to 2.43% succinic acid

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improved muscle quality by increasing muscle antioxidant capacity and hardness, changing muscle amino acid and fatty acid composition, and regulating muscle glucose and lipid metabolism.

1. Introduction

With the scarcity and sudden rise in the price of fish meal, enhancing the utilization of non-protein nutrients in aquatic animals is of vital importance (Aragão et al., 2022). Carbohydrates are abundantly available and affordable, and the optimum dietary carbohydrate level might enhance growth and feed efficiency (Kamalam et al., 2017). Therefore, high-carbohydrate diets (HCD) have been extensively used in both omnivorous (Boonanuntanasarn et al., 2018b) and herbivorous (Tan et al., 2009) fish cultures to minimize protein and lipid catabolism in fish (Krogdahl et al., 2005). Nonetheless, the ability of fish to utilize carbohydrates is limited, and they are considered "glucose intolerant" (Polakof et al., 2012). Excessive dietary carbohydrate ingestion causes metabolic disorders, reduced growth performance (Li et al., 2016), prolonged hyperglycemia (Kostyniuk et al., 2019), and extreme lipid deposition (Prisingkorn et al., 2017; Viegas et al., 2016). Moreover, excessive carbohydrate intake decreased the content of polyunsaturated fatty acids (PUFA) and affected the amount of essential amino acids (EAA) in muscle (Wang et al., 2017; Wu et al., 2021; Li et al., 2014), resulting in poor fish flesh quality. Hence, the pursuit of effective methods to improve the utilization of carbohydrates and reduce metabolic disorders and muscle quality degradation caused by HCD has attracted increasing attention.

Long-term consumption of HCD has been found to lead to changes in concentrations of intermediate metabolites from the tricarboxylic acid cycle (TCA), such as cis-aconitate and malic acid (Zakim et al., 1967; Zhang et al., 2021). Succinic acid, situated at the core position of various metabolic pathways, can be produced by glycolysis and fatty acid β-oxidation pathways, as well as by intestinal microbial fermentation of dietary fiber (Cummings et al., 1987; De Vadder et al., 2016). Additionally, succinic acid is also a downstream product of the α-ketoglutarate dehydrogenase complex and is involved in the formation and elimination of endogenous reactive oxygen species (ROS) (Liang, 2018). The above results raise speculation that supplementation with exogenous succinic acid may be an effective way to enhance the utilization of HCD. Studies have indicated that dietary supplementation with 0.5% succinic acid promotes the growth, feed utilization, and immunity of white shrimp (*Litopenaeus vannamei*) (Duan et al., 2018). Dietary supplementation with 0.15% succinate also promoted growth, feed intake, and protein deposition in juvenile zebrafish (*Danio rerio*) (Ding et al., 2022). Succinic acid has also been reported to induce the conversion of fast-twitch muscle fibers to slow-twitch muscle fibers in the skeletal muscle of mice, change the proportion of muscle fibers, and significantly improve muscle quality (Wang et al., 2019). However, the role of succinic acid in improving the muscle quality of farmed fish remains unexplored.

Nile tilapia (*Oreochromis niloticus*) is a crucial economic fish farmed globally, playing a significant role in addressing the issue of fish scarcity in developing nations due to its ease of cultivation, substantial flesh, and high protein content (Deng et al., 2010; He et al., 2021). Tilapia have been found to tolerate 330 g/kg of carbohydrate (Boonanuntanasarn et al., 2018a), and a diet with a carbohydrate/lipid ratio of 2:1 to 6.5:1 was most conducive to growth (Kabir et al., 2020). Diets are classified as HCD when the dietary carbohydrate content exceeds 380 g/kg (Ning et al., 2023). Prior research has indicated that diets with carbohydrate levels of 420 and 449 g/kg decrease muscle quality (Wu et al., 2021), systemic lipid accumulation, and glucose metabolism disorders (Liu et al., 2018). Therefore, the aim of the current study was to investigate the effects of succinic acid supplementation in a HCD containing 55% corn starch on the growth performance, flesh quality, glucose, and lipid metabolism of Nile tilapia and the underlying mechanisms. Our study might provide a new method for alleviating HCD stress and improving the quality of fish flesh.

2. Materials and methods

2.1. Animal ethics statement

In China, tilapia is widely farmed and is neither an endangered nor a protected species. The Yangtze River Fisheries Research Institute's Institutional Animal Care and Use Committee gave its approval for this work (Permit Number: YFI 2018-40).

2.2. Experimental diets

Five iso-nitrogenous and iso-lipidic diets were designed for this experiment. Succinic acid (99% purity) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). In the current study, casein and gelatin were utilized as protein sources, fish and soybean oils were utilized as lipid sources, and corn starch was utilized as a carbohydrate source. The experimental diets were divided into a HCD (CON group, containing 550 g/kg corn starch and 60 g/kg lipid) and four supplementary diets supplemented with 0.5% (S0.5), 1.0% (S1.0), 2.0% (S2.0), and 4.0% (S4.0) succinic acid (Table 1). All components were precisely weighed, ground through a 60-mesh screen, and mixed evenly. Oil and water (about 30% of the dry ingredients) were added and mixed thoroughly. The well-mixed ingredients were then extruded (2.0 mm in diameter) using a laboratory pellet mill (TY-432, Shanghai Taiyi Machinery, China). The resultant noodle-like diets were dried in a convection oven at 60 °C for 3 h. For later usage, the feeds were crushed into tiny pellets and kept at −20 °C.

2.3. Experimental fish and feeding trial

The feeding study was carried out at the Yangtze River Fisheries Research Institute (Wuhan, China) with an indoor recirculating aquaculture system. Tilapia were purchased from the Guangxi Tilapia National Breeding Station (Nanning, China) and cultured in an indoor recirculating aquaculture system with commercial tilapia feed (supplied by Shenzhen Alpha Feed Co., Ltd.) for 2 weeks to acclimate to the environment. Prior to the feeding trial, the experimental fish were fasted for 24 h and anesthetized with 50 mg/L of MS-222 (tricaine methane sulfonate). About 150 healthy fish with initial body weights (204.90 ± 1.23 g) were chosen, weighed, and arranged in 15 tanks with a total water volume of 400 L and 10 fish each. Three tanks were selected at random for

Table 1
Composition and nutrient levels of the diets (DM basis, g/kg).

Item	Groups				
	CON	S0.5	S1.0	S2.0	S4.0
Ingredients					
Casein	256.00	256.00	256.00	256.00	256.00
Gelatin	64.00	64.00	64.00	64.00	64.00
Fish oil	30.00	30.00	30.00	30.00	30.00
Soybean oil	30.00	30.00	30.00	30.00	30.00
Corn starch	550.00	550.00	550.00	550.00	550.00
Succinic acid		5.00	10.00	20.00	40.00
Cellulose	40.00	35.00	30.00	20.00	
Vitamin premix[1]	5.00	5.00	5.00	5.00	5.00
Mineral premix[2]	5.00	5.00	5.00	5.00	5.00
$Ca(H_2PO_4)_2$	15.00	15.00	15.00	15.00	15.00
Choline chloride	2.50	2.50	2.50	2.50	2.50
Vitamin C	2.50	2.50	2.50	2.50	2.50
Total	1,000.00	1,000.00	1,000.00	1,000.00	1,000.00
Nutrient levels[3]					
Dry matter	958.42	952.95	960.02	964.94	961.88
Ash	19.76	19.36	19.96	19.96	19.51
Crude protein	320.06	319.19	316.94	316.48	317.82
Crude lipid	61.46	61.13	60.76	60.75	58.49
Gross energy, MJ/kg	20.58	20.58	20.55	20.52	20.36

[1] One kilogram of vitamin premix contained the following: vitamin A 0.8 g, vitamin D_3 0.08 g, vitamin E 20 g, vitamin K_3 18.3 g, vitamin B_1 10 g, vitamin B_2 12.5 g, vitamin B_6 8 g, nicotinic acid 12 g, biotin 3 g, calcium pantothenate 20 g, folic acid 3.2 g, inositol 406 g. All ingredients were diluted with micro-cellulose to 1 kg.
[2] One kilogram of mineral premix contained the following: $C_6H_{10}CaO_6$ 50 g, $FeSO_4$ 20 g, $MgSO_4$ 100 g, NaH_2PO_4 100 g, NaCl 20 g, $AlCl_3$ 0.6 g, KIO_3 0.6 g, KCl 40 g, $CuSO_4$ 2 g, $MnSO_4$ 4 g, $ZnSO_4$ 20 g, $CoCl_2$ 2 g. All ingredients were diluted with micro-cellulose to 1 kg.
[3] Nutrient levels were measured values.

each diet. The fish were hand-fed 3 times a day, at 08:30, 12:30, and 17:30, until their behavior demonstrated signs of satiety. The water temperature, feeding behavior, and mortality of tilapia were recorded daily. Approximately 30% of the water in each tank was exchanged every 2 days. The parameters of the water throughout the experiment were as follows: pH 6.8 to 7.2; dissolved oxygen content > 5 mg/L; ammonia nitrogen mass content < 0.05 mg/L. The temperature of the water was between 28 and 33 °C. The feeding trial lasted for 8 weeks.

2.4. Sample collection

At the end of the feeding trial, fish were fasted for 24 h. All fish were anesthetized with 80 mg/L of MS-222 before sampling. To calculate the specific growth rate (SGR), weight gain rate (WGR), survival rate (SR), and feed conversion ratio (FCR), the number and total weight of fish in each tank were recorded. Two fish were randomly selected from each tank for whole-body composition determination. The other six fish from each tank were randomly chosen to measure the body weight and length to calculate the condition factor (CF). Then, blood samples were drawn from the caudal vein of these 6 fish using a 2-mL syringe. Blood samples were centrifuged (960 × *g*, 4 °C) to obtain serum samples and stored at −80 °C prior to analysis. The above six fish were disinfected with 75% alcohol before dissection. The viscera, liver, and mesenteric fat were weighed to calculate the viscerosomatic index (VSI), hepatosomatic index (HSI), and mesenteric fat index (MFI). Then, the dorsal muscle was separated, immediately frozen in liquid nitrogen, and stored at −80 °C for RNA isolation. To examine the texture, the muscles (10 mm × 10 mm × 5 mm) at the third to fifth dorsal fins above the lateral line of two fish from each tank were cut and stored at 4 °C. The muscle (5 mm × 5 mm × 5 mm) of the other two fish per tank was collected and then fixed in 4% phosphate buffer saline (PBS) for histology determination. The muscle and liver of the remaining fish were isolated and stored at −20 °C for further nutritional value analysis.

2.5. Growth indices and biochemical analyses

2.5.1. Growth indices

The following formulae were used to calculate the experimental fish's SR, WGR, SGR, feeding rate (FR), feeding intake (FI), FCR, HSI, VSI, CF, MFI, and protein efficiency ratio (PER) based on the measured data.

$$\mathrm{WGR}\ (\%) = [(W_t - W_0)/W_0] \times 100;$$

$$\mathrm{SR}\ (\%) = N_t/N_0 \times 100;$$

$$\mathrm{SGR}\ (\%/\mathrm{d}) = [(\ln W_t - \ln W_0)/t] \times 100;$$

$$\mathrm{FR}\ (\%) = [W_f/(W_t + W_0) \times 2/t] \times 100;$$

$$\mathrm{FI}\ (\mathrm{g/fish}) = W_f/N_t;$$

$$\mathrm{FCR} = W_f/(W_t N_t - W_0 N_0 + W_d);$$

$$\mathrm{HSI}\ (\%) = W_h/W_t \times 100;$$

$$\mathrm{VSI}\ (\%) = W_v/W_t \times 100;$$

$$\mathrm{CF}\ (\%) = W_t/L^3 \times 100;$$

$$\mathrm{MFI}\ (\%) = W_m/W_t \times 100;$$

$$\mathrm{PER}\ (\%) = [(W_t - W_0)/(W_f \times W_p)] \times 100;$$

where W_t is final body weight (g); W_0 is initial body weight (g); L is body length (cm); N_t is final fish number; N_0 is initial individual fish number; t is experimental days (d); W_f is dry feed consumed (g); W_d is total death weight (g); W_h is hepatopancreas weight (g); W_v is viscerosomatic weight (g); W_p is feed crude protein content (%); W_m is mesenteric fat weight (g).

2.5.2. Composition analysis

The standard methodology was used to determine the proximate composition of diets, liver, muscle, and whole fish (AOAC, 2005). Diets were dried at 105 °C to a consistent weight according to AOAC method 2001.12 in order to measure their moisture content. The whole-body, muscle, and liver were freeze-dried for 72 h in a vacuum freeze dryer (Christ Beta 2-4 LD plus LT, Marin Christ Corporation, Osterode, Germany) to determine their moisture content. The crude protein content was measured with an auto Kjeldahl system (Kjelflex K-360; BUCHI Labortechnik AG, Flawil, Switzerland) according to the AOAC method 2001.11. Crude lipid content was determined by petroleum ether Soxhlet extraction (Sox606, Hanon Advanced Technology Group Co., Ltd., China) according to AOAC method 920.39. The ash content was determined by calcination in a muffle furnace (SX-4-10, Nanbei Instrument Limited, China) for 10 h at 550 °C according to AOAC method 942.05. An adiabatic bomb calorimeter (SDC311; Hunan Sundy Science and Technology Development Co., Ltd., China) was used to measure the gross energy of the meals by direct combustion.

