Hindawi
Aquaculture Nutrition
Volume 2022, Article ID 2717003, 10 pages
https://doi.org/10.1155/2022/2717003

Research Article

Evaluation of Chlorogenic Acid Supplementation in Koi (*Cyprinus carpio*) Diet: Growth Performance, Body Color, Antioxidant Activity, Serum Biochemical Parameters, and Immune Response

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Received 4 March 2022; Revised 21 April 2022; Accepted 26 April 2022; Published 12 May 2022

Academic Editor: Xiangjun Leng

A ten-week feeding trial was performed to evaluate the effects of chlorogenic acid (CGA) on the growth, body color, antioxidant activity, serum biochemical parameters, and immune response of koi. Five diets were formulated to contain 0 (control), 200 (CGA-2), 400 (CGA-4), 600 (CGA-6), and 800 (CGA-8) mg/kg CGA for the rearing of experimental fish. By means of an analysis by polynomial orthogonal contrasts, it was determined that dietary supplementation with CGA had no significant effects on the growth performance of koi ($P > 0.05$), while body color indices (redness, yellowness, and lightness) showed a linear order polynomial ($P < 0.05$). A quadratic response was found for the relationship between total carotenoid in scale of koi and dietary CGA levels, with an optimum level of 381.84 mg/kg. Dietary CGA levels and lysozyme activity showed a cubic relationship polynomial model, with an optimum level of 383.89-975.73 mg/kg. TGF-β expression in the head-kidney of koi was obviously improved by 600 mg/kg CGA diet ($P < 0.05$). And in regards to effect in serum, SOD and CAT activities was best explicated by a linear order polynomial, while the quadratic and cubic relationship polynomial models represented the GSH and GSH-Px activities, which the optimal supplementation level of CGA was estimated at 550.78 and 231.74-782.66 mg/kg, respectively. The relationship between ALT activity and CGA supplementation was best explicated by quadratic relationship polynomial, with an optimum level of 522.78 mg/kg. Based on the model observed, 522.78-600 mg/kg CGA was the recommended supplementation in diet that improved the body color, antioxidant activity, biochemical parameters, and immune response of koi.

1. Introduction

Koi (*Cyprinus carpio*), including Showa Sanshoku, Kohaku, Yamabuki ogon, and other strains [1], has already become one of the most popular pet hobbies in the world due to its superiority, such as bright color, graceful body form, and fast reproduction [2, 3]. Moreover, koi is becoming a very important culture species of ornamental fish in China [4]. However, rapid development of intensive aquaculture increased the susceptibility of infectious diseases in koi, which could cause ornamental value decreasing and mortality increasing, eventually leading to lower price and substantial economic losses in the koi farming [4, 5]. Unfortunately, antibiotics and chemical disinfectants, as a kind of traditional control drug, are no longer recommended to treat these fish diseases due to their serious harmful effects including the spread of drug resistant pathogens, suppression of aquatic animal's immune system, and environmental pollution [6]. Hence, the use of new preventive approaches is becoming increasingly important in koi farming such as immunostimulants to improve their innate defense and resistance against diseases.

Plant extracts are good sources of natural immunostimulants attracting much attention in aquaculture [7–9], because they have the multiple target effects, excellent biodegrade ability, and few side effects [10–12]. Chlorogenic acid

(CGA) is a well-known phenolic acid and widely distributed in natural plants, such as honeysuckle, eucommia, chrysanthemum, and sunflower [13]. Numerous studies have demonstrated that CGA have a variety of biological activities, including antioxidant, antibacterial, anti-inflammation, antiglycation, antitumor, and immune regulation[14–16], which indicated that it may have potential to maintain the health of animals [17].

At present, CGA has been served as a feed supplement to improve the immunity and growth performance in livestock and poultry husbandry [18–22]. In aquaculture, the application of CGA is mostly focused on the growth, flesh quality, and lipid metabolism of the food fish [23–27]. Of course, CGA is a novel natural antioxidant which can be applied in the storage of aquatic products [28, 29]. Nevertheless, it has received little attention about the use of CGA to stimulate disease resistance and maintain the health in ornamental fish especially koi. Given the functional properties of CGA, the purpose of this study was to evaluate the effects of CGA on the growth, body color, antioxidant activity, serum biochemical parameters, and immune response of koi.

