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Effects of three active components in *Eucommia ulmoides* on growth and flesh quality of grass carp (*Ctenopharyngodon idellus*) based on transcriptomics

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Abstract

The study investigated the effects of three active components in *Eucommia ulmoides* on growth, flesh quality and muscle transcriptomics of grass carp (*Ctenopharyngodon idella*). Quercetin (QC), chlorogenic acid (CGA) and geniposide (GP) were individually supplemented into basal diet (control) at 400 mg/kg, and then, the four diets were fed to grass carp (18.50 ± 0.40 g) for 60 days. The results indicated that dietary CGA rather than QC and GP significantly improved growth performance. Compared to the control, dietary QC decreased the flesh centrifugal loss and thawing loss, and increased hardness and free amino acid level ($p < .05$), while dietary CGA only increased the free amino acids in flesh ($p < .05$). Dietary QC and CGA also increased the n-3 polyunsaturated fatty acid level and n-3/n-6 ratio in flesh ($p < .05$). Transcriptome profiling of muscle, the changes of genes and pathways in the QC group are speculated to be involved in energy production and myofibril composition and contraction, which may affect the flesh quality. The changes of fatty acid composition and free amino acid content induced by dietary QC and CGA may be realized by lipid and energy metabolism. In conclusion, dietary CGA improved the growth and flesh nutritional value, and QC improved the flesh quality of grass carp.

KEYWORDS

chlorogenic acid | flesh quality | geniposide | grass carp | growth | quercetin | transcriptomics,

1 | INTRODUCTION

Grass carp (*Ctenopharyngodon idellus*) is an important cultured freshwater fish with the highest production of 5.52 million tons in 2017, accounting for 6.89% of the finfish aquaculture in the world (FAO, 2019). With the increase in intensive aquaculture, the flesh quality of grass carp showed a declining trend, which has become a growing public concern. In the future, the production increase will not be the primary goal in aquaculture, while obtaining high-quality fish products based on a sustainable aquaculture is becoming an important task for the industry (Kobayashi et al., 2015). As a traditional herb widely used in East Asia, *Eucommia ulmoides* (EU) has been found many functions including enhancing bones' strength, antioxidant capacity and immunity and reducing body fattiness (Hussain et al., 2016). In recent years, EU has been used as feed additive in pig (Lee et al., 2009; Zou, Xiang, Wang, Wei, & Peng, 2016), chicken (Hu,

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Wang, Zhou, & Liu, 2006; Wang, Du, Ye, Tao, & Wang, 2012), lamb (Liu, Li, Zhao, & Deng, 2018; Liu, Zhao, Li, & Deng, 2018) and fish (Sun et al., 2017a; Sun, Xu, Li, Pan, & Leng, 2018; Wang et al., 2018), and the improved growth performance and flesh quality were observed in these studies.

There are many active components in EU, and they could be classified into several groups according to the structural characteristics, including phenylpropanoids (chlorogenic acid and caffeic acid), flavonoids (quercetin and rutin), iridoids (geniposidic acid and geniposide) and others (He et al., 2014). Quercetin (QC) belongs to the subclass of flavonols and exists in many fruits and vegetables, and it has been demonstrated to be an excellent antioxidant (Boots, Haenen, & Bast, 2008). Studies have shown that the supplementation of QC improved the growth and flesh quality of pig (Zou et al., 2016), chicken (Goliomytis et al., 2014) and lamb (Andrés et al., 2014). Chlorogenic acid (CGA) is an ester of caffeic acid and quinic acid, which is a polyphenol derivative widely existing in EU, fruits and teas. It is well known for its functions of antioxidant, anti-inflammatory and anticancer (Meng, Cao, Feng, Peng, & Hu, 2013). In addition, dietary CGA was reported to improve the growth and flesh quality of fish (Sun et al., 2019), shrimp, *Penaeus vannamei* (Wang et al., 2015), and turtle, *Trionyx sinensis* (Wen, Shu, & Xiao, 2010). Geniposide (GP) is a water-soluble iridoid glycoside, which has been reported to have antioxidant, anti-inflammatory and hypolipidaemic effects in rats (Fu et al., 2012; Liao et al., 2014). Sun et al. (2017b) found that dietary GP improved the lipid metabolism and flesh quality in grass carp, which is the only study reported in fishes.

The development of high-throughput sequencing technologies has realized the molecular investigation of species with complex genomes. Among these technologies, the RNA sequencing (RNA-Seq) can rapidly and comprehensively investigate the transcriptome level of variations and provide valuable information about gene function, cell responses and evolution (Wang, Gerstein, & Snyder, 2009). With the popularization of this technology and the reduction of cost, RNA-Seq has been used in various aspects of aquatic animals such as nutrition, immunization and genetic breeding. In large yellow croaker (*Larimichthys crocea*), Wei et al. (2018) analysed the effects of dietary hydroxyproline on the flesh quality by transcriptomics. In grass carp, transcriptomics was also used to evaluate the effects of stocking density on muscle structure and lipid metabolism (Zhao et al., 2019).

As many active components contained in EU, what active components are involved in the growth-promoting and flesh quality-improving effects of EU? How about the acting mechanism of these active components? Therefore, three active components of EU including chlorogenic acid (phenylpropanoids), quercetin (flavonoids) and geniposide (iridoids) were selected based on the previous studies to investigate their effects on growth and flesh quality of grass carp by transcriptomics, biochemical analysis and flesh quality evaluation. These results would provide theoretical basis for explaining the acting mechanism of EU and guide the green aquaculture in the future.