2.5.3. Serum biochemical analysis

The contents of total protein (TP) (Sysmex, 290618), total cholesterol (TCHO) (Sysmex, 290723, 290724), triglyceride (TG) (Sysmex, 80945, 80946), glucose (GLU) (Sysmex, 290713, 290714), and albumin (ALB) (Sysmex, 290615) in serum were determined by

the methods of biuret, cholesterol oxidase-peroxidase amino-antipyrine (CHOD-PAP), glycerol kinase-glycerol phosphate oxidase-peroxidase (GK-GPO-POD), hexokinase, and bromocresol green (BCG), respectively. The activities of aspartate aminotransferase (AST) (Sysmex, 290705, 290706), alanine aminotransferase (ALT) (Sysmex, 290703, 290704), and alkaline phosphatase (ALP) (Sysmex, 290701, 290702) in serum were determined by the methods of lactate dehydrogenase-ultraviolet (LDH-UV), malate dehydrogenase-ultraviolet (MDH-UV), and nitrophenyl-disodium phosphate -amino methy propanol (NPP-AMP), respectively. The kits were acquired from Sysmex Corporation in order to use an automated biochemical analyzer (BX-3010, Sysmex Corporation, Tokyo, Japan) to identify serum indicators.

2.5.4. Activities of liver and intestinal digestive enzymes

The fresh liver and intestine samples (about 0.6 g per sample) were accurately weighed and put into 10 mL centrifuge tubes. The samples were homogenized with ice-cold physiological saline solution (1:9, w/v) on ice using a portable homogenizer and then centrifuged at 960 × *g* for 30 min at 4 °C. The resulting supernatants were collected and kept at −80 °C to analyze the enzyme activity. The lipase (LPS, A054-2-1) activity, amylase (AMS, C016-1-1) activity, and total protein (TP, A045-4) content of the liver and intestine were determined by commercial kits (Nanjing Jiancheng Bioengineering Institute, China). The protease activity was determined by the Folin-phenol method as follows: 0.25 mL of tissue homogenate supernatant was added with 0.5 mL of 0.5% casein preheated in advance and reacted in a water bath for 15 min. Then 0.75 mL of 10% trichloroacetic acid was added and centrifuged (960 × *g*, 10 min) to remove precipitation. Then 1.25 mL of sodium carbonate (0.55 mol/L) and 0.25 mL of Folin reagent were added to 0.25 mL of the resultant supernatant, respectively. The color was developed in a water bath for 15 min. The corresponding optical density (OD) values were recorded at 680 nm wave length for subsequent calculations.

2.5.5. Determination of glycogen content of liver and muscle

The fresh liver and muscle tissue were weighted (about 0.1 g), and 3 times the volume of alkali solution was added and hydrolyzed in a boiling water bath for 20 min. The resultant hydrolysate was collected, and the glycogen contents of the liver and muscle were determined by commercial kits (A043-1-1, Nanjing Jiancheng Bioengineering Institute, China).

2.5.6. Muscle texture properties analysis (TPA)

Complete fresh back muscle blocks were selected and cut into 1 cm × 1 cm × 1 cm squares. Texture profile analysis was used to measure muscle hardness, springiness, gumminess, chewiness, and resilience with the TVT-300XP texture instrument (Perten Instruments (Beijing) Co., Ltd., Beijing, China). The P-cy5s cylindrical probe was used for two tests; the test speed was 1 mm/s, the interval was 5 s, the deformation was 60%, and the data acquisition rate was 200 pps. The test speed was 2 mm/s before and after the test. Each sample was pressed twice.

2.5.7. Determination of collagen content

The hydroxyproline (Hyp) content was used to determine the collagen concentration. The muscle alkaline-soluble Hyp and alkaline-insoluble Hyp were separated by the method provided by Li et al. (2005). In brief, following the homogenization of muscle samples, sodium hydroxide (0.2 mol/L) and 8 mol/L hydrochloric acid (HCl) were used to extract the alkaline-soluble and alkaline-insoluble Hyp in an ice bath, respectively. The Hyp was determined by the method provided by Zhang et al. (2013). The Hyp content was calculated using a standard curve.

2.5.8. Determination of muscle antioxidant capacity

The fresh muscle samples (about 0.6 g per sample) were accurately weighed and put into 10 mL centrifuge tubes. The samples were homogenized with ice-cold physiological saline solution (1:9, w/v) on ice using a portable homogenizer and then centrifuged at 960 × *g* for 30 min at 4 °C. The resulting supernatants were collected and kept at −80 °C to analyze the enzyme activity. Using assay kits acquired from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China), the total protein (TP, A045-4) content, total antioxidant capacity (T-AOC, A015-2-1), catalase (CAT, A007-1-1) activity, superoxide dismutase (SOD, A001-3) activity, and malondialdehyde (MDA, A003-1) content of muscle were determined.

2.5.9. Histological properties analysis

Following ethanol dehydration and xylene cleaning, muscle samples were embedded in paraffin. Hematoxylin and eosin were used to stain the 5 μm tissue sections, followed by observation and photography using the light microscope DM2500 (Leica DM2500, Leica, Solms, Germany). The myofiber characteristics, including the number of muscle fiber and density, were measured using Image-J Launcher software. The muscle fiber density (N/mm^2) was expressed as the rate of all the fibers in the entire region.

2.5.10. Analysis of amino acid and fatty acid contents

Bound amino acids were determined using an automated amino acid analyzer (HITACHI L-8900, Tokyo, Japan). Samples of muscle were prepared using the following method: freeze-dried muscle samples weighing about 0.1 g were put into glass tubes that were sealed, and 12 mL of HCl (6 mol/L) was added to hydrolyze the samples for 24 h at 110 °C. The hydrolysate was then filtered and diluted with distilled water to a volume of 100 mL. A vacuum drier was used for 24 h to remove the HCl from the filtrate (2 mL), which was then evaporated to dryness at 60 °C. A second 24 h evaporation of the 2 mL of distilled water was added. Then, 8 mL of 0.1 mol/L HCl was used to deliquesce the sedimentation and filter the mixture through a 0.22 μm millipore membrane, leaving 1 mL of the supernatant for analysis of bound amino acids.

The free amino acids were determined using an automated amino acid analyzer (HITACHI L-8900, Tokyo, Japan). Samples of muscle were prepared using the following method: fresh muscle tissues weighing approximately 1.0 g were homogenized with 3 mL of 10% sulfosalicylic acid and then centrifuged (18,000 × *g*, 4 °C, 15 min). Then 1 mL of supernatant was filtered through a 0.22 μm millipore membrane and used for the measurement of free amino acids.

The fatty acids were isolated from the muscle in accordance with the Chinese standard (GB 5009.168-2016). Briefly, approximately 0.4 g of muscle tissue was put into a 5 mL centrifuge tube, then 4 mL of isooctane was added, vortexed, and mixed for 30 s, and then shaken in a shaker at 25 °C overnight for extraction. Then, 8 mL of 2% sodium hydroxide in methanol solution was added to the resultant extraction solution and gently boiled for 40 min. Following this, 20 min of gentle boiling was spent adding 7 mL of 15% boron trifluoride to the methanol solution. Twenty milliliters of n-heptane were added and boiled gently for 1 min. The mixture was allowed to stratify after adding the saturated sodium chloride solution. Five mL of the upper n-heptane extraction solution was taken. Then 5 g of anhydrous sodium sulfate was added and stirred for 1 min and stood for 5 min. The resulting supernatants were put in a 2 mL centrifuge tube and kept at −20 °C until analysis. Gas chromatography (GC, Agilent 7890A, Agilent Technologies, Santa Clara, USA) was used to determine individual fatty acids. A polydicyanopropyl siloxane capillary column with a strong polar stationary phase separated the lipids under the following GC

conditions: the injection volume was 1 μL, and the temperatures of the injection port and the detection port were 270 and 280 °C, respectively. The temperature schedule was set up as follows: after 13 min at 100 °C, the temperature was increased to 180 °C at a rate of 10 °C per min and maintained for 6 min. After that, the temperature increased to 200 °C at a rate of 1 °C per min and stayed there for 20 min. After reaching 230 °C at a rate of 4 °C per min, the temperature was maintained for 10.5 min. The carrier was nitrogen gas, and the split ratio was 100:1. The ratio of the peak areas of various fatty acids to the internal standard's peak areas (C11:0) was used to compute the fatty acid content.

2.5.11. cDNA synthesis and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

TRIzol reagent (Life Technologies, Carlsbad, CA, USA) was used to extract total RNA from muscle, and PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Biotech, Dalian, China) instructions were followed to generate cDNA. TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Biotech, Dalian, China) was utilized to quantify the mRNA expression of genes. The RT-qPCR was carried out according to published papers (Xie et al., 2021). The primers are listed in Table 2 (synthesized by Sangon Biotech). β-actin was selected as the housekeeping gene. Using the $2^{-\Delta\Delta Ct}$ method, relative quantification of transcript expression was computed, with the relative mRNA expressions of the CON group serving as the reference.

2.6. Statistical analysis

The experiment data were analyzed with SPSS 26.0 software (SPSS Inc., Chicago, IL, USA). All results were presented as the means ± standard deviation (SD). Normality and homoscedasticity were confirmed initially. Then, a one-way analysis of variance was performed on all the data, and Tukey's multiple range tests were performed. Furthermore, orthogonal polynomial contrasts were used to evaluate all the data in order to determine if the tendency (or pattern) was linear or second-order polynomial. Statistical differences were evaluated at $P < 0.05$. GraphPad Prism 8 was used to create the column charts (GraphPad Software, Inc., La Jolla, CA, USA). The quadratic regression graphs were made using OriginLab 2019 (OriginLab Inc., Massachusetts, USA).

3. Results

3.1. Growth performance and feed utilization

The SR of tilapia was 100%. With the increase in succinic acid supplementation in the CON diet, the WGR, SGR, PER, FCR, VSI and CF of tilapia demonstrated a significantly second-order polynomial relationship ($P < 0.05$), and the HSI and MFI revealed a significantly linear and second-order polynomial relationship ($P < 0.05$). The minimum of VSI and HSI and the maximum of CF were recorded in the S1.0 group. The maximum of WGR, SGR, and PER and the minimum of FCR were recorded in the S2.0 group (Table 3). Dietary supplementation with succinic acid had no considerable effect on the FR and FI ($P > 0.05$). Quadratic curve model analysis based on WGR, SGR, PER, and FCR demonstrated that the optimal supplementation with succinic acid in HCD ranged from 1.83% to 2.43% for tilapia (Fig. 1).

3.2. Proximate composition and muscle collagen content

With the increase in succinic acid supplementation in the CON diet, the contents of whole fish moisture and muscle crude protein exhibited a significantly linear relationship ($P < 0.05$), and the contents of whole fish crude lipid, muscle crude lipid, muscle glycogen, liver moisture, and collagen alkali-soluble hydroxyproline demonstrated a significantly second-order polynomial relationship ($P < 0.05$). The contents of liver crude lipid, collagen alkali-insoluble hydroxyproline, and total hydroxyproline revealed a significantly linear and second-order polynomial relationship ($P < 0.05$). The maximum contents of muscle ash, muscle crude protein, and total hydroxyproline appeared in the S0.5 group; the minimum crude lipid contents of the whole fish, muscle, and liver were recorded in the S1.0 group; and the maximum contents of muscle glycogen and collagen alkali-soluble hydroxyproline were presented in the S2.0 group. Dietary supplementation with succinic acid had no considerable effect on the contents of whole fish ash, muscle moisture, and liver crude protein ($P > 0.05$, Table 4).

3.3. Serum biochemical indices

With the increase in succinic acid supplementation in the CON diet, the activities of AST, ALT, and TG content in serum exhibited a significantly second-order polynomial relationship ($P < 0.05$), and the contents of TP, ALB, TCHO, and GLU demonstrated a significantly linear and second-order polynomial relationship ($P < 0.05$). The maximum contents of TP and ALB in serum were recorded in the S1.0 group, and the minimum amounts of AST and ALT activities and the contents of ALP and TG appeared in the S1.0 group. The minimum amount of serum TCHO content was presented to group S4.0 (Table 5).

3.4. Activities of liver and intestinal digestive enzymes

With the increase in succinic acid supplementation in the CON diet, the activities of AMS and LPS in the liver demonstrated a significantly second-order polynomial relationship ($P < 0.05$), and the activity of LPS in the intestine revealed a significantly linear ($P < 0.001$) and second-order polynomial relationship ($P = 0.001$). The highest AMS activity in the intestine was recorded in the S0.5 group, the maximum AMS activity in the liver was recorded in the S1.0 group, the maximum LPS activity in the liver appeared in the S2.0 group, and the maximum LPS activity in the intestine was presented in the S4.0 group. Dietary supplementation with succinic acid had no considerable effect on the activity of protease in the liver and intestine ($P > 0.05$, Table 6).