2. Materials and Methods

2.1. Experimental Diets. Ingredients and proximate composition of five experimental diets are presented in Table 1, which was formulated to contain 41% crude protein, 6.6% crude fat, and gross energy 16.2 MJ/kg. All experimental diets were prepared with the level of 0 (control), 200 (CGA-2), 400 (CGA-4), 600 (CGA-6), and 800 (CGA-8) mg/kg CGA in basal diet, respectively. CGA was purchased from Shanghai Sunny Biotech Co., Ltd. (Shanghai, China) with purity higher than 98.5%. All feed ingredients were fully mixed after a grinder and extruded pellets by an extruder (MY56X2A, Mu Yang Group, Jiangsu, China) with a particle size of 2.0 mm module. All diets were air-dried and then stored in plastic bags at 4°C until use.

Table 1: Feed formulation and composition of five diets used in the feeding trial∗.

Ingredient (g/kg diet)	Control	CGA-2	CGA-4	CGA-6	CGA-8
Fish meal (CP 60%)	100.00	100.00	100.00	100.00	100.00
Soybean meal	240.00	240.00	240.00	240.00	240.00
Rapeseed meal	240.00	240.00	240.00	240.00	240.00
Wheat gluten	103.00	103.00	103.00	103.00	103.00
Wheat flour	263.00	262.80	262.60	262.40	262.20
Soybean oil	35.00	35.00	35.00	35.00	35.00
$Ca(H_2PO_4)_2$	10.00	10.00	10.00	10.00	10.00
Vitamin premix[a]	1.50	1.50	1.50	1.50	1.50
Mineral premix[b]	1.50	1.50	1.50	1.50	1.50
Choline chloride (50%)	2.00	2.00	2.00	2.00	2.00
Lysine (98.5%)	3.00	3.00	3.00	3.00	3.00
Methionine	1.00	1.00	1.00	1.00	1.00
Chlorogenic acid	0.00	0.20	0.40	0.60	0.80
Proximate composition (g/kg)					
Crude protein	413.30	416.20	412.80	410.80	415.80
Crude fat	66.00	64.70	68.00	65.30	63.00
Moisture	59.70	62.70	61.30	65.00	59.70
Ash	64.00	64.00	64.00	66.00	63.30
Gross energy (MJ/kg)	16.25	16.21	16.31	16.12	16.19

[a]Vitamin premix (mg/kg diet): vitamin B_1, 12 mg; vitamin B_2, 5 mg; vitamin B_5, 30 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.05 mg; vitamin D_3, 5 mg; vitamin E, 40 mg; vitamin K_3, 5 mg; inositol, 100 mg; niacin acid, 35 mg; folic acid, 2 mg, biotin, 0.05 mg; retinol acetate, 25 mg; ascorbic acid, 500 mg; ethoxyquin 150 mg, corn protein powder 585 mg. [b] Mineral premix (mg/kg diet): KCl, 10 mg; KI (1%), 3 mg; $CoCl_2 \cdot 6H_2O$ (1%), 0.35 mg; $CuSO_4 \cdot 5H_2O$, 0.7 mg; $FeSO_4 \cdot H_2O$, 20 mg; $ZnSO_4 \cdot H_2O$, 10 mg; $MnSO_4 \cdot H_2O$, 4 mg; $Na_2SeO_3 \cdot 5H_2O$ (1%), 3.25 mg; $MgSO_4 \cdot 7H_2O$, 150 mg; $Ca(H_2PO_4)_2 \cdot H_2O$, 1000 mg; NaCl, 6.8 mg; Zoelite, 292 mg. ∗All diets were produced at Beijing Sanyou Chuangmei Feed Technology Co., Ltd, Beijing, China, as extruded pellets.