2 | MATERIALS AND METHODS

2.1 | Experimental design and diets

Four diets were designed with the supplementation of QC, CGA and GP in basal diet (control) at an inclusion of 400 mg/kg, respectively. CGA and GP were supplied by ChengDu ConBon Bio-Tech Co., LTD with purity higher than 98%, and QC was purchased from ChengDu Desite Bio-Tech Co., LTD with purity higher than 95%. The supplemental level of QC, CGA and GP referred to the studies of Zhai and Liu. (2013), Sun et al. (2017a) and Sun et al. (2017b), respectively. All ingredients were ground with a grinder and sifted using a 40-μm sieve, and then weighed and mixed to form a homogenous mixture. Soybean oil and deionized water (about 300 g/kg mixture) were then added into the mixture and thoroughly mixed to achieve a homogeneity for pelleting with an extruder (SLP45; Chinese Fishery Machinery and Instrument Research Institute). The pelleting temperature was 90–95°C. All diets were air-dried and stored at 4°C until use. The diet formula and proximate composition are presented in Table 1.

2.2 | Fish and feeding management

Grass carp were obtained from Jinshan Aquaculture Farm, Shanghai, China. A total of 240 grass carp with an initial body weight of 18.50 ± 0.40g were randomly allocated into 12 cages ($1.5 \times 1.0 \times 1.2$ m) with 20 fish per cage. The cages were hung in indoor cement pools without direct sunshine. The feeding trial was conducted at Binhai Aquaculture Station of Shanghai Ocean University (Shanghai, China) and lasted for 60 days. During the feeding period, the fish were fed with one of the four diets three times daily (8:00, 13:00 and 18:00) with a daily feeding rate of 30–50 g/kg of body weight. The feed intake was adjusted appropriately according to the feeding behaviour and water temperature to ensure no feed residue left after feeding (in 10 min). All cages were maintained with a similar amount of feed intake. About one third of the cultured water was renewed by pond water after filtration and dark sedimentation, and the waste in the cement pools was cleared by siphoning every 5 days. Water temperature, pH, dissolved oxygen and ammonia nitrogen levels were 27–32, 7.5–8.0, >5mg/L and < 0.2mg/L, respectively. All the procedures were strictly carried out in accordance with the Regulations of the Experimental Animal Ethics Committee of Shanghai Ocean University and in compliance with regulations by the Institutional Animal Care and Use Committee.

2.3 | Sample collection and proximate composition analysis

At the end of the feeding trial, all fish were deprived of diets for 24 hr, then anaesthetized with MS-222 (30 mg/L) and measured body weight to calculate weight gain (WG) and feed conversion ratio (FCR). Three fish per cage were selected randomly to individually measure body weight, body length, liver weight and visceral weight; then,

TABLE 1 Ingredients and proximate composition of the experimental diets (g/kg diet)

	Experimental diets			
Ingredients[a]	Control	QC	CGA	GP
Fish meal	20.0	20.0	20.0	20.0
Soybean meal	180.0	180.0	180.0	180.0
Cottonseed meal	160.0	160.0	160.0	160.0
Rapeseed meal	180.0	180.0	180.0	180.0
Wheat bran	100.0	100.0	100.0	100.0
Rice bran	100.0	100.0	100.0	100.0
Wheat middling	224.5	224.1	224.1	224.1
Soybean oil	10.0	10.0	10.0	10.0
Choline chloride (500 g/kg)	5.0	5.0	5.0	5.0
Vitamin premix[b]	2.5	2.5	2.5	2.5
Mineral premix[c]	3.0	3.0	3.0	3.0
Monocalcium phosphate	15.0	15.0	15.0	15.0
Quercetin	0.0	0.4	0.0	0.0
Chlorogenic acid	0.0	0.0	0.4	0.0
Geniposide	0.0	0.0	0.0	0.4
Total	1,000.0	1,000.0	1,000.0	1,000.0
Proximate composition				
Crude protein	325.4	325.3	325.1	325.5
Crude lipid	56.1	55.8	56.2	55.7
Ash	66.7	66.5	66.4	67.3
Moisture	105.1	106.1	105.8	104.5

Abbreviations: CGA, chlorogenic acid; GP, geniposide; QC, quercetin.

[a]The ingredients were purchased from the Yuehai Feed Company, and the protein contents of ingredients are as follow: fish meal (630.0 g/kg), soybean meal (442.0 g/kg), cottonseed meal (500.0 g/kg), rapeseed meal (377.0 g/kg), wheat middling (169.0 g/kg) and rice bran (143.0 g/kg).

[b]Vitamin premix (mg or IU/kg diet): VA, 10,000 IU; VD_3, 3,000 IU; VE, 150 IU; VK_3, 12.17 mg; VB_1, 20 mg; VB_2, 20 mg; VB_3, 100 mg; VB_6, 22 mg; VB_{12}, 0.15 mg; VC, 1,000 mg; biotin, 0.6 mg; folic acid, 8 mg; inositol, 500 mg.

[c]Mineral premix (mg/kg diet): I, 1.5 mg; Co, 0.6 mg; Cu, 3 mg; Fe, 63 mg; Zn, 89 mg; Mn, 11.45 mg; Se, 0.24 mg; Mg, 180 mg.

condition factor (CF), hepatosomatic index (HSI) and viscerosomatic index (VSI) were calculated by the methods of Sun et al. (2019).

The dorsal muscle from the left side of the body of the three fish was sampled for determining water-holding capacity (WHC) immediately referring to the study of Sun et al. (2018). A block of flesh (0.8 cm × 0.8 cm × 0.8 cm) from dorsal muscle on the right side of body was used to perform the texture profiles analysis (TPA) using a Universal TA device (Tengba Company). Test conditions were as follows: a 25 mm × 25 mm flat-bottomed cylindrical probe, a test speed of 1 mm/s and a post-test speed of 2 mm/s with the stay time of 2 s.

The rest of dorsal muscle was stored at −80°C for the analysis of crude protein, lipid, ash, moisture, free amino acids and fatty acid. The proximate composition was analysed according to AOAC. The crude protein content was determined using the Kjeldahl system method (2300 Auto analyser; FOSS Tecator, AB). The crude lipid content was estimated after ether extraction using SOXTHERM (SOX 416 Macro). The ash content was estimated by combusting samples in a muffle furnace at 550°C for 6 hr. The moisture content was determined by drying the samples to a constant weight at 105°C in a drying oven.