3.5. Muscle texture analysis

With the increase in succinic acid supplementation, muscle springiness and chewiness displayed a significantly second-order polynomial relationship ($P < 0.05$), and muscle hardness and resilience demonstrated a significantly linear and second-order polynomial relationship ($P < 0.05$). The maximum muscle hardness, gumminess, and chewiness were recorded in the S1.0 group, and the maximum muscle springiness and resilience were present in the S2.0 group (Table 7).

3.6. Histological properties analysis

Compared with the CON group, the density of muscle fibers in the S1.0 group was higher, and the gap between muscle fibers was smaller (Fig. 2). In comparison to the CON group, the muscle fiber density of the S1.0 and S2.0 groups was significantly greater ($P = 0.001$). In contrast to the other groups, the number of muscle fibers with a diameter of 70 to 110 μm was significantly increased in the S1.0 group ($P < 0.001$), and the number of muscle fibers with a diameter of less than 70 μm was significantly decreased in the S1.0 group ($P < 0.001$) (Table 8).

Table 2
Primer sequences for real-time qPCR.

Gene	Accession number	Primer sequence (5′ to 3′)	Product length, bp	Melting temperature, °C
β-actin	XM_003443127.5	Forward: TCGTGCGTGACATCAAGGAGAAG Reverse: CAAGGAAGGAAGGCTGGAAGAGG	180	60
MyoD	NM_001279720.1	Forward: CCTCCTCTTCATCCTCATCCTCC Reverse GCGTTGGTCGTCTTCCTCTTG	181	60
MyoG	NM_001279526.1	Forward GAGGAGCACGCTGATGAACC Reverse CGCTTGACGACGACACTCTG	185	59
Myf5	XM_005456634.3	Forward GGCGGCTGAAGAAGGTGAAC Reverse AGGCTCTCGATGTACTGGATGG	121	58
MRF4	NM_001282891.1	Forward CCTCCGCTGACCATTCCACTT Reverse GCTGTCGTTGGTGATGCTGTC	116	60
Nrf2	XM_003447296.5	Forward GCTGGACTCGCTGAAGGAAGA Reverse GCCATCCGTTGACTGCTGAAG	184	58
CAT	XM_003447521.5	Forward CCCATCCTTCGTTCATTCCCAGAA Reverse GGTGTGAGAGCCGTAGCCATTC	175	58
SOD	XM_003446807.4	Forward GCCCACACTTCAATCCCTACAA Reverse GGCTCTCTTCATTTCCTCCTTT	219	56
FASN	XM_031753129.2	Forward TGAAACTGAAGCCTTGTGTGCC Reverse TCCCTGTGAGCGGAGGTGATTA	141	60
LPL	XM_031725752.2	Forward TGCTAATGTGATTGTGGTGGAC Reverse GCTGATTTTGTGGTTGGTAAGG	217	59
ACC	XM_031755303.2	Forward CCACCACAGGCTGAACTGAGAG Reverse GCCAGACTAGCATAGACCGAATCC	197	60
CPT1B	XM_031737259.2	Forward TTGTCCACCAGCCAGACTCCA Reverse ACCATAACCATCGTCAGCCACAG	114	59
GLUT1	XM_031739850.2	Forward GTTGGAACTGCGGTGATTGGCT Reverse ATAGCAACAGCGATGGACCACAC	167	60
GLUT4	XM_031727154.2	Forward GCTGTCCGTTGCCATCTTCTCC Reverse TGTCAAGCCAGATGCCAATCCA	223	60
PFKMA	XM_031746855.2	Forward CCGTGAGAGCCACAGTCAGAGT Reverse TGCCGTCTCCACCGATTACACA	264	59
PFKMB	XM_031739988.2	Forward GGTCGCATCTTCGCCAACTCTC Reverse AGCCTTAGCCACCACTGTGTCTT	143	58
HK1A	XM_039611531.1	Forward TGCCACTGCTACACTGAAGATGC Reverse TCCTCGGGCGTGTCGTAGATTT	183	60
HK1B	XM_019360229.2	Forward TTCTCTTTCCCGTGTGCCCAAAC Reverse GTGACGCAGTTCCTCCATGTAGC	273	60
PCK	XM_031730149.2	Forward	146	58

(*continued on next page*)

Table 2 (*continued*)

Gene	Accession number	Primer sequence (5′ to 3′)	Product length, bp	Melting temperature, °C
GYS	XM_031732663.2	GCACCTCCAACAAGACCAACC Reverse ATGCCAATCTGTGAGCGTGATG Forward GAGGCATCTACACCGTCATCCA Reverse GCTCGCTCTTCCAGGAGTCTAA	276	59

MyoD = myogenic differentiation antigen; *MyoG* = myogenin; *Myf5* = myogenic factor 5; *MRF4* = muscle regulatory factor 4; *Nrf2* = nuclear factor-erythroid 2 related factor 2; *CAT* = catalase; *SOD* = superoxide dismutase; *FASN* = fatty acid synthase; *LPL* = lipoprotein lipase; *ACC* = acetyl-CoA carboxylase; *CPT1B* = carnitine palmitoyltransferase-1B; *GLUT1* = glucose transporter 1; *GLUT4* = glucose transporter 4; *PFKMA* = phosphofructokinase muscle-A; *PFKMB* = phosphofructokinase muscle-B; *HK1A* = hexokinase-1A; *HK1B* = hexokinase-1B; *PCK* = phosphoenolpyruvate carboxykinase; *GYS* = glycogen synthase.

Table 3
Effects of dietary supplementation with succinic acid levels on growth performance of Nile tilapia.[1]

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
IBW, g	204.79 ± 0.873	205.41 ± 0.518	203.27 ± 1.558	205.63 ± 1.150	205.39 ± 0.372	0.087	0.376	0.656
FBW, g	459.36 ± 4.026^{b}	476.69 ± 4.918^{c}	492.83 ± 1.336^{d}	504.52 ± 4.939^{d}	442.61 ± 6.716^{a}	<0.001	0.395	0.018
WGR, %	124.31 ± 2.008^{b}	132.07 ± 2.867^{c}	142.46 ± 1.312^{d}	145.35 ± 2.641^{d}	115.49 ± 3.257^{a}	<0.001	0.195	<0.001
SGR, %/d	1.44 ± 0.016^{b}	1.50 ± 0.022^{c}	1.58 ± 0.010^{d}	1.60 ± 0.019^{d}	1.37 ± 0.027^{a}	<0.001	0.175	<0.001
FCR	1.27 ± 0.026^{c}	1.22 ± 0.013^{b}	1.23 ± 0.007^{bc}	1.16 ± 0.023^{a}	1.21 ± 0.006^{b}	<0.001	0.076	<0.001
FR, %	1.64 ± 0.079	1.69 ± 0.075	1.62 ± 0.089	1.66 ± 0.046	1.48 ± 0.118	0.082	0.016	0.022
FI, g/fish	295.26 ± 32.060	318.16 ± 18.565	295.59 ± 31.580	318.98 ± 4.030	259.03 ± 32.905	0.103	0.077	0.044
VSI, %	7.93 ± 0.212^{c}	7.22 ± 0.155^{b}	6.86 ± 0.146^{a}	7.49 ± 0.272^{b}	7.81 ± 0.321^{c}	<0.001	0.128	<0.001
HSI, %	2.15 ± 0.061^{b}	1.91 ± 0.097^{a}	1.83 ± 0.079^{a}	1.86 ± 0.067^{a}	1.90 ± 0.069^{a}	<0.001	0.012	<0.001
MFI, %	2.83 ± 0.077^{b}	2.16 ± 0.082^{a}	2.21 ± 0.080^{a}	2.26 ± 0.103^{a}	2.26 ± 0.090^{a}	<0.001	0.008	<0.001
CF, %	3.97 ± 0.149^{a}	4.10 ± 0.135^{a}	4.31 ± 0.158^{b}	4.15 ± 0.095^{ab}	4.07 ± 0.134^{a}	<0.001	0.810	0.003
PER, %	2.35 ± 0.020^{a}	2.45 ± 0.020^{ab}	2.47 ± 0.050^{b}	2.63 ± 0.060^{c}	2.49 ± 0.010^{b}	0.001	0.070	<0.001

IBW = initial body weight; FBW = final body weight; WGR = weight gain rate; SGR = specific growth rate; FCR = feed conversion ratio; FR = feed rate; FI = feed intake; VSI = viscerosomatic index; HSI = Hepatosomatic index; MFI = mesenteric fat index; CF = condition factor; PER = protein efficiency ratio.
[a-d] Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

3.7. Muscle antioxidant capacity

With the increase in succinic acid supplementation, the activity of CAT and T-AOC in muscle displayed a significantly second-order polynomial relationship ($P < 0.05$), while the MDA content demonstrated a significantly linear ($P = 0.005$) and second-order polynomial relationship ($P = 0.003$). The maximum muscular SOD activity and the minimum MDA content appeared in the S0.5 group. The maximum muscular CAT activity was recorded in the S1.0 group, and the maximum T-AOC was recorded in the S2.0 group (Table 9). The mRNA expression levels of muscular *CAT*, *SOD*, and *Nrf2* in the succinic acid supplementation group were significantly up-regulated ($P < 0.05$) (Fig. 3).

3.8. Fatty acid profiles of the muscle

There were 22 different types of fatty acids found in muscle tissue: 7 types of saturated fatty acids (SFA), 7 types of monounsaturated fatty acids (MUFA), and 8 types of PUFA (Table 10). With the increase in succinic acid supplementation, the contents of C18:2n-6c and ΣMUFA exhibited a significantly linear relationship ($P < 0.05$), and the contents of C18:3n-3 (LA) and Σn-3PUFA demonstrated a significantly second-order polynomial relationship ($P < 0.05$). The contents of C16:0, C18:1n-9c, C20:3n-6, C20:4n-6 (ARA), C20:5n-3 (EPA), C24:1n-9, and ΣSFA demonstrated a significantly linear and second-order polynomial relationship ($P < 0.05$). The maximum contents of ΣPUFA and Σn-6PUFA were present in the S0.5 group; the minimum content of ΣMUFA was recorded in the S1.0 group; the maximum contents of Σn-3PUFA and DHA appeared in the S1.0 group; and the maximum content of ΣSFA was recorded in the S2.0 group. The maximum contents of ARA and EPA were present in the S4.0 group.

3.9. Free and bound amino acid profiles in muscle

A total of 17 kinds of bound amino acids were detected in this study, including 9 kinds of EAA (except Try) and 8 kinds of non-essential amino acids (NEAA) (Table 11). With the increase in succinic acid supplementation, Arg content demonstrated a significantly second-order polynomial relationship ($P = 0.017$), and His, Ile, Lys, and Met contents displayed a significantly linear and second-order polynomial relationship ($P < 0.05$). The maximum content of Thr was recorded in the S0.5 group, the maximum content of Arg and the minimum content of Leu were recorded in the S1.0 group, the maximum content of Met was recorded in the S2.0 group, and the maximum contents of His, Ile, and Lys were recorded in the S4.0 group. Among the NEAA, the content of Tyr demonstrated a significantly linear relationship ($P = 0.048$), the content of Ala demonstrated a significantly second-order polynomial relationship ($P = 0.003$), and the contents of Asp and Cys demonstrated a significantly linear and second-order polynomial relationship ($P < 0.05$). The maximum contents of Pro and ΣNEAA were recorded in the S1.0 group; the maximum contents of Ala and Cys were recorded in the S2.0 group; and the maximum contents of Asp, ΣEAA and total amino acids (ΣTAA) were recorded in the S4.0 group. Dietary supplementation with succinic acid had no discernible effect on the content of Phe, Val, Gly, Glu, and Ser ($P > 0.05$).

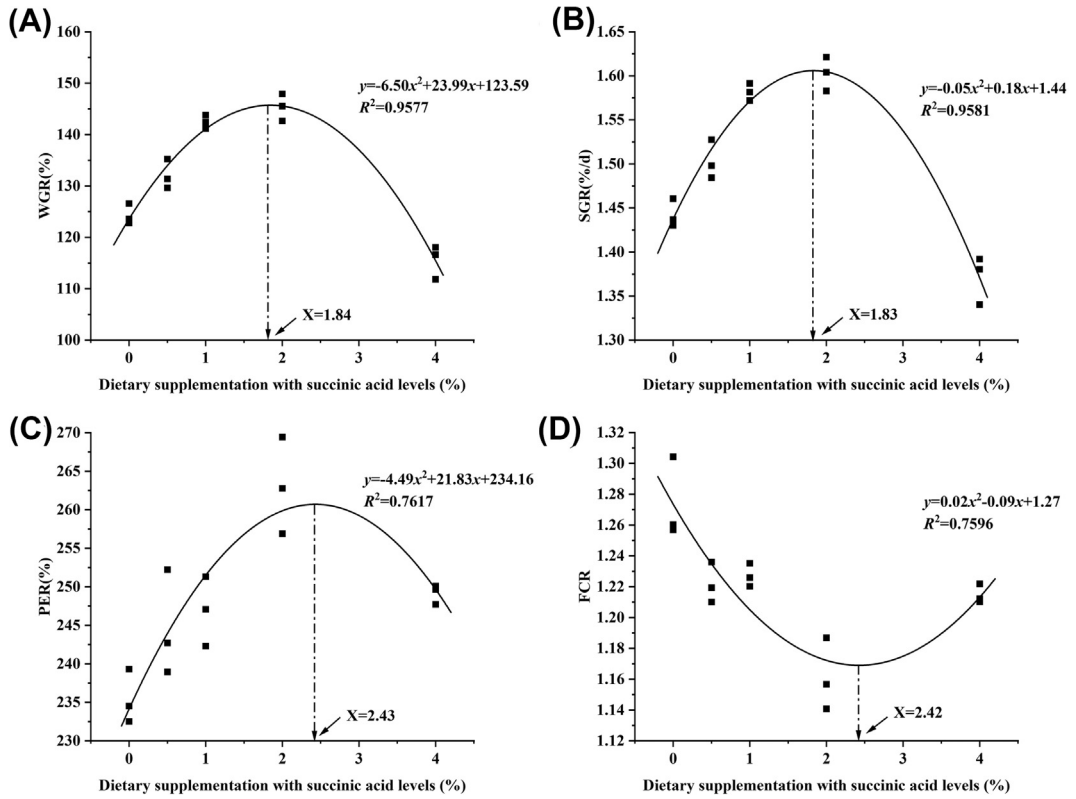

Fig. 1. Quadratic curve model analysis of weight gain rate (WGR) (A), specific growth rate (SGR) (B), protein efficiency ratio (PER) (C) and feed conversion ratio (FCR) (D) of Nile tilapia.