2.2. Experimental Animals and Husbandry. Healthy koi (Yamabuki ogon), a body color of yellow, was purchased from a commercial farm (Tongzhou, Beijing, China). Before the beginning of feeding trial, fish were fed with the basal diets for two weeks to acclimatize the experimental conditions. Then, a total of 450 healthy fish (average initial weight 53.33 ± 1.22 g) were randomly distributed to one control and four CGA groups (CGA-2 to CGA-8). Each group was designed with three repetitive ponds with 30 fish per ponds (2 m × 1.2 m × 1.5 m), and the water volume is 3 m^3 for each pond. In order to ensure the water quality, the experimental conditions are controlled at temperature 22.33 ± 1.35°C, dissolved oxygen 8.83 ± 0.58 mg/L, pH 8.26 ± 0.08, ammonia-nitrogen 0.05 ± 0.01 mg/L, and nitrite 0.004 ± 0.001 mg/L.

The feeding trial lasted for 70 days, and fish were hand-fed to apparent satiation four times (8 : 00, 11 : 00, 14 : 00, and 17 : 00) daily.

2.3. Sampling Procedures. After being fasted for 24 h, all fish in each pond were anaesthetized by immersion in a chloretone (0.30 mL L^{-1} water) solution. Nine anaesthetized fish were captured randomly in each pond. The brightness, redness, and yellowness of body surface in each fish were measured by a spectrophotometer. Then, the weight and body length of each fish were measured individually. Blood was taken from anaesthetized fish by the caudal venipuncture with 2 mL disposable syringes and centrifuged at 4000 r/min for 10 min at 4°C. The serum samples were ($n = 9$ per group) stored at −20°C until subsequent analyses. After blood sampling, the scales, epidermis, and muscles were removed under the dorsal fin and above the lateral line and stored at -20°C until analyses for pigment. Then, the head-kidney samples were collected and stored at −80°C until analysis for cytokine mRNA expression.

All rest fish in each pond were weighted and counted at the end of all sampling. Growth indexes were calculated according to the diet consumption, the weight, and number of all fish.

2.4. Chemical Analyses. All experimental diets were analyzed according to their chemical compositions [30, 31]. Samples were dried to a constant weight at 105°C and then used to

determine moisture. Crude protein was analyzed by measuring nitrogen (N × 6.25) (Kjeltec 2300 Protein Analyzer; Foss) by the Kjeldahl method. Crude lipid was measured by acid hydrolysis with a Sotex System Hotplate 2022 Hydrolyzing Unit (Foss), followed by Soxhlet extraction using a Sotex system 2050 (Foss). Ash was examined by combustion in a muffle furnace at 550°C for 16 h. Gross energy was determined by Parr 1281 Automatic Bomb Calorimeter (Parr).

2.5. Body Color Analysis. According to the method of Leng, Shi, Li, Xu, & Yang [32], the brightness ($L*$), redness ($a*$), and yellowness ($b*$) of the body surface above the widest lateral line on the side of fish were measured by a portable Chroma Meter (CM-600d, Konica Minolta, Japan) calibrating with a white standard, whereby $L*$ values describe relative lightness ranging from black to white (where $L* = a$ negative value represents black and $L* = a$ positive value represents white). The values of $a*$ describe relative variation from green to red, green ($a* = a$ negative value) and red ($a* = a$ positive value). And $b*$ values describe relative variation from blue to yellow, blue ($b* = a$ negative value) and yellow ($b* = a$ positive value) (http://en.wikipedia.org/wiki/Colorfulness).

Besides, the total carotenoid in the scales, epidermis, and muscles of koi was, respectively, measured according to the method of Wang, Xiong, Zhang, Ren, & Zhang [33]. Briefly, the grinded sample (0.1 g) was attained constant volume 5 mL with acetone. Next, the sample was put into an ultrasonic cleaning machine for ultrasonic shock at low temperature for 40 min. Then, the sample was centrifuged at 4000 r/min for 10 min. And the pigment extract of sample was stored at 4°C for 24 h. The pigment extract was placed in a 1 cm colorimetric dish with acetone as blank control. And the absorbance value of each pigment extract was determined by UV spectrophotometer.

$$\text{Carotenoid}\left(\frac{mg}{kg}\right) = \frac{(A \times K \times V)}{E \times G}, \quad (1)$$

where A is the absorbance value, K is a constant (10^4), V is the volume of extracted liquid (mL), E is the molar extinction coefficient (2500), and G is the sample weight (G).