For determination of free amino acid in muscle, samples were homogenized with 30 volumes of extract liquid (methanol:water = 4:1) and centrifuged at 12,900 *g* and 4°C for 30 min. The supernatants were analysed using Ultra Performance Liquid Chromatography, UPLC (Waters ACQUITY, USA). The fatty acid composition of the muscle was determined with boron trifluoride method according to the description of Zou et al. (2012) with GC-MS (7890B gas chromatograph–mass spectrometer; Agilent Technologies). For the transcriptomic assay, about 0.2 g dorsal muscle from the left side was collected into sterile tubes and frozen in liquid N_2, and then stored at −80°C until use. The muscle of three fish per cage was pooled as one sample with a total of 12 samples when performing the transcriptomic assay.

2.4 | Transcriptomic assay

Total RNA was extracted from the tissue using TRIzol® reagent according to the manufacturer's instructions (Invitrogen), and genomic DNA was removed using DNase I (Takara). Then, RNA quality was determined by 2100 Bioanalyzer (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). RNA-Seq transcriptome library was prepared following TruSeq™ RNA Sample Preparation Kit from Illumina using 5 μg of total RNA, and paired-end RNA-Seq sequencing library was sequenced with the Illumina HiSeq 4000 (2 × 150 bp read length). The raw paired-end reads were trimmed, and quality was controlled by SeqPrep and Sickle with default parameters. Then, clean reads were separately aligned to reference genome with orientation mode using TopHat software.

To identify differentially expressed genes (DEGs) between the control and treatment groups, the expression level of each transcript was calculated according to the FPKM (fragments per kilobase of exon per million mapped reads) method. RSEM (RNA-Seq by Expectation-Maximization) was used to quantify gene abundances. R statistical package software edgeR was utilized for differential expression analysis. In addition, functional enrichment analysis includes the GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway. KEGG and GO were significantly enriched at Bonferroni-corrected *p*-value ≤ .05. GO functional enrichment and KEGG pathway analysis were carried out by Blast2GO and KOBAS software.

2.5 | Statistical analysis

All data are presented as mean ± *SD* (standard deviation). Statistical analysis was performed using SPSS 22.0 statistical software. All data were subjected to one-way ANOVA. If significant differences were detected, the Duncan multiple range test was used for determining

TABLE 2 Growth performance of grass carp fed diets with three active components for 60 days

Parameters	Control	QC	CGA	GP
IBW/g	18.50 ± 0.50	18.50 ± 0.40	18.50 ± 0.50	18.50 ± 0.30
FBW/g	82.97 ± 1.69^{b}	82.85 ± 2.10^{b}	86.57 ± 1.42^{a}	81.68 ± 0.48^{b}
FCR	1.56 ± 0.05^{a}	1.56 ± 0.05^{a}	1.47 ± 0.03^{b}	1.57 ± 0.02^{a}
WG/%	348.53 ± 9.11^{b}	345.55 ± 2.73^{b}	367.91 ± 7.76^{a}	347.93 ± 11.33^{b}
CF/(g/cm^3)	1.83 ± 0.04	1.88 ± 0.06	1.86 ± 0.04	1.85 ± 0.02
HSI/%	2.17 ± 0.17	2.08 ± 0.14	2.07 ± 0.20	2.00 ± 0.13
VSI/%	8.18 ± 0.48	7.99 ± 0.55	8.03 ± 0.66	8.16 ± 0.18
SR/%	100.00	100.00	100.00	100.00

Note: Values in the same row with different superscript alphabets indicate significant differences ($p < .05$).

Abbreviations: CF, condition factor (g/cm^3); FBW, final body weight (g); FCR, feed conversion ratio; HSI, hepatosomatic index (%); IBW, initial body weight (g); SR, survival rate (%); VSI, viscerosomatic index (%); WG, weight gain (%).

TABLE 3 Proximate composition in muscle of grass carp fed diets with three active components for 60 days (g/kg, wet weight)

Parameters	Control	QC	CGA	GP
Moisture	785.8 ± 4.8	780.5 ± 4.7	780.2 ± 4.8	781.1 ± 4.1
Crude protein	190.1 ± 2.7	192.2 ± 2.9	192.1 ± 3.3	189.6 ± 3.1
Crude lipid	16.4 ± 0.1	15.7 ± 1.3	15.5 ± 0.9	17.0 ± 0.8
Crude ash	12.2 ± 0.3	12.0 ± 0.2	12.1 ± 0.1	12.1 ± 0.4

Note: Values in the same row with different superscript alphabets indicate significant differences ($p < .05$).

the statistical significance among groups. Mean values were considered significantly different if the p value was <.05.

3 | RESULTS

3.1 | Growth performance and physical indices

Compared to the control, WG was increased by 5.56% ($p < .05$), and FCR was decreased by 0.09 ($p < .05$) by the supplementation of CGA in diet, but the growth was not significantly affected by dietary QC and GP ($p > .05$). HSI, VSI, CF and SR showed no significant differences among all the treatments ($p > .05$) (Table 2).

3.2 | Muscle composition

As shown in Table 3, there were no significant differences in the contents of muscle moisture, crude lipid, crude protein and crude ash among all the groups ($p > .05$).

3.3 | Water-holding capacity and texture characteristics of flesh

Dietary QC significantly decreased the thawing loss and centrifugal loss, and increased the hardness of flesh ($p < .05$). The other two groups showed no significant differences ($p > .05$) with the control group (Table 4).

3.4 | Free amino acids in muscle

In Table 5, a total of 19 free amino acids were detected in the muscle, and Ala had the highest level. The supplementation of QC and CGA significantly increased the contents of Gln, Arg, His, Lys, non-essential amino acids (NEAAs), essential amino acids (EAAs) and total free amino acids (TFAAs). In addition, Glu, Ala and delicious amino acids (DAAs) in the QC group, and Pro and Ser in the CGA group were significantly higher ($p < .05$), while the Asp in the CGA group was significantly lower than that of the control ($p < .05$).