Table 4
Effects of dietary supplementation with succinic acid levels on proximate composition and muscle collagen content of Nile tilapia[1] (g/kg).

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
Whole fish								
Moisture	701.81 ± 8.228^{ab}	684.06 ± 5.124^{a}	695.35 ± 7.452^{ab}	704.79 ± 8.146^{b}	708.51 ± 6.566^{b}	0.015	0.041	0.123
Ash	39.81 ± 2.459	42.34 ± 2.745	41.56 ± 2.353	39.62 ± 1.286	40.56 ± 2.809	0.261	0.619	0.884
Crud protein	156.37 ± 2.759^{ab}	161.36 ± 3.568^{b}	156.95 ± 4.174^{ab}	154.98 ± 2.278^{a}	156.65 ± 2.302^{ab}	0.018	0.268	0.437
Crud lipid	98.80 ± 1.647^{b}	92.14 ± 2.183^{ab}	85.10 ± 6.532^{a}	85.63 ± 3.981^{a}	85.94 ± 5.118^{a}	<0.001	0.002	<0.001
Muscle								
Moisture	765.67 ± 7.751	762.54 ± 3.699	766.42 ± 4.898	766.61 ± 5.602	765.96 ± 7.942	0.784	0.616	0.834
Ash	11.95 ± 0.455^{a}	12.68 ± 0.438^{b}	12.25 ± 0.271^{ab}	12.20 ± 0.420^{ab}	12.36 ± 0.236^{ab}	0.037	0.648	0.832
Crud protein	185.89 ± 3.585^{a}	196.97 ± 7.555^{b}	190.78 ± 5.825^{ab}	180.95 ± 6.155^{a}	183.48 ± 5.420^{a}	0.001	0.029	0.091
Crud lipid	23.55 ± 0.949^{b}	20.35 ± 0.629^{a}	20.06 ± 0.854^{a}	21.18 ± 0.727^{a}	20.53 ± 1.132^{a}	<0.001	0.052	0.008
Glycogen	0.76 ± 0.075^{a}	1.00 ± 0.069^{b}	1.10 ± 0.107^{b}	1.12 ± 0.112^{b}	1.01 ± 0.093^{b}	<0.001	0.050	<0.001
Liver								
Moisture	733.07 ± 11.781^{c}	682.29 ± 5.905^{ab}	671.51 ± 19.431^{ab}	651.73 ± 24.298^{a}	694.45 ± 6.526^{bc}	0.001	0.343	<0.001
Crud protein	92.99 ± 3.738	91.54 ± 3.917	92.79 ± 3.037	93.07 ± 2.774	92.17 ± 4.072	0.935	0.917	0.961
Crud lipid	90.77 ± 4.171^{c}	84.20 ± 5.377^{bc}	72.79 ± 6.280^{a}	79.85 ± 4.421^{ab}	77.42 ± 3.808^{ab}	<0.001	0.016	0.001
Glycogen	107.30 ± 4.980^{bc}	108.57 ± 4.563^{bc}	116.95 ± 8.601^{c}	102.06 ± 6.555^{b}	72.31 ± 1.659^{a}	<0.001	<0.001	<0.001
Collagen								
Alkali-soluble hydroxyproline	1.65 ± 0.037^{a}	1.71 ± 0.053^{abc}	1.73 ± 0.036^{bc}	1.77 ± 0.041^{c}	1.65 ± 0.065^{ab}	<0.001	0.760	<0.001
Alkali-insoluble hydroxyproline	1.42 ± 0.042^{bc}	1.52 ± 0.051^{c}	1.36 ± 0.048^{ab}	1.29 ± 0.105^{a}	1.29 ± 0.061^{a}	<0.001	<0.001	0.001
Total hydroxyproline	3.06 ± 0.066^{a}	3.23 ± 0.081^{b}	3.09 ± 0.051^{ab}	3.06 ± 0.133^{a}	2.94 ± 0.107^{a}	0.001	0.002	0.005

$^{a-c}$ Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

A total of 14 kinds of free amino acids were detected in the muscle tissue, including 8 kinds of EAA and 6 kinds of NEAA (Table 12). With the increase in dietary succinic acid supplementation, the contents of Leu and Phe demonstrated a significantly linear relationship ($P < 0.05$), and the contents of Ile, Met, Asp, Gly, Pro, and total bitter amino acid (ΣBAA) followed a significantly second-order polynomial relationship ($P < 0.05$). The content of Arg followed a significantly linear ($P = 0.001$) and second-order

Table 5
Effects of dietary supplementation with succinic acid levels on serum biochemical indices of Nile tilapia.[1]

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
TP, g/L	35.82 ± 0.947[b]	36.92 ± 1.609[b]	37.31 ± 1.661[b]	35.95 ± 2.571[b]	32.78 ± 1.408[a]	0.001	0.001	<0.001
ALB, g/L	8.72 ± 0.329[ab]	9.13 ± 0.321[b]	9.14 ± 0.400[b]	8.76 ± 0.2333[ab]	8.34 ± 0.227[a]	0.001	0.002	0.001
AST, U/L	48.83 ± 2.927[b]	36.83 ± 1.941[a]	36.33 ± 1.633[a]	38.83 ± 2.483[a]	39.33 ± 2.582[a]	<0.001	0.113	0.001
ALT, U/L	30.17 ± 2.041[b]	28.17 ± 1.472[ab]	26.50 ± 0.837[a]	27.17 ± 1.472[a]	27.67 ± 0.816[a]	0.002	0.096	0.002
ALP, U/L	26.33 ± 1.366[b]	24.00 ± 0.632[a]	23.00 ± 1.265[a]	24.83 ± 0.983[ab]	24.00 ± 1.549[a]	0.001	0.214	0.116
TG, mmol/L	3.21 ± 0.157[d]	2.09 ± 0.146[ab]	1.89 ± 0.194[a]	2.23 ± 0.102[bc]	2.37 ± 0.095[c]	<0.001	0.202	<0.001
TCHO, mmol/L	4.18 ± 0.163[b]	3.79 ± 0.271[ab]	3.74 ± 0.261[a]	3.60 ± 0.285[a]	3.45 ± 0.158[a]	<0.001	<0.001	<0.001
GLU, mmol/L	6.07 ± 0.502[a]	5.77 ± 0.408[a]	6.12 ± 0.638[ab]	6.23 ± 0.545[ab]	7.06 ± 0.747[b]	0.010	0.001	0.002

TP = total protein; ALB = albumin; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; TG = triglyceride; TCHO = total cholesterol; GLU = glucose.
[a-d] Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

Table 6
Effects of dietary supplementation with succinic acid levels on digestive enzymes of Nile tilapia.[1]

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
Liver								
Amylase, U/mg prot	23.48 ± 0.532[a]	27.30 ± 0.965[b]	28.48 ± 1.042[b]	28.41 ± 0.390[b]	27.04 ± 0.898[b]	<0.001	0.177	0.001
Lipase, U/g prot	0.32 ± 0.016[a]	0.38 ± 0.016[b]	0.38 ± 0.026[b]	0.45 ± 0.033[c]	0.32 ± 0.003[a]	<0.001	0.773	<0.001
Protease, U/mg prot	152.73 ± 2.835	145.99 ± 5.202	148.80 ± 6.651	150.26 ± 1.199	150.80 ± 6.577	0.572	0.776	0.781
Intestine								
Amylase, U/mg prot	30.36 ± 1.346[a]	32.78 ± 0.600[b]	30.15 ± 0.654[a]	29.16 ± 0.655[a]	29.84 ± 0.311[a]	0.002	0.111	0.194
Lipase, U/g prot	0.33 ± 0.025[a]	0.40 ± 0.021[a]	0.42 ± 0.017[ab]	0.43 ± 0.055[ab]	0.51 ± 0.059[b]	0.004	<0.001	0.001
Protease, U/mg prot	188.62 ± 7.458	179.69 ± 8.103	186.79 ± 8.353	182.09 ± 5.567	176.05 ± 9.548	0.347	0.098	0.267

[a,b] Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

Table 7
Effects of dietary supplementation with succinic acid levels on muscle texture of Nile tilapia.[1]

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
Hardness, g	2,594.00 ± 58.716[ab]	2,650.70 ± 80.000[bc]	2,726.90 ± 97.974[c]	2,671.40 ± 41.183[bc]	2,509.80 ± 50.988[a]	<0.001	0.002	<0.001
Springiness	0.52 ± 0.032[a]	0.53 ± 0.032[ab]	0.55 ± 0.020[ab]	0.56 ± 0.036[b]	0.51 ± 0.033[a]	0.005	0.725	0.001
Gumminess, g	1,088.77 ± 44.597[a]	1,116.57 ± 78.151[ab]	1,171.91 ± 64.082[b]	1,091.96 ± 54.321[a]	1,080.75 ± 33.862[a]	0.005	0.174	0.110
Chewiness, g	563.90 ± 18.691[a]	598.04 ± 19.371[b]	621.57 ± 10.230[c]	600.86 ± 10.786[bc]	570.74 ± 25.309[a]	<0.001	0.321	<0.001
Resilience	0.21 ± 0.017[a]	0.29 ± 0.022[b]	0.30 ± 0.021[b]	0.33 ± 0.017[c]	0.30 ± 0.011[b]	<0.001	0.001	<0.001

[a-c] Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

polynomial relationship ($P < 0.001$). The minimum contents of Ile and Leu were found in the S0.5 group; the maximum contents of Ala, ΣEAA, ΣNEAA, and TAA were recorded in the S0.5 group; the maximum contents of His, Lys, Pro, and ΣFAA were recorded in the S1.0 group; and the minimum contents of Met, Val, Gly, and Tyr were recorded in the S1.0 group. The maximum content of Asp and the minimum content of ΣBAA appeared in the S2.0 group, and the minimum content of Arg was recorded in the S4.0 group. The level of dietary supplementation with succinic acid had no discernible effect on the content of Glu ($P = 0.077$).

3.10. Muscle regulatory factor (MRF) gene expression in the muscle

The mRNA expression levels of *MyoD*, *MyoG*, *Myf5*, and *MRF4* were significantly up-regulated in the succinic acid supplementation group ($P < 0.05$). The S2.0 group had a significantly greater *MyoD* mRNA expression level compared to the other groups ($P < 0.001$). The S0.5 group had the highest *MyoG* mRNA expression level. With the increase in succinic acid supplementation, the mRNA expression levels of *Myf5* and *MRF4* demonstrated a significantly second-order polynomial relationship ($P < 0.05$), and the maximum was recorded in the S1.0 group (Fig. 4).

3.11. Glucose lipid metabolism in the muscle

The mRNA expression level of *FASN* was significantly down-regulated in the succinic acid supplementation group ($P = 0.004$). With the increase in succinic acid supplementation, the mRNA expression levels of *CTP1B* and *HSL* demonstrated a significantly second-order polynomial relationship ($P < 0.05$), and the maximum

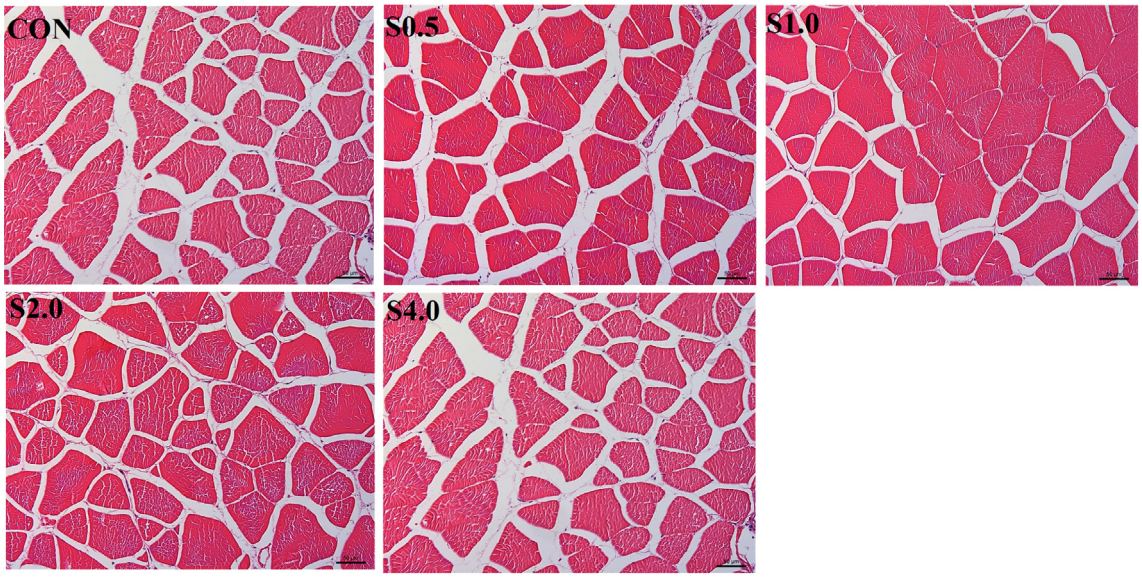

Fig. 2. Effects of dietary supplementation with succinic acid levels on the morphology of Nile tilapia. Myofiber microstructure of cross-section (200 ×). Scale bar = 50 μm. CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

Table 8
Effects of dietary supplementation with succinic acid levels on the number of muscle fiber and fiber density of Nile tilapia.[1]

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
Number of muscle fiber								
Muscle fiber diameter <70 μm	27.66 ± 1.229^{c}	22.09 ± 1.513^{b}	15.01 ± 0.531^{a}	21.69 ± 1.136^{b}	30.43 ± 1.089^{c}	<0.001	0.126	<0.001
Muscle fiber diameter 70–110 μm	47.85 ± 1.023^{b}	54.97 ± 1.807^{c}	62.99 ± 1.531^{d}	46.24 ± 0.719^{b}	38.16 ± 1.278^{a}	<0.001	0.007	0.004
Muscle fiber diameter >110 μm	24.50 ± 2.055^{a}	22.94 ± 0.664^{a}	22.00 ± 1.474^{a}	32.07 ± 0.558^{b}	31.41 ± 1.035^{b}	<0.001	0.001	0.004
Muscle fiber density, N/mm^2	155.95 ± 5.832^{a}	164.88 ± 4.175^{ab}	171.43 ± 5.051^{b}	166.67 ± 4.325^{b}	162.50 ± 6.682^{ab}	0.001	0.592	0.003

$^{a-d}$ Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

Table 9
Effects of dietary supplementation with succinic acid levels on muscle antioxidant indices of Nile tilapia.[1]

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
CAT, U/mg prot	25.70 ± 1.976^{a}	36.99 ± 1.293^{c}	51.12 ± 3.379^{d}	32.08 ± 1.990^{b}	33.17 ± 1.765^{b}	<0.001	0.856	0.031
T-AOC, U/mg prot	0.19 ± 0.014^{a}	0.20 ± 0.011^{a}	0.45 ± 0.034^{b}	0.48 ± 0.039^{b}	0.19 ± 0.016^{a}	0.005	0.975	<0.001
SOD, U/mg prot	10.63 ± 0.813^{a}	12.53 ± 0.824^{b}	12.01 ± 1.014^{ab}	11.10 ± 0.956^{a}	11.91 ± 0.485^{ab}	<0.001	0.544	0.765
MDA, nmol/mg prot	0.16 ± 0.013^{b}	0.13 ± 0.006^{a}	0.13 ± 0.016^{a}	0.16 ± 0.008^{b}	0.17 ± 0.013^{b}	<0.001	0.005	0.003

CAT = catalase; T-AOC = total antioxidant capacity; SOD = superoxide dismutase; MDA = malondialdehyde.
$^{a-d}$ Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

was reflected in the S1.0 group and S2.0 group, respectively. The mRNA expression level of *LPL* demonstrated a significantly linear and second-order polynomial relationship ($P < 0.05$), and the minimum was recorded in the S4.0 group (Fig. 5).

The mRNA expression levels of genes related to glycolysis (*PFKMA* and *PFKMB*), glycogen synthesis (*GYS*), and gluconeogenesis (*PCK*) were significantly increased in the succinic acid supplementation group ($P < 0.05$). The mRNA expression levels of *HK1A*, *HK1B*, *HK2*, and *GLUT4* demonstrated a significantly linear and second-order polynomial relationship ($P < 0.05$). The maximum mRNA expression levels of *HK1B* and *HK2* were present in the S0.5 group, and the maximum mRNA expression level of *HK1A* was recorded in the S1.0 group. The maximum mRNA expression level of *GLUT4* was recorded in the S2.0 group. The *GLUT1* mRNA expression level revealed a significantly second-order polynomial relationship ($P = 0.004$), and the maximum appeared in the S1.0 group (Fig. 6).

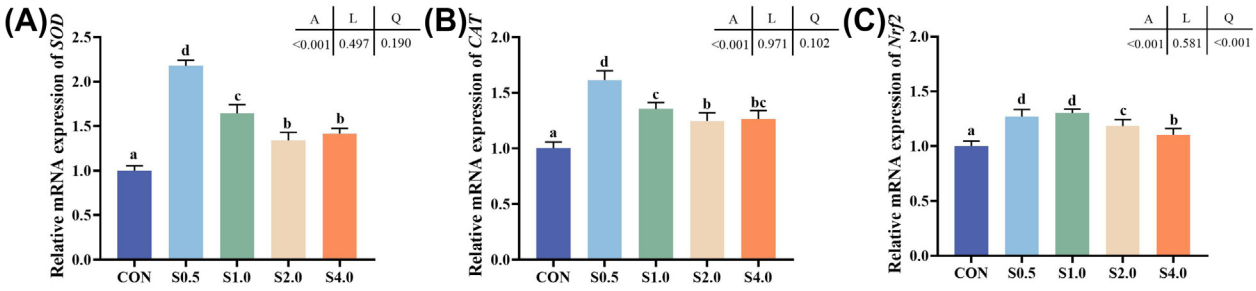

Fig. 3. Effects of dietary supplementation with succinic acid levels on the relative mRNA expression of antioxidant genes in muscle of Nile tilapia. CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid. All values are means ± SD ($n = 6$). A: *P*-value of one-way analysis of variance; L: *P*-value of linear trend analyzed by orthogonal polynomial contrasts; Q: *P*-value of quadratic trend analyzed by orthogonal polynomial contrasts. *SOD* = superoxide dismutase; *CAT* = catalase; *Nrf2* = nuclear factor-erythroid 2 related factor 2.[a-d] Bars with different letters are significantly different at $P < 0.05$.

Table 10
Effects of dietary supplementation with succinic acid levels fatty acid composition in muscle of Nile tilapia[1] (g/kg).

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
C12:0	0.03 ± 0.004	0.03 ± 0.005	0.03 ± 0.004	0.03 ± 0.003	0.03 ± 0.001	0.956	0.638	0.889
C14:0	0.67 ± 0.052^{a}	0.85 ± 0.066^{b}	0.73 ± 0.046^{ab}	0.83 ± 0.038^{b}	0.74 ± 0.057^{ab}	0.010	0.712	0.143
C14:1n-5	0.04 ± 0.003	0.04 ± 0.007	0.03 ± 0.007	0.03 ± 0.002	0.04 ± 0.004	0.391	0.498	0.572
C15:0	0.05 ± 0.011	0.05 ± 0.005	0.05 ± 0.005	0.05 ± 0.002	0.04 ± 0.005	0.966	0.541	0.835
C16:0	4.30 ± 0.163^{a}	4.64 ± 0.327^{a}	4.60 ± 0.147^{a}	5.23 ± 0.182^{b}	5.23 ± 0.094^{b}	0.001	<0.001	<0.001
C16:1n-7	0.92 ± 0.097	1.03 ± 0.067	1.03 ± 0.030	1.05 ± 0.096	0.96 ± 0.094	0.273	0.958	0.095
C17:0	0.05 ± 0.011	0.05 ± 0.005	0.05 ± 0.008	0.05 ± 0.003	0.05 ± 0.012	0.825	0.286	0.564
C17:1n-7	0.03 ± 0.009	0.04 ± 0.002	0.03 ± 0.004	0.03 ± 0.004	0.04 ± 0.011	0.560	0.302	0.479
C18:0	1.36 ± 0.125^{a}	1.31 ± 0.025^{a}	1.33 ± 0.041^{a}	1.60 ± 0.030^{b}	1.35 ± 0.044^{a}	0.001	0.482	0.059
C18:1n-9t	0.07 ± 0.003	0.07 ± 0.011	0.07 ± 0.008	0.09 ± 0.010	0.07 ± 0.001	0.056	0.617	0.108
C18:1n-9c	7.51 ± 0.363^{b}	6.22 ± 0.326^{a}	5.81 ± 0.236^{a}	6.45 ± 0.276^{a}	5.66 ± 0.342^{a}	<0.001	0.017	0.026
C18:2n-6c	2.16 ± 0.116^{a}	2.43 ± 0.050^{b}	2.14 ± 0.028^{a}	2.08 ± 0.103^{a}	2.06 ± 0.060^{a}	0.001	0.044	0.137
C20:0	0.06 ± 0.005	0.06 ± 0.006	0.04 ± 0.010	0.05 ± 0.006	0.06 ± 0.014	0.189	0.467	0.177
C18:3n-6	0.08 ± 0.019	0.08 ± 0.005	0.08 ± 0.012	0.09 ± 0.006	0.08 ± 0.013	0.823	0.417	0.630
C20:1n-9	0.28 ± 0.022^{ab}	0.22 ± 0.006^{a}	0.28 ± 0.059^{ab}	0.33 ± 0.006^{b}	0.29 ± 0.013^{ab}	0.018	0.150	0.198
C18:3n-3 (LA)	0.19 ± 0.018	0.192 ± 0.014	0.213 ± 0.005	0.215 ± 0.010	0.195 ± 0.001	0.061	0.654	0.018
C20:2n-6	0.118 ± 0.016	0.107 ± 0.004	0.12 ± 0.018	0.119 ± 0.016	0.112 ± 0.010	0.740	0.815	0.857
C20:3n-6	0.13 ± 0.003^{a}	0.13 ± 0.006^{a}	0.13 ± 0.007^{a}	0.15 ± 0.008^{b}	0.15 ± 0.007^{b}	0.003	0.002	0.004
C20:4n-6 (ARA)	0.27 ± 0.013^{a}	0.29 ± 0.011^{ab}	0.29 ± 0.011^{ab}	0.30 ± 0.007^{ab}	0.31 ± 0.017^{b}	0.040	0.003	0.014
C20:5n-3 (EPA)	0.177 ± 0.005^{a}	0.181 ± 0.002^{ab}	0.173 ± 0.003^{a}	0.192 ± 0.003^{b}	0.213 ± 0.006^{c}	<0.001	<0.001	<0.001
C24:1n-9	0.04 ± 0.003	0.04 ± 0.004	0.04 ± 0.003	0.04 ± 0.005	0.04 ± 0.006	0.150	0.012	0.047
C22:6n-3 (DHA)	1.19 ± 0.018^{a}	1.25 ± 0.030^{ab}	1.32 ± 0.059^{b}	1.26 ± 0.045^{ab}	1.26 ± 0.041^{ab}	0.039	0.395	0.104
ΣSFA	6.51 ± 0.329^{a}	6.97 ± 0.284^{ab}	6.82 ± 0.147^{a}	7.85 ± 0.227^{c}	7.51 ± 0.052^{bc}	<0.001	0.004	0.001
ΣMUFA	8.88 ± 0.458^{b}	7.65 ± 0.393^{a}	7.29 ± 0.158^{a}	8.02 ± 0.346^{ab}	7.11 ± 0.343^{a}	0.001	0.023	0.055
ΣPUFA	4.30 ± 0.078^{a}	4.66 ± 0.102^{b}	4.46 ± 0.065^{ab}	4.39 ± 0.101^{a}	4.38 ± 0.084^{a}	0.005	0.468	0.578
Σn-3PUFA	1.55 ± 0.039^{a}	1.63 ± 0.042^{ab}	1.70 ± 0.064^{b}	1.67 ± 0.045^{ab}	1.67 ± 0.040^{ab}	0.024	0.105	0.022
Σn-6PUFA	2.75 ± 0.107^{a}	3.03 ± 0.065^{b}	2.75 ± 0.034^{a}	2.72 ± 0.117^{a}	2.71 ± 0.097^{a}	0.007	0.138	0.346
Total FA	19.69 ± 0.728^{ab}	19.28 ± 0.738^{ab}	18.58 ± 0.159^{a}	20.27 ± 0.484^{b}	19.00 ± 0.383^{ab}	0.022	0.043	0.056

ΣSFA = total saturated fatty acid; ΣMUFA = total monounsaturated fatty acid; ΣPUFA = total polyunsaturated fatty acid; FA = fatty acid.
[a-c] Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

4. Discussion

4.1. Succinic acid supplementation in HCD improves the growth and feed utilization of tilapia

Carbohydrates are used in fish diets as the cheapest energy source to enhance growth performance and promote protein-sparing action (Li et al., 2020). The majority of research has revealed that the optimal dietary carbohydrate level improves growth, but excessive dietary carbohydrate negatively affects fish health (Han et al., 2021). In recent years, glucose metabolism intermediates or derivatives have been used as feed additives to improve growth performance and feed utilization in aquatic animals (Duan et al., 2018). For example, dietary supplementation with 0.2% malic acid and 0.2% citric acid significantly improved the growth performance of Gibel carp (*Carassius auratus gibelio*) (Zhang et al., 2020), and dietary supplementation with 0.15% succinate promoted the growth and whole-body protein deposition of zebrafish (Ding et al., 2022). Our study demonstrated that supplementation with 2.0% succinic acid in HCD significantly improved the WGR, SGR, and PER of tilapia and reduced the FCR, indicating that succinic acid ameliorated the adverse effects of HCD on tilapia. Previous studies in zebrafish have reported that the growth promotion effect of succinate was related to the increase in FI (Ding et al., 2022). Nevertheless, succinic acid had no impact on the FI of tilapia, which might be related to different feeding strategies, fish size, and cultivation density. Quadratic curve model analysis based on WGR, SGR, PER, and FCR demonstrated that the optimal

Table 11
Effects of dietary supplementation with succinic acid levels on bound amino acid contents in muscle of Nile tilapia[1] (g/kg).