3.5 | Fatty acid composition in muscle

As shown in Table 6, there were no significant differences in saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) among all the treatments ($p > .05$), but the supplementation of QC and CGA significantly increased C18:3, C22:6, n-3 PUFAs and n-3/n-6 PUFA ratio in flesh ($p < .05$).

3.6 | Functional enrichment analyses of DEGs

The volcano plots showed the significant differences in muscle transcriptomes induced by the supplementation of three active components (Figure S1). Compared to the control, a total of 318, 199 and 119 up-regulated DEGs (FC > 2) and 181, 105 and 141

TABLE 4 The flesh water-holding capacity and texture characteristics of grass carp fed diets with three active components for 60 days

Parameters	Control	QC	CGA	GP
Steaming loss/%	28.59 ± 0.55	28.62 ± 0.26	28.19 ± 0.51	28.71 ± 0.58
Thawing loss/%	6.89 ± 0.38^{a}	6.09 ± 0.58^{b}	6.34 ± 0.44^{a}	6.79 ± 0.36^{a}
Centrifugal loss/%	19.60 ± 0.97^{a}	16.19 ± 0.43^{b}	18.68 ± 1.16^{a}	19.03 ± 1.66^{a}
Hardness/gf	337.60 ± 31.84^{b}	372.67 ± 15.01^{a}	350.00 ± 18.00 ^{ab}	340.00 ± 35.30^{b}
Springiness	0.55 ± 0.04	0.59 ± 0.05	0.59 ± 0.03	0.58 ± 0.03
Chewiness/gf	120.28 ± 18.17	127.67 ± 6.51	124.45 ± 4.47	122.38 ± 2.23
Cohesiveness/gf	0.60 ± 0.06	0.61 ± 0.03	0.61 ± 0.03	0.61 ± 0.05
Resilience	1.02 ± 0.05	1.05 ± 0.10	1.05 ± 0.10	1.02 ± 0.06

Note: Values in the same row with different superscript alphabets indicate significant differences ($p < .05$).

TABLE 5 Muscle free amino acid of grass carp fed diets with three active components for 60 days (mg/100 g, wet weight)

Parameters	Control	QC	CGA	GP
Glu	2.25 ± 0.20^{a}	3.36 ± 0.23^{b}	2.39 ± 0.32^{a}	2.30 ± 0.13^{a}
Ala	28.55 ± 2.56^{a}	34.08 ± 0.51^{b}	30.29 ± 2.04^{ab}	27.54 ± 1.98^{a}
Asp	1.50 ± 0.09^{b}	1.57 ± 0.16^{b}	1.16 ± 0.20^{a}	1.33 ± 0.07^{ab}
Gln	21.25 ± 1.16^{a}	26.87 ± 0.99^{b}	26.90 ± 0.77^{b}	22.99 ± 0.41^{a}
Arg	5.17 ± 0.18^{a}	6.45 ± 0.30^{b}	6.68 ± 0.16^{b}	5.42 ± 0.44^{a}
His	17.48 ± 0.06^{a}	19.38 ± 0.23^{b}	19.05 ± 0.56^{b}	17.49 ± 0.61^{a}
Asn	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
Pro	6.93 ± 0.79^{a}	7.83 ± 0.70^{ab}	8.76 ± 0.90^{b}	7.73 ± 0.81^{ab}
Ser	7.02 ± 0.42^{a}	7.75 ± 0.22^{ab}	8.86 ± 0.52^{b}	7.85 ± 1.41^{ab}
Tyr	2.09 ± 0.07	2.1 ± 0.02	2.02 ± 0.26	2.15 ± 0.28
Cys	0.19 ± 0.03	0.19 ± 0.02	0.18 ± 0.01	0.18 ± 0.04
Trp	2.85 ± 0.49^{ab}	2.72 ± 0.44^{ab}	2.26 ± 0.24^{a}	3.14 ± 0.19^{b}
Met	0.38 ± 0.07	0.46 ± 0.05	0.48 ± 0.02	0.45 ± 0.07
Phe	2.40 ± 0.17^{b}	2.66 ± 0.14	2.62 ± 0.11	2.64 ± 0.08
Lys	19.84 ± 0.62^{a}	24.94 ± 0.29^{b}	24.53 ± 2.49^{b}	20.36 ± 1.11^{a}
Thr	5.48 ± 0.74	6.15 ± 0.16	5.69 ± 0.33	5.83 ± 0.42
Val	0.38 ± 0.01	0.39 ± 0.03	0.37 ± 0.01	0.41 ± 0.03
Leu	0.37 ± 0.03	0.36 ± 0.07	0.36 ± 0.03	0.39 ± 0.05
Ile	0.41 ± 0.17	0.49 ± 0.01	0.52 ± 0.08	0.48 ± 0.06
DAAs	32.29 ± 2.73^{a}	39.01 ± 0.85^{b}	33.85 ± 2.16^{a}	31.20 ± 3.00^{a}
EAAs	32.12 ± 1.89^{a}	38.17 ± 0.07^{b}	36.83 ± 2.21^{b}	33.70 ± 1.18^{a}
NEAAs	90.17 ± 3.65^{a}	107.32 ± 1.87^{b}	104.13 ± 3.59^{b}	92.68 ± 3.79^{a}
TFAAs	124.57 ± 4.69^{a}	147.78 ± 1.77^{b}	143.15 ± 5.67^{b}	126.38 ± 4.55^{a}

Note: Values in the same row with different superscript alphabets indicate significant differences ($p < .05$).

Abbreviations: DAAs, delicious amino acids (Gly was not tested in this analysis); EAAs, essential amino acids; NEAAs, non-essential amino acids; TFAAs, total free amino acids.

down-regulated (FC < 0.5) DEGs were identified in the QC, CGA and GP groups, respectively.