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
Arg	12.68 ± 0.491^{a}	14.54 ± 0.833^{ab}	15.05 ± 1.295^{b}	14.83 ± 0.619^{b}	15.19 ± 0.366^{b}	0.017	0.036	0.017
His	5.39 ± 0.361^{a}	6.81 ± 0.768^{ab}	7.03 ± 0.841^{b}	7.01 ± 0.509^{b}	7.42 ± 0.296^{b}	0.016	0.017	0.013
Ile	9.60 ± 0.340^{a}	10.49 ± 0.500^{ab}	10.62 ± 0.366^{b}	11.10 ± 0.086^{b}	11.23 ± 0.280^{b}	0.001	0.001	<0.001
Leu	16.19 ± 0.878^{ab}	17.03 ± 0.566^{b}	14.96 ± 0.773^{a}	17.54 ± 0.875^{b}	17.72 ± 0.446^{b}	0.005	0.051	0.154
Lys	15.16 ± 0.753^{a}	19.26 ± 0.805^{b}	19.56 ± 1.183^{b}	19.74 ± 0.529^{b}	19.97 ± 0.637^{b}	<0.001	0.021	0.002
Met	5.64 ± 0.465^{a}	6.70 ± 0.507^{b}	6.97 ± 0.151^{b}	7.37 ± 0.137^{b}	7.34 ± 0.179^{b}	<0.001	0.004	<0.001
Phe	9.16 ± 0.768	10.32 ± 0.610	10.56 ± 1.013	10.72 ± 0.545	10.89 ± 0.404	0.080	0.035	0.026
Thr	10.54 ± 0.576^{a}	14.15 ± 1.168^{b}	11.64 ± 0.827^{a}	11.88 ± 0.441^{a}	12.03 ± 0.340^{a}	0.002	0.927	0.827
Val	10.84 ± 0.576	11.81 ± 0.519	11.95 ± 0.766	12.31 ± 0.767	12.21 ± 0.313	0.091	0.049	0.021
Ala	11.64 ± 0.576^{a}	12.99 ± 0.638^{ab}	13.46 ± 0.594^{b}	13.50 ± 0.080^{b}	13.29 ± 0.429^{b}	0.006	0.063	0.003
Asp	18.43 ± 0.888^{a}	19.55 ± 0.787^{ab}	19.91 ± 0.607^{ab}	20.17 ± 0.643^{ab}	20.39 ± 0.552^{b}	0.046	0.015	0.010
Cys	1.61 ± 0.097^{a}	1.80 ± 0.056^{ab}	1.85 ± 0.024^{ab}	2.26 ± 0.119^{c}	1.99 ± 0.137^{b}	<0.001	0.022	<0.001
Gly	9.92 ± 0.333	10.71 ± 0.309	11.05 ± 0.608	10.84 ± 0.733	10.71 ± 0.105	0.114	0.314	0.077
Glu	25.33 ± 1.135	26.76 ± 1.227	27.06 ± 1.733	27.82 ± 1.368	27.92 ± 0.764	0.172	0.036	0.037
Pro	8.76 ± 0.595^{a}	10.01 ± 0.278^{b}	12.01 ± 0.235^{c}	10.09 ± 0.500^{b}	9.90 ± 0.248^{b}	<0.001	0.769	0.073
Ser	8.62 ± 0.507	9.48 ± 0.539	9.66 ± 0.748	9.90 ± 0.464	10.02 ± 0.273	0.057	0.020	0.014
Tyr	3.89 ± 0.264^{b}	2.13 ± 0.118^{a}	3.66 ± 0.284^{b}	2.26 ± 0.142^{a}	2.33 ± 0.115^{a}	<0.001	0.048	0.088
ΣEAA	95.20 ± 4.235^{a}	111.11 ± 4.928^{b}	108.34 ± 5.411^{b}	112.51 ± 4.035^{b}	114.01 ± 3.023^{b}	0.002	0.015	0.007
ΣNEAA	88.20 ± 3.835^{a}	93.43 ± 3.678^{ab}	98.67 ± 3.527^{b}	96.85 ± 3.747^{ab}	96.54 ± 2.160^{ab}	0.028	0.093	0.017
TAA	183.40 ± 7.896^{a}	204.53 ± 8.597^{b}	207.02 ± 8.681^{b}	209.36 ± 7.762^{b}	210.55 ± 5.164^{b}	0.009	0.026	0.006

ΣEAA = total essential amino acids; ΣNEAA = total non-essential amino acids; TAA = total amino acids.
$^{a-c}$ Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

Table 12
Effects of dietary supplementation with succinic acid on levels of free amino acid contents in muscle of Nile tilapia[1] (g/kg).

Item	Groups[2]					*P*-value		
	CON	S0.5	S1.0	S2.0	S4.0	ANOVA	L	Q
Arg	71.16 ± 0.561^{d}	52.47 ± 0.782^{c}	41.02 ± 1.407^{b}	38.93 ± 0.848^{ab}	36.63 ± 0.313^{a}	<0.001	0.001	<0.001
His	206.19 ± 7.585^{a}	225.22 ± 5.377^{bc}	238.14 ± 3.698^{c}	220.34 ± 1.068^{ab}	233.29 ± 8.429^{bc}	<0.001	0.080	0.108
Ile	13.33 ± 1.129^{c}	8.21 ± 0.464^{a}	9.47 ± 0.675^{ab}	10.08 ± 0.085^{b}	12.31 ± 0.387^{c}	<0.001	0.457	0.025
Leu	20.32 ± 0.368^{c}	15.03 ± 0.574^{a}	17.66 ± 0.360^{b}	20.20 ± 1.344^{c}	21.63 ± 1.022^{c}	<0.001	0.027	0.057
Lys	165.69 ± 2.886^{ab}	187.06 ± 4.625^{c}	215.18 ± 13.138^{d}	154.59 ± 9.460^{a}	179.18 ± 4.281^{bc}	<0.001	0.677	0.897
Met	10.81 ± 0.650^{c}	10.06 ± 0.441^{bc}	8.37 ± 0.610^{a}	9.38 ± 0.241^{ab}	9.49 ± 0.454^{abc}	0.002	0.246	0.028
Phe	10.73 ± 0.881^{ab}	10.89 ± 0.334^{ab}	9.11 ± 0.864^{a}	11.58 ± 1.085^{ab}	12.32 ± 1.260^{b}	0.019	0.036	0.081
Val	12.45 ± 0.655^{c}	9.66 ± 0.610^{ab}	8.21 ± 0.880^{a}	12.14 ± 0.362^{c}	11.49 ± 0.958^{bc}	<0.001	0.487	0.448
Ala	345.83 ± 10.908^{a}	428.55 ± 12.553^{b}	367.20 ± 17.554^{a}	348.48 ± 6.623^{a}	421.38 ± 12.794^{b}	<0.001	0.176	0.218
Asp	5.53 ± 0.302^{a}	6.65 ± 0.377^{ab}	6.93 ± 0.711^{b}	7.43 ± 0.457^{b}	6.41 ± 0.248^{ab}	0.005	0.357	0.001
Gly	452.26 ± 13.302^{c}	412.00 ± 5.467^{a}	397.58 ± 11.761^{a}	418.04 ± 6.626^{ab}	443.74 ± 12.978^{bc}	<0.001	0.505	0.007
Glu	41.12 ± 1.263	42.54 ± 2.653	48.14 ± 3.189	43.07 ± 3.846	43.85 ± 1.601	0.077	0.657	0.428
Pro	1,007.58 ± 25.822^{a}	1,094.72 ± 42.108^{bc}	1,143.76 ± 17.634^{c}	1,122.52 ± 16.131^{bc}	1,055.81 ± 17.148^{ab}	0.001	0.829	0.001
Tyr	25.72 ± 1.568^{d}	18.54 ± 0.796^{b}	15.35 ± 1.417^{a}	22.11 ± 0.456^{c}	17.65 ± 1.117^{ab}	<0.001	0.186	0.251
ΣEAA	657.07 ± 7.367^{a}	714.71 ± 5.416^{b}	701.61 ± 16.178^{b}	652.55 ± 16.530^{a}	663.35 ± 2.051^{a}	<0.001	0.202	0.413
ΣNEAA	1,878.05 ± 34.086^{a}	2,003.01 ± 44.542^{b}	1,978.96 ± 41.856^{b}	1,961.65 ± 28.576^{ab}	1,988.84 ± 18.186^{b}	0.010	0.155	0.165
ΣFAA	46.65 ± 1.563^{a}	49.19 ± 2.692^{ab}	55.07 ± 3.091^{b}	50.50 ± 4.250^{ab}	50.25 ± 1.387^{ab}	0.047	0.555	0.175
ΣBAA	334.26 ± 5.399^{c}	320.65 ± 3.638^{ab}	322.86 ± 5.200^{abc}	311.05 ± 3.166^{a}	324.85 ± 6.175^{bc}	0.003	0.340	0.001
TAA	2,535.12 ± 35.560^{a}	2,717.71 ± 49.935^{b}	2,680.57 ± 56.502^{b}	2,614.2 ± 38.965^{ab}	2,652.19 ± 18.356^{b}	0.003	0.587	0.504

ΣEAA = total essential amino acids; ΣNEAA = total non-essential amino acids; ΣFAA = total flavor amino acid; ΣBAA = total bitter amino acid; TAA = total amino acids.
$^{a-d}$ Different letters in the same line show significant differences ($P < 0.05$).
[1] All values are means ± SD ($n = 6$). L: linear trend; Q: second order polynomial trend.
[2] CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid.

supplementation level with succinic acid in HCD ranged from 1.83% to 2.43% for tilapia, which was higher than that of zebrafish (0.15%) (Ding et al., 2022) and white shrimp (0.5%) (Duan et al., 2018), which might be due to fish needing more succinic acid to increase body metabolism in HCD and alleviate HCD stress.

Generally, herbivorous and omnivorous fish have a stronger capacity to utilize dietary carbohydrates, which may be related to enhanced AMS activity in vivo (Bautista et al., 1988). Organic acids have the capacity to slightly reduce the pH of the intestinal environment, which further facilitates the improvement of digestive enzyme activity and boosts the digestion and absorption of nutrients (Li et al., 2006; Luckstadt, 2012). Prior research revealed that the addition of organic acids enhanced the digestive enzyme activity in red drum (*Sciaenops ocellatus*) (Castillo et al., 2014). Furthermore, dietary supplementation with 0.5% succinic acid promoted both digestive enzyme activity and feed utilization in white shrimp (Duan et al., 2018). In the current study, dietary supplementation with 0.5% succinic acid enhanced the activities of AMS and LPS, indicating that succinic acid might enhance the ability to utilize carbohydrates and promote the digestion and absorption of nutrients by improving digestive enzyme activity to adapt to a HCD, which was conducive to the growth of tilapia.

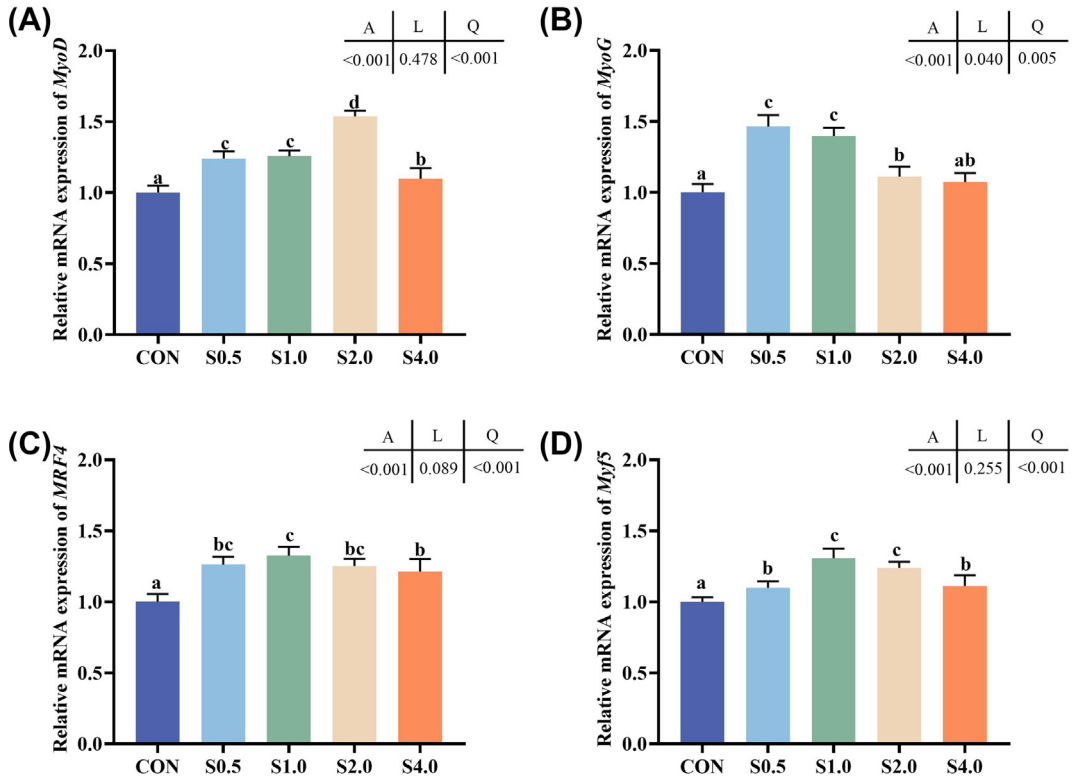

Fig. 4. Effects of dietary supplementation with succinic acid levels on mRNA expression levels of MRF of Nile tilapia. CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid. All values are means ± SD ($n = 6$). A: *P*-value of one-way analysis of variance; L: *P*-value of linear trend analyzed by orthogonal polynomial contrasts; Q: *P*-value of quadratic trend analyzed by orthogonal polynomial contrasts. *MyoD* = myogenic differentiation antigen; *MyoG* = myogenin; *MRF4* = muscle regulatory factor 4; *Myf5* = myogenic factor 5. [a-d] Bars with different letters are significantly different at $P < 0.05$.

4.2. Succinic acid supplementation in HCD improves the muscle antioxidant capacity of tilapia

Excessive dietary carbohydrate induces excessive production of free radicals, especially ROS, which leads to the oxidation of lipids and proteins in muscle, resulting in a decline in muscle quality (Cai et al., 2022; Wang et al., 2015). In vitro studies on the muscle cells of olive flounder (*Paralichthys olivaceus*) found that HCD induced oxidative stress and led to the apoptosis of muscle cells (Liu et al., 2022). In addition, oxidative stress reduced the physicochemical and textural properties of common carp (*Cyprinus carpio*) muscle (Morachis-Valdez et al., 2015). Organisms have an antioxidant defense system, which includes enzymatic and non-enzymatic antioxidants like SOD and CAT, to avoid oxidative damage (Cai et al., 2021). Dietary supplementation with organic acids enhances the antioxidant capacity of aquatic animals (Duan et al., 2017; Wang et al., 2015). Dietary supplementation with 0.5% succinic acid increased the antioxidant capacity of the hepatopancreas of white shrimp (Duan et al., 2018). In this study, dietary supplementation with 0.5% to 1.0% succinic acid significantly enhanced the antioxidant capacity of tilapia by improving the activity of endogenous antioxidant enzymes, such as CAT, SOD, and T-AOC. The exogenous addition of succinic acid stimulates the activity of succinate dehydrogenase (SDH), a common antioxidant, which simultaneously links the TCA with oxidative phosphorylation and imports electrons from the TCA into the electron transport chain (Adisa et al., 2019). Dietary succinic acid might accelerate the flow of paired electrons in the electron transport chain and accelerate the generation of electrochemical transmembrane potential to prevent the leakage of unpaired electrons, causing ROS production and the formation of lipid peroxides (Grishina et al., 2015). Therefore, succinic acid improves muscle quality after slaughter and during storage by reducing oxidative damage in muscle.

4.3. Succinic acid supplementation in HCD improves the muscle hardness of tilapia

Muscle texture is one of the most important parameters for reflecting muscle quality. The assessment of muscle texture is commonly based on its textural properties (Liu et al., 2020). Muscle hardness is the main indicator for consumers to evaluate aquatic products, and is positively correlated with flesh quality (Cai et al., 2022). Long-term intake of HCD significantly reduces muscle hardness, gumminess, and chewiness (Bao et al., 2022; Wu et al., 2021). In the current study, dietary supplementation with 1.0% succinic acid significantly increased muscle hardness, gumminess, chewiness, and resilience, indicating that succinic acid improves muscle texture, thereby improving taste. Our results are consistent with studies on mammals such as cattle (Gao et al., 2014) and fattening pigs (Liang, 2018).

The textural properties of fish flesh are significantly influenced by the diameter and density of muscle fibers (Koganti et al., 2020). Studies have shown that the muscle fiber diameter of Atlantic salmon (*Salmo salar*) (Johnston et al., 2000), sea bass (*Dicentrarchus labrax*) (Periago et al., 2005), and gilthead sea bream (*Sparus aurata*) (Valente et al., 2011) were negatively correlated with muscle hardness, while muscle fiber density was positively correlated with muscle hardness, chewiness, and collagen content (Bao et al., 2022). Dietary supplementation with succinic acid promoted the transformation of fast-twitch muscle fibers into slow-twitch muscle fibers in mice by activating the Ca^{2+} signaling pathway mediated by the succinate receptor 1 (*SUCNR1*) gene (Wang et al., 2019).

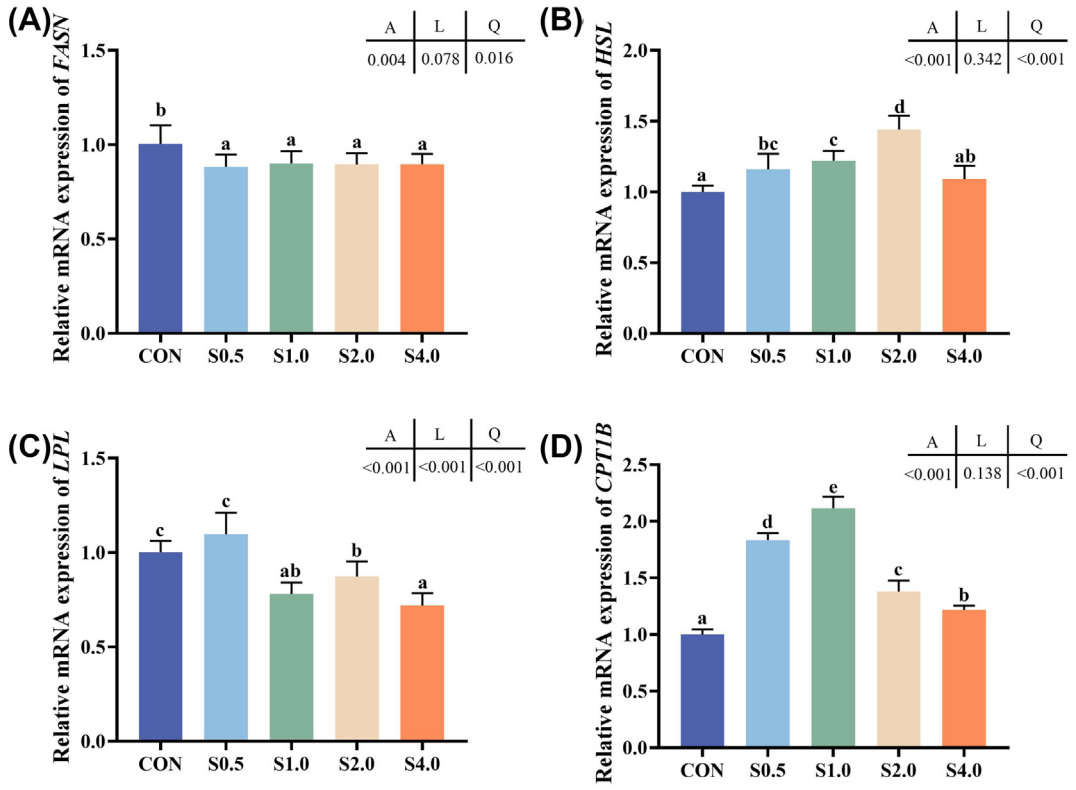

Fig. 5. Effects of dietary supplementation with succinic acid levels on mRNA expression levels of genes related to fatty acid anabolism of Nile tilapia. CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid. All values are means ± SD ($n = 6$). A: *P*-value of one-way analysis of variance; L: *P*-value of linear trend analyzed by orthogonal polynomial contrasts; Q: *P*-value of quadratic trend analyzed by orthogonal polynomial contrasts. *FASN* = fatty acid synthase; *HSL* = hormone sensitive lipase; *LPL* = lipoprotein lipase; *CPT1B* = carnitine palmitoyltransferase-1B. $^{a\text{-}d}$ Bars with different letters are significantly different at $P < 0.05$.

In the present study, dietary supplementation with 1.0% succinic acid increased the muscle fiber density and the number of muscle fibers with a diameter ranging from 70 to 110 μm, and decreased the number of muscle fibers greater than 110 μm, which might promote the transformation of muscle fiber type, change the proportion of muscle fibers and thus significantly improve muscle quality.

The development of muscle fibers is regulated by *MRF* genes; MyoD and Myf5 regulate myoblast proliferation, while MyoG and MRF4 regulate myoblast differentiation (Koganti et al., 2020). Prior research has confirmed that excessive carbohydrate intake reduces the mRNA expression level of *MRF* gene and inhibits muscle fiber hyperplasia (Bao et al., 2022; Chapalamadugu et al., 2009). In the current study, dietary supplementation with 0.5% to 2.0% succinic acid significantly up-regulated the mRNA expression levels of *MyoD*, *MyoG*, *Myf5*, and *MRF4*, indicating that succinic acid promotes the development of muscle fibers. Myogenic regulatory factors are regulated by the mammalian target of rapamycin (mTOR) signaling pathway, and the anabolic process of mTOR signaling requires adequate energy from the TCA (Meng et al., 2023; Periago et al., 2005). Therefore, succinic acid might promote the mTOR signaling pathway by accelerating the oxidative phosphorylation process, thereby up-regulating the expression level of *MRF* gene, stimulating muscle cell development, and thereby increasing muscle fiber density. The aforementioned statement provides additional clarification regarding the potential mechanism underlying succinate-induced alterations in muscle fiber diameter and density.

Collagen content, which is directly proportional to muscle hardness, is a crucial determinant of fish flesh quality (Dong et al., 2022). The concentration of hydroxyproline is used to measure collagen (Hofman et al., 2011). Hydroxyproline is a metabolite of Pro catalyzed by proline hydroxylase (PHD). Proline hydroxylase is a type of dioxygenase dependent on oxygen and α-ketoglutarate, which is also the basis for collagen to maintain its triple helix structure (Yang et al., 2021). In addition, collagen is mainly synthesized by fibroblasts using Gly and Pro (Li and Wu, 2018). In this study, supplementation with 0.5% succinic acid significantly increased the contents of Pro, Gly, and hydroxyproline in muscle, suggesting that succinic acid might increase the content of muscle collagen by increasing the raw materials for collagen synthesis. On the other hand, succinic acid might activate PDH to promote collagen synthesis by increasing the concentration of α-ketoglutarate. Other research revealed that the content of hydroxyproline in muscle was positively correlated with the muscle texture of Atlantic salmon (Li et al., 2005) and Atlantic halibut (*Hippoglossus hippoglossus*) (Hagen et al., 2007), which improves muscle hardness. In the current study, fish fed with 0.5% to 1.0% succinic acid had higher muscle fiber density and total hydroxyproline content, which might be one of the reasons for the increase in the muscle hardness of tilapia.