To find out the function and analyse the potential biological pathways, GO enrichment and KEGG pathway enrichment were performed for the DEGs. In general, GO enrichment displayed that 23 GO terms are significantly enriched ($p < .05$) in the QC group (Figure 1), but there are no significant enrichment GO terms in the CGA and GP groups. As shown in Figure 1, most of GO terms were related to muscle movement and myofilament structure, such as motor activity, myosin complex, cytoskeletal part, actin binding and troponin complex, and the other GO terms were involved in muscle energy metabolism. The results of KEGG showed that there were 37, 4 and 5 KEGG pathways significantly enriched in QC, CGA and GP ($p < .05$), respectively (Table 7). The majority of 37 pathways in the

TABLE 6 Muscle fatty acid composition of grass carp fed diets with three active components for 60 days (percentage of fatty acids, %)

Parameters	Control	QC	CGA	GP
C14:0	0.72 ± 0.04	0.75 ± 0.02	0.75 ± 0.04	0.73 ± 0.02
C16:0	19.11 ± 0.08	19.03 ± 0.24	19.10 ± 0.14	19.33 ± 0.17
C18:0	6.49 ± 0.21	6.27 ± 0.18	6.23 ± 0.21	6.36 ± 0.29
C22:0	0.67 ± 0.02	0.64 ± 0.02	0.64 ± 0.03	0.65 ± 0.05
SFAs	26.98 ± 0.12	26.68 ± 0.58	26.72 ± 0.32	27.06 ± 0.49
C16:1	2.60 ± 0.36	2.52 ± 0.19	2.64 ± 0.17	2.69 ± 0.34
C18:1	33.48 ± 1.18	33.65 ± 1.38	34.42 ± 1.56	34.77 ± 1.32
MUFAs	36.08 ± 1.52	36.17 ± 1.42	37.07 ± 1.72	37.4 5 ± 1.00
C18:2	21.48 ± 0.28	20.85 ± 0.30	20.82 ± 0.57	20.88 ± 0.50
C20:2	0.99 ± 0.06	0.95 ± 0.05	0.96 ± 0.01	0.94 ± 0.02
C20:3	1.52 ± 0.01	1.46 ± 0.03	1.42 ± 0.03	1.49 ± 0.01
C20:4	6.42 ± 0.15	5.80 ± 0.25	5.50 ± 0.07	6.43 ± 0.10
n−6 PUFAs	30.42 ± 0.39	29.06 ± 0.12	28.70 ± 0.48	29.74 ± 0.09
C18:3	1.73 ± 0.03^{a}	1.86 ± 0.03^{b}	1.86 ± 0.06^{b}	1.79 ± 0.08^{ab}
C20:5	0.47 ± 0.02	0.51 ± 0.03	0.50 ± 0.06	0.48 ± 0.02
C22:6	3.97 ± 0.13^{a}	4.24 ± 0.02^{b}	4.22 ± 0.17^{b}	3.88 ± 0.18^{a}
n−3 PUFAs	6.17 ± 0.16^{a}	6.62 ± 0.20^{b}	6.58 ± 0.22^{b}	6.14 ± 0.21^{a}
PUFAs	36.59 ± 1.17	35.68 ± 1.13	35.28 ± 1.36	35.88 ± 1.23
n−3/n−6	0.20 ± 0.01^{a}	0.23 ± 0.01^{b}	0.23 ± 0.02^{b}	0.21 ± 0.01^{ab}

Note: Values in the same row with different superscript alphabets indicate significant differences ($p < .05$).

SFAs, saturated fatty acids; MUFAs, monounsaturated saturated fatty acids; PUFAs, polyunsaturated fatty acids.

QC group were involved in muscle contraction, lipid metabolism, immunity and antioxidation. Three of four pathways in the CGA group were related to lipid metabolism, and four of five pathways in the GP group were related to immunity.

4 | DISCUSSION

4.1 | Effects of EU active compounds on the growth performance

In the present study, the supplementation of CGA increased WG and decreased FCR of grass carp ($p < .05$). The similar findings have been reported in our previous studies for this fish (Li et al., 2014; Sun et al., 2019). In addition, dietary CGA was also found to improve the growth of turtle (Wen et al., 2010), pig (Chen et al., 2018) and rat (Ruan et al., 2014). But in another study on grass carp (Sun et al., 2017a), the fish with a body weight of 95.1 g did not show the positive effect of CGA on the growth. Obviously, even in the same fish of grass carp, dietary CGA also showed various growth responses. It seems that growing period is an important factor, and the younger and smaller fish may be more sensitive to dietary CGA. Moreover, the inconsistent results were probably due to the differences in diet composition, environment, feeding period, etc. Polyphenols can selectively inhibit intestinal pathogenic bacteria, promote the growth of beneficial bacteria and balance the intestinal flora composition, thereby increasing the intestinal absorption of nutrients (Hidalgo et al., 2012; Parkar, Stevenson, & Skinner, 2008). The metabolism of CGA mainly occurs in the intestine, which may optimize the composition of the intestinal flora (Kang et al., 2004) and provide more nutrients for the growth. In the future, it is necessary to enhance the research of CGA on the intestinal microbes of grass carp.

Studies in chicken (Goliomytis et al., 2014; Simitzis, Spanou, Glastra, & Goliomytis, 2018), rabbit (North, Dalle, & Hoffman, 2018) and rat (Hollinger, Shanely, Quindry, & Selsby, 2015) showed that dietary QC did not significantly improve the growth of these animals. In the previous study, the supplementation of GP in diet did not affect the growth of grass carp (Sun et al., 2017b). The same result was found in the present study, and no growth improvement was observed in the QC and GP groups. Therefore, QC and GP may not be the growth-promoting components in EU.