4.4. Succinic acid supplementation in HCD improves the muscular nutritional value and flavor of tilapia

The levels of protein and amino acids in muscle determine its nutritional value and flavor (Jiang et al., 2016). In the present research, dietary supplementation with 1.5% succinic acid significantly increased muscle crude protein content, which might be related to the activation of Akt/mTOR/S6 and other signaling

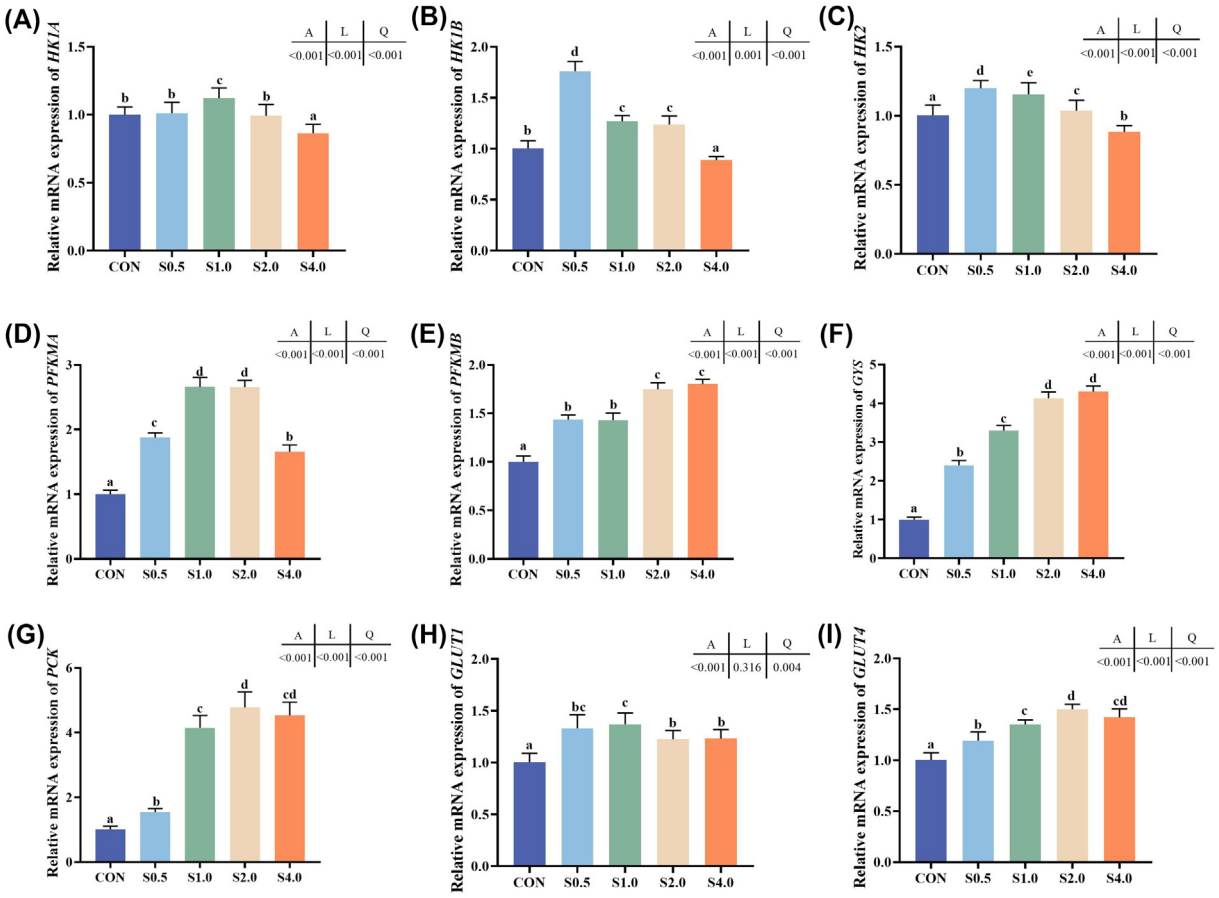

Fig. 6. Effects of dietary supplementation with succinic acid levels on mRNA expression levels of genes related to glucose metabolism and transport of Nile tilapia. CON: high-carbohydrate diet (HCD); S0.5: the HCD was supplemented with 0.5% succinic acid; S1.0: the HCD was supplemented with 1.0% succinic acid; S2.0: the HCD was supplemented with 1.5% succinic acid; S4.0: the HCD was supplemented with 2.0% succinic acid. All values are means ± SD ($n = 6$). A: *P*-value of one-way analysis of variance; L: *P*-value of linear trend analyzed by orthogonal polynomial contrasts; Q: *P*-value of quadratic trend analyzed by orthogonal polynomial contrasts. *HK1A* = hexokinase 1A; *HK1B* = hexokinase 1B; *HK2* = hexokinase 2; *PFKMA* = phosphofructokinase, muscle-A; *PFKMB* = phosphofructokinase, muscle-B; *GYS* = glycogen synthase; *PCK* = phosphoenolpyruvate carboxykinase; *GLUT1* = glucose transporter 1; *GLUT4* = glucose transporter 4. [a-d] Bars with different letters are significantly different at $P < 0.05$.

pathways related to protein synthesis (Liang, 2018). Protein is composed of amino acids, and EAA content is an important indicator of nutritional value in muscle. Studies have shown that TCA cycle intermediate metabolites are precursors for the synthesis of some amino acids. For example, oxaloacetate is a precursor for the synthesis of aspartate (Ling et al., 2023). Exogenous addition of α-ketoglutaric acid significantly increased the contents of amino acids such as Arg, Ala, Gly, and Glu in cells (Mühling et al., 2010). In the current study, dietary supplementation with 0.5% to 4.0% succinic acid significantly increased the contents of muscle ΣEAA and TAA, suggesting that succinic acid might promote the synthesis of bound amino acids and increase protein content by increasing the concentration of TCA cycle intermediate metabolites, thereby improving the nutritional value of tilapia muscle.

Muscle fatty acid contents and composition are important for human health and are important indicators of the nutritional value of muscle (Wood et al., 2008). According to the World Health Organization (WHO) report, reducing the intake of SFA and increasing the intake of unsaturated fatty acids (especially n-3PUFA and n-6PUFA) in the human diet effectively prevents chronic disease (Diet nutrition and the prevention of chronic diseases, 2003). The human body cannot synthesize EPA and DHA, so fish is the preferred source of high-quality PUFA for consumers. Studies have shown that dietary supplementation with 1.2% citrate significantly increases PUFA content in muscle (Xu et al., 2020). In addition, unsaturated fatty acids (especially n-3PUFA) are prone to lipid peroxidation, which reduces the content of PUFA in the body (Kamal-Eldin and Yanishlieva, 2002). In the current study, fish fed with 1.0% succinic acid had higher n-3PUFA content and lower total fatty acid content, suggesting that succinic acid changes the composition of fatty acids and increases the nutritional value of tilapia muscle by reducing lipid peroxidation, which was also confirmed by the significant reduction of MDA content in muscle.

Free amino acids are the main basis of muscle flavor in aquatic animals, including umami and bitterness (Herranz et al., 2006). Among the 14 free amino acids detected in this study, Asp and Glu were related to umami, Ala and Gly were related to sweetness, and Val, Met, Ile, Leu, His, and Arg were associated with bitterness. The delicious taste of fish depends on the composition and contents of free amino acids in muscle, especially the contents of free Asp, Glu, Gly, and Ala (Cheng, 2021). Studies have shown that dietary supplementation with 1.5% α-ketoglutaric acid significantly increased the contents of Thr, Ala, and Pro, as well as TAA and EAA, in the muscle of mirror carp (Wang et al., 2017). Furthermore, supplementation with 1.0% α-ketoglutaric acid significantly increased the contents of Glu, Lys, Ala, and Pro in the skeletal muscle of growing pigs (Chen et al., 2018). Succinic acid is a natural flavor enhancer that increases muscle umami (Subbaraj et al., 2016). In the present research, dietary supplementation with 1.0% succinic acid increased the contents of ΣFAA (Asp and Glu) and sweet amino acids (Ala and

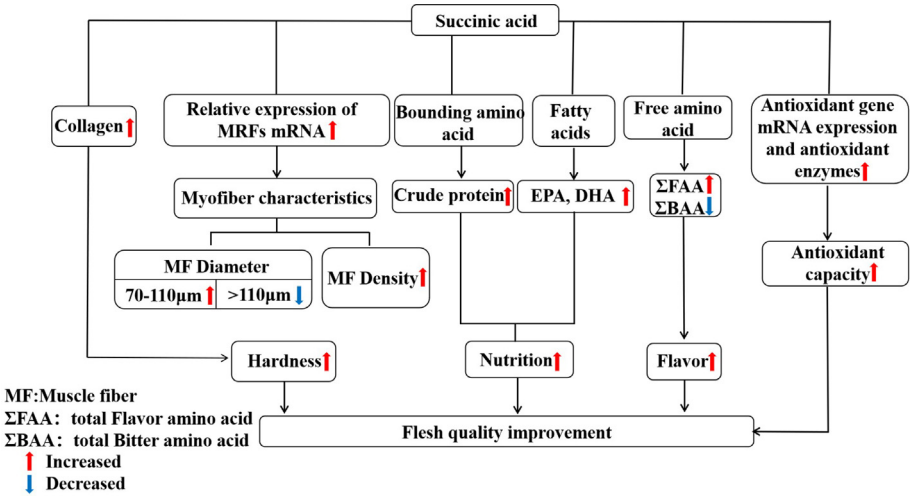

Fig. 7. Summary figure of dietary supplementation with succinic acid levels affecting the flesh quality of Nile tilapia. Optimal succinic acid level ultimately improves the flesh quality of Nile tilapia by increasing muscle hardness, nutritional value, flavor, and decreasing oxidative stress. MRF = myogenic regulatory factors; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; ΣFAA = total flavor amino acid; ΣBAA = total bitter amino acid; MF = muscle fiber.

Gly) and decreased the ΣBAA (Val, Met, Ile, Leu, His, and Arg). Therefore, succinic acid might increase the flavor of muscle by increasing the content of flavor amino acids.

4.5. Succinic acid supplementation in HCD affects the glucose and lipid metabolism of tilapia muscle

Gluconeogenesis and glycolysis are two crucial metabolic processes for carbohydrate metabolism. Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) are three key enzymes in the glycolytic pathway, and phosphoenolpyruvate carboxykinase (PCK), fructose-1, 6-diphosphatase (FBPase) and glucose-6-phosphatase (G-6-pase) are the rate-limiting enzymes of gluconeogenesis (Enes et al., 2009; Polakof et al., 2011). Studies have shown that succinate significantly reduced liver G6Pase activity by regulating intestinal gluconeogenesis in mice, thereby reducing liver glucose production (De Vadder et al., 2016). In the current study, dietary supplementation with 1.0% succinic acid up-regulated the mRNA expression levels of glycolytic genes (*HK2*, *HK1A*, *HK1B*, *PFKMA* and *PFKMB*), indicating that succinic acid might promote glycolysis to accommodate HCD. In addition, excessive carbohydrates were converted into glycogen, which was stored in the muscles and liver to maintain blood glucose balance (Enes et al., 2009). In the current study, dietary supplementation with 1.0% succinic acid increased the contents of liver and muscle glycogen and significantly up-regulated the expression levels of glycogen synthesis (*GYS*) and glucose transporters (*GLUT1* and *GLUT4*) genes, which was consistent with the results of previous studies in zebrafish (Ding et al., 2022). Therefore, succinic acid might promote glycolysis and glycogen deposition to maintain glucose homeostasis.

Long-term feeding of HCD might result in metabolic problems that cause fat storage and impair the growth performance of fish (Prisingkorn et al., 2017; Viegas et al., 2016). Thus, lipid anabolism plays a crucial role in maintaining glucose homeostasis. Fatty acid synthase is a key enzyme involved in lipid synthesis. Carnitine palmitoyltransferase-1 is considered to be the rate-limiting enzyme in fatty acid β-oxidation (Morash et al., 2008). Hormone sensitive lipase is an intracellular neutral lipase that hydrolyzes lipids such as triglycerides and cholesterol esters (Lampidonis et al., 2011). In this study, dietary supplementation with 0.5% succinic acid reduced the crude lipid content of the whole fish and liver, MFI, and muscle total fatty acid content, down-regulated the expression of the lipid synthesis gene (*FASN*), and up-regulated the expression of lipid catabolic genes (*CTP1B*, *HSL*, *LPL*), indicating that succinic acid might induce a change in muscle fatty acid content by promoting fatty acid decomposition and β-oxidation and inhibiting fatty acid synthesis. Furthermore, dietary supplementation with 0.5% to 2.0% succinic acid significantly reduced the HSI and VSI of tilapia, confirming that succinic acid reduces abnormal fat deposition, thereby increasing the proportion of edible parts (muscle). In addition, the contents of serum TG and TCHO reflected the status of lipid metabolism (Zhang et al., 2021). In this study, fish fed 1.0% to 4.0% succinic acid had lower contents of serum TG and TCHO. These results further confirm the promoting effect of succinic acid on lipid catabolism. Therefore, succinic acid might reduce muscle total fatty acid content by promoting fatty acid β-oxidation and inhibiting the fatty acid synthesis process.

5. Conclusion

Dietary supplementation with 1.83% to 2.43% succinic acid promoted the growth performance, digestion, absorption, and antioxidant capacity of Nile tilapia. Additionally, succinic acid changed the proportion of muscle fibers by up-regulating muscle regulatory factor genes to increase muscle hardness and altering muscle amino acid and fatty acid composition to improve muscle flavor and nutritional value (Fig. 7). Succinic acid also promoted glycolysis and fatty acid β-oxidation, inhibited lipogenesis, decreased the accumulation of fat in the whole-body, and maintained glucose homeostasis.

Author contributions

Juan Tian, Hua Wen: Conceptualization, Funding acquisition, Resources. **Manxia Cao**, **Ningning Xie**, **Jianmin Zhang**, **Lixue Dong**, **Ming Jiang**, **Feng Huang**, **Xing Lu:** Formal analysis, Investigation, Visualization. **Manxia Cao**, **Ningning Xie**, **Juan Tian:** Software, Methodology, Writing - Original draft, Writing - Reviewing & Editing. All the authors have read and approved the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately

influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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