4.2 | Effects of QC on the flesh quality

The physical properties of the muscle include pH, water-holding capacity (WHC) and texture characteristics such as hardness, springiness and chewiness. WHC refers to the ability of flesh to retain moisture, and it is possibly influenced by pH (Theofania, Dimitra, & Petros, 2020). Hardness is considered as the maximum resistance of flesh to the penetration, and springiness is related to the height

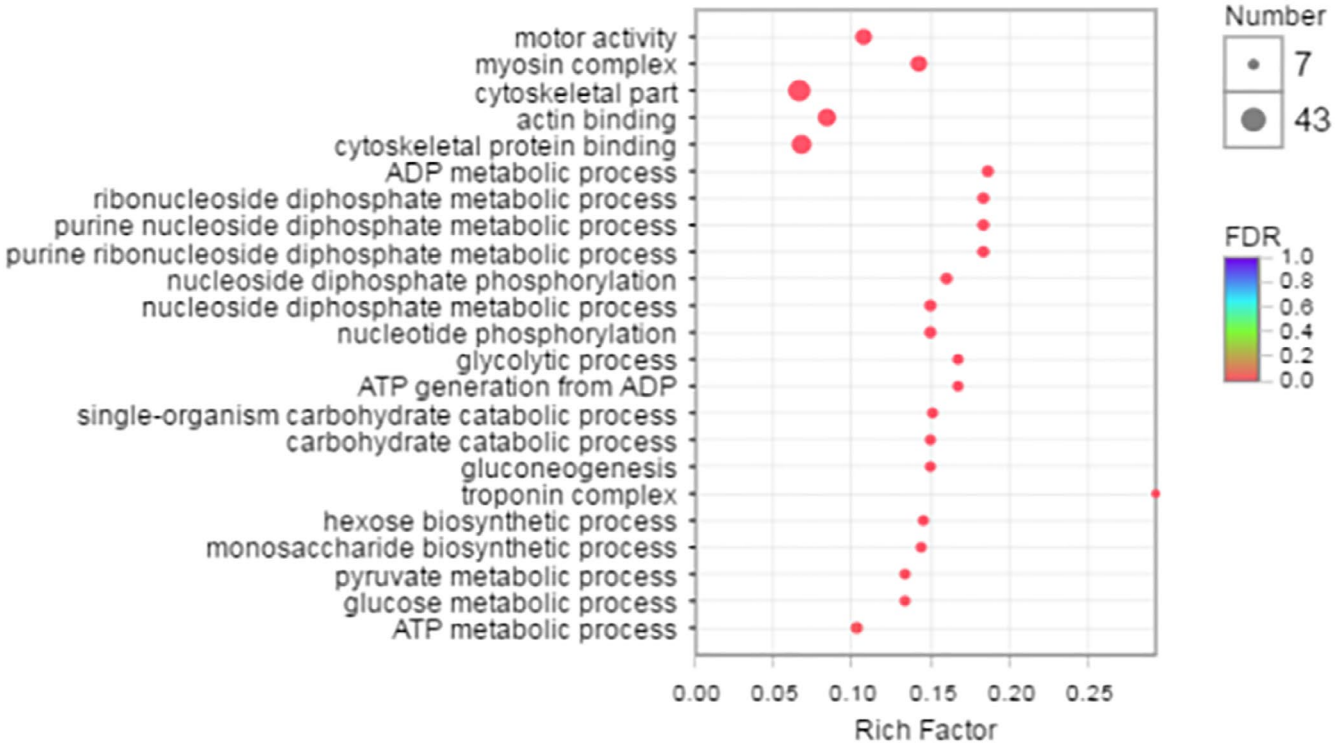

FIGURE 1 Scatterplot of enriched GO terms for differentially expressed genes (DEGs) in QC group. *X*-axis: the percentages of DEGs belonging to the corresponding pathway, *Y*-axis: the top 23 GO terms. The sizes of bubble represent the number of DEGs in the corresponding term, and the colors of the bubble represent the enrichment *p*-value of the corresponding term

that the flesh recovers in the time frame between the end of the first bite and the start of the second bite. In fish, the hardness is an texture parameter more important than springiness, which is closely related to muscle density, muscle fibre type and muscle collagen (Yu et al., 2014). In general, the flesh with high WHC and hardness is more readily accepted by consumers (Theofania & Petros, 2017). Studies have shown that EU promoted the flesh quality by improving the WHC and texture characteristics (Sun et al., 2017b; Xu et al., 2010; Zhou et al., 2016). In the present study, the hardness, centrifugal loss and thawing loss of flesh were significantly affected only by dietary QC ($p < .05$), but not by GP and GCA, which indicated that QC may be the main component in flesh quality-improving effect of EU.

Larsson et al. (2012) suggested that glycolysis is the major source of energy in muscle, and the down-regulation of related genes may be involved in the increase in flesh hardness. In this study, flesh hardness was significantly increased by dietary QC, and the expressions of 10 glycolytic enzymes also were significantly down-regulated, including *pyruvate kinase*, *enolase* and *lactate dehydrogenase* (Table S1), which indicated that the flesh of the QC group has lower glycolysis velocity. In addition, flesh hardness is also closely related to calcium signalling pathways (Yu et al., 2014). The calcium signalling pathway in the QC group was highly enriched, and 11 genes including *VDC*, *RyR1 and CRAC* were significantly up-regulated in the 17 significantly changed genes in the pathway (Table S2). *Ryanodine receptor 1* (*RyR1*) is a calcium release channel existing in the *endoplasmic reticulum/sarcoplasmic reticulum* (*ER/SR*), which could rapidly release Ca^{2+} from *ER/SR* to participate in the contraction of muscle (Feng et al., 2011). *Voltage-dependent calcium channel L type* (*VDCC*) can bind with *RyR1* in skeletal muscle and then trigger the release of Ca^{2+} from the sarcoplasmic reticulum and cause the contraction of muscle, thus playing an important role in excitation–contraction coupling (Yuen, Campiglio, Tung, Flucher, & Petegem, 2017). *Calcium release-activated calcium* (*CRAC*) channel protein 1 mediates Ca^{2+} influx following depletion of intracellular Ca^{2+} stores and channel activation by the Ca^{2+} sensor (Soboloff et al., 2006). The opening of calcium channels not only increased the calcium ion concentration, but also caused the release of neurotransmitters, and thereby enhances the contraction of muscle (Santulli, Nakashima, Yuan, & Marks, 2017). The present study found that many signalling pathways relating to muscle contraction (cardiac muscle contraction, vascular smooth muscle contraction and so on) and affecting muscle contractility (GnRH signalling pathway, insulin signalling pathway, thyroid hormone signalling pathway and so on) were highly enriched, which indicated that dietary QC may increase the contractility of muscle. *Myosin light chain kinase* (*MYLK*) and *striated muscle preferentially expressed protein kinase* (*SPEG*) play important roles in muscle contraction, and the up-regulated expression of the two enzymes in the present study also proved the above viewpoint.

In the studies of Macrae and Mefferd. (2006) and Borghi et al. (2016), QC was found to increase the aerobic capacity of muscle, thereby enhance the athletic performance. The increased muscle contractility could promote the collagen cross-linking and muscle hardness (Zimmerman,

TABLE 7 The detailed information of significant KEGG pathways enriched in the QC, CGA and GP groups

Pathway ID	Term	Number	*p*-Value
QC group			
map04260	Cardiac muscle contraction	19	5.21E−08
map04261	Adrenergic signalling in cardiomyocytes	24	3.16E−07
map05416	Viral myocarditis	17	1.19E−06
map00010	Glycolysis/gluconeogenesis	10	4.89E−06
map04530	Tight junction	28	2.27E−05
map05414	Dilated cardiomyopathy (DCM)	13	4.51E−04
map05231	Choline metabolism in cancer	12	4.79E−04
map04014	Ras signalling pathway	17	1.43E−03
map04066	HIF−1 signalling pathway	10	1.45E−03
map04510	Focal adhesion	18	1.52E−03
map05410	Hypertrophic cardiomyopathy (HCM)	10	2.27E−03
map04020	Calcium signalling pathway	17	4.09E−03
map04711	Circadian rhythm-fly	3	7.08E−03
map04310	Wnt signalling pathway	11	7.77E−03
map00564	Glycerophospholipid metabolism	8	1.11E−02
map04919	Thyroid hormone signalling pathway	10	1.30E−02
map04664	Fc epsilon RI signalling pathway	8	1.14E−02
map00565	Ether lipid metabolism	5	1.60E−02
map04072	Phospholipase D signalling pathway	13	1.62E−02
map04145	Phagosome	12	1.84E−02
map04710	Circadian rhythm	4	2.22E−02
map04520	Adherens junction	9	2.57E−02
map04151	PI3K-Akt signalling pathway	20	2.54E−02
map04910	Insulin signalling pathway	9	2.70E−02
map00030	Pentose phosphate pathway	3	2.93E−02
map04912	GnRH signalling pathway	8	3.24E−02
map04370	VEGF signalling pathway	6	3.68E−02
map04611	Platelet activation	10	3.83E−02
map05322	Systemic lupus erythematosus	8	4.22E−02
map04810	Regulation of actin cytoskeleton	13	4.27E−02
map04640	Hematopoietic cell lineage	7	4.31E−02
map05202	Transcriptional misregulation in cancer	11	4.39E−02
map00730	Thiamine metabolism	2	4.59E−02
map04152	AMPK signalling pathway	8	4.61E−02
map05418	Fluid shear stress and atherosclerosis	9	4.76E−02
map04750	Inflammatory mediator regulation of TRP channels	8	4.93E−02
map04270	Vascular smooth muscle contraction	9	4.96E−02
CGA group			
map04911	Insulin secretion	6	1.34E−02
map00564	Glycerophospholipid metabolism	5	2.42E−02
map05216	Thyroid cancer	3	4.03E−02
map00565	Ether lipid metabolism	3	4.57E−02
GP group			
map05164	Influenza A	11	8.85E−05

(Continues)

TABLE 7 (Continued)

Pathway ID	Term	Number	*p*-Value
map05162	Measles	8	4.52E−04
map04622	RIG-I-like receptor signalling pathway	4	4.65E−03
map04974	Protein digestion and absorption	6	7.17E−03
map04610	Complement and coagulation cascades	4	2.62E−02

Mccormick, Vadlamudi, & Thomaset, 1993). As a strong antioxidant, QC can play a synergistic role with vitamin C to increase cross-linking (Askari et al., 2013). In addition, the flesh hardness was also affected by the proteolytic enzymes in the muscle. Cathepsin is the major member of the lysosomal semi-deacetylase family and the major protein catabolic pathway in muscle degradation, especially for *cathepsin L* (Salem, Kenney, Rexroad, & Yao, 2006). The activity changes of *cathepsin L* showed significantly negative correlation with muscle hardness and pH (Bahuaud et al., 2010). In this study, the expression of *cathepsin L* in the QC group was significantly decreased, which corresponded to the increase in hardness of muscle. It was reported that the supplementation of QC or plant extracts rich in QC significantly increased the pH and WHC of pork (Zou et al., 2016) and chicken (Cao, Zhang, Yu, Zhao, & Wang, 2012).

Most of moisture in muscle is held by the subcellular structure of the muscle (myofibril), and the moisture between actin and myosin accounts for about 85%-96% of total muscle moisture. So the change of WHC indicated the structure change of myofibrillar fibres (Bertram, Purslow, & Andersen, 2002). The expression of a series of genes including *slow myosin heavy chains* (*s-MyHCs*), *MYH-6/7* and slow troponin genes was significantly up-regulated, and the fast troponin gene expression was down-regulated in the QC group, which could explain this result (Table S3). *MYH-7* and *MYH-6* encode the intron miR-208b, while miR-208b drives the conversion of fast myofilament to slow myofilament (Rooij et al., 2009). The up-regulation of *s-MyHCs* and slow troponins and the down-regulation of fast troponins genes indicated the growth of slow filament (Magnoni et al., 2013). Moreover, the high expression of GO terms such as motor activity, myosin complex and actin binding also suggested the changes in myofilament structure and function. Compared with fast muscle fibres, slow muscle fibres have higher aerobic exercise capacity, tolerance and insulin sensitivity. The conclusion was consistent with the above result in which quercetin could improve muscle contractility (Hyatt et al., 2016; Lim, Sutton, & Rao, 2015). In summary, QC could improve the flesh hardness and WHC by changing the composition of myofilament to reduce anaerobic metabolism and promote aerobic metabolism.

4.3 | Effects of EU active compounds on the flesh fatty acids and free amino acids

As a candidate gene for detecting flesh quality, *heart-type fatty acid binding protein* (*H-FABP*) widely distributes in cytosol, and it preferentially binds to long-chain unsaturated fatty acids and then transports from the cell membrane to the site exerting regulatory function, and promotes the fatty acid uptake by the cell (Hanhoff, Lücke, & Spener, 2002). *Solute carrier family 27* (fatty acid transporter) member 1 (*Slc27a1*) is a fatty acid transporter, and it transports long-chain and super-long-chain fatty acids into cells and is involved in fatty acid uptake, transport and metabolism (Coe, Smith, Frohnert, Watkins, & Bernlohr, 1999). In this study, the expression of *H-FABP* in the QC and CGA groups was significantly decreased, and the expression of lipid metabolism-related genes such as *Slc27a1*, *lipid phosphate phosphohydrolase 2-like* (*PPAR2*) and *diacylglycerol kinase iota-like isoform X1* (*DGKA*) was up-regulated. Such a result may be concerned with the changes in fatty acid composition (Table S4).

As important part of unsaturated fatty acids, n-3 PUFAs play important roles in preventing cardiovascular and cerebrovascular diseases, and in lowering serum lipid, blood pressure and inflammation (Russo, 2009). Toufektsian, Salen, Laporte, Tonelli, and Lorgeril. (2010) once reported that dietary polyphenols increased the content of n-3 PUFAs in rats. When used for the nutrition evaluation of flesh, the ratio of n-3/n-6 PUFA is more important than the content of n-3 PUFAs or n-6 PUFAs (Zhao et al., 2019). The studies of Andrés et al. (2013) and Starčević et al. (2015) showed that polyphenolic compounds (quercetin and gallic acid) increased the n-3/n-6 ratio in lamb and chicken, respectively. Lorgeril, and Salen. (2010) also found that dietary polyphenols (anthocyanins) increased serum n-3 PUFA level and n-3/n-6 ratio in mouse. This study showed that dietary QC and CGA significantly increased the n-3 PUFA level and n-3/n-6 ratio in muscle. All the results may be related to the increased intestinal absorption for n-3 PUFAs, which needs a further study in the future.

QC could modulate the TCA cycle and increase the energy synthesis in rat (Wu, 2010). In the present study, the higher free amino acid contents in the QC group than in control may be attributed to the increase in oxaloacetate and ketoglutarate in TCA cycle, which could produce delicious amino acids and other non-essential amino acids by transamination. In the study of Wu et al. (2018), dietary CGA changed the diversity of intestinal flora, improved intestinal mucosal barrier function and increased the serum levels of free essential, non-essential and total amino acids in pig. The serum free amino acids are the main source for free amino acids and protein synthesis in muscle. Therefore, the increase in flesh free amino acids in the CGA group may be related to the change of intestinal flora and the increase in serum free amino acids.

4.4 | Effects of EU active compounds on antioxidant and immunity

Polyphenolic compound could improve the antioxidant and immune capacity besides increasing the free amino acid and

fatty acid in muscle (Zhang & Tsao, 2016). When exposed to environmental stress, the body would synthesize more protective proteins, heat-shock proteins, to maintain cell homeostasis (Roberts, Agius, Saliba, Bossier, & Sung, 2010). Roussou, Lambropoulos, Pagoulatos, Fotsis, and Roussis. (2004) and Kanagaraj, Panneerselvam, Govindarajan, Ameeramja, and Perumal. (2015) once reported that polyphenolic compounds reduced the expression of HSP in tumour cells and liver in mouse. In this study, the expression of *heat-shock protein 27* (*HSP27*) in the CGA group and *heat-shock 27 kDa protein 3* (*HSPB3*) in the QC group was also significantly decreased, while the expression of *PPARγ*, a key gene in multiple antioxidant pathways, was significantly up-regulated in the QC and CGA groups. Polyphenols are the activators of *PPARγ*, and it could increase the activity of *PPARγ* (Tian et al., 2018), thereby improving antioxidant indices such as ALP and SOD, which has been verified in our previous study experiments (Sun et al., 2017a).

Some studies have demonstrated that QC (Li et al., 2016), CGA (Naveed et al., 2018) and GP (Song et al., 2014) could enhance the immunity of the body. The present results of transcriptome showed that many immune-related signalling pathways were significantly enriched in the two active component groups (Table 7), such as phospholipase D, Ras and HIF-1 signalling pathway in the QC group, and influenza A, complement and coagulation cascades and RIG-I-like receptor signalling pathway in the GP group. In the CGA group, no enriched immune-related pathways were found, but immune-related genes such as *IL7RA*, *Nfil3*, *NBL1* and *IRF-7* were also significantly expressed in the "immune system" pathway (Table S5). The relevant signalling pathways of these genes did not show significances in enrichment analysis, which may be due to the incomplete functional annotation of compared *C. idellus* reference database.

5 | CONCLUSION

In conclusion, the supplementation of CGA improved the growth, and the supplementation of QC improved the flesh quality of grass carp in the present study. The improved flesh quality by dietary QC may be achieved by changing myofibril structure and regulating muscle glycolytic pathways and calcium signalling pathways to increase flesh WCH and hardness. QC and CGA could affect fatty acid composition and free amino acid levels by modulating lipid metabolism and carbohydrate metabolism. In addition, QC, CGA and GP may enhance the immunity through different signalling pathways of immune response and inflammatory.

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CONFLICT OF INTEREST

There are no conflicts of interest.

AUTHOR CONTRIBUTIONS

H. Y. and X. L. completed the experiment and prepared the manuscript; Z. X. and Z. C. carried out the growth experiment and analysed the samples; and X. L. designed the experiment and revised the manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this article.

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