2.6. Antioxidant Activity Analysis. According to the method described by Draper et al. [34], Zhou et al. [35] and Kong et al. [36], superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), glutathione (GSH), and glutathione peroxidase (GSH-Px) activities were determined by the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer instructions.

2.7. Biochemical Analysis. Alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST) were performed by an automatic biochemical analyzer HITACHI 7160 (Hitachi, Ltd.) according to manufacturer's instructions from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China).

Table 2: Sequences of primers used in this study.

Gene name	Sequences of primers (5′ to 3′)	Amplification size/bp
IL8	F: AAGGAATGAGTCTTAGCGGTCT R: TCTTAACCCAGGGTGCAGTAG	198.00
TNF-α	F: CGATTCCTATGGCGGCTAC R: GGAGTTCTTCCTTCGTTTTGG	161.00
IL-1β	F: GGCACGGATTACAACACTTT R: TGGCACCTGGGACATTTC	99.00
TGF-β	F: CCAGGGAACATCTCCAGTCA R: TCTTTCATCAGACGCTCCATC	125.00
β-Actin	F: TCTGAAGTACCCCATTGAGCA R: CTGTGTCATCTTTTCCCTGTTG	166.00

2.8. Immunological Parameters Analysis. Complement 3 (C_3) and complement 4 (C_4) were determined by commercial kits (Jiancheng Bioengineering Institute, Nanjing, China), which included measuring the increase in turbidity after immune response of C_3 or C_4 and the increase in C_3 or C_4 antibodies [37, 38].

Using turbidimetric assay, we tested the decrease in turbidity after lysis of *Micrococcus lysodeikticus* (lysozyme detection kit, Jiancheng Bioengineering Institute, Nanjing, China), which represented lysozyme (LZM) activity [39].

2.9. RNA Extraction and cDNA Synthesis. Using the TRNzol total RNA extraction kit (No. DP424; Tiangen Biotech Co., Ltd., Beijing, China), we extracted total RNA from the head-kidney tissue (approximately 30 mg of each sample). RNA quality was examined by 1.5% agarose gel electrophoresis. Then, A260/280 absorbance ratio was determined by a NanoDrop 2000 (Thermo Scientific, USA) to check the RNA purity. All extracted samples had an A260/280 ratio of approximately 1.8 to 2.1. Afterwards, cDNA synthesis was performed with reverse transcriptase and 1 μg of total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (No. RR047B; TaKaRa, Japan) following the manual [40]. The cDNA templates were stored at −80°C until analysis.

2.10. Quantitative Real-Time PCR (qRT-PCR). The primer sequences for interleukin-8 (IL-8), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and β-actin are given in Table 2. The β-actin gene was used as the housekeeping gene and was amplified using its specific primers. The qRT-PCR was carried out with SYBR®Premix Ex Taq™ II (TliRNaseH Plus) kit (No. RR82LR; TaKaRa, Beijing, China) in a 20 μL reaction volume (ABI, USA). The total reaction volume contained 10 μl of 2×SYBR®Premix Ex Taq™ II Master Mix, 2 μL of cDNA, 0.5 μL each of forward and reverse primers and 7.0 μL of nuclease-free water. The qRT-PCR thermocycling parameters were set as following: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 40 s. In order to establish the PCR product melting curve, the temperature was set at 95°C, 10 s; 60°C, 60 s; and 95°C, 15 s and slowly heated from 60°C to 99°C (Ramp Rate at 0.05°C/s) after the

Table 3: Growth performance of koi fed the experimental diets for ten weeks.

Item	Control	CGA-2	CGA-4	CGA-6	CGA-8	ANOVA	Regression[1]			
						P value	Model	*P* value	R^2	Op. level
Initial body weight (g)	54.17 ± 0.83	52.50 ± 0.00	54.17 ± 0.83	52.50 ± 0.00	53.33 ± 0.83	0.23	NR	—	—	—
Final body weight (g)	201.38 ± 2.54	199.50 ± 7.49	203.90 ± 2.97	202.65 ± 4.24	210.51 ± 4.42	0.55	NR	—	—	—
WGR (%)[2]	265.64 ± 6.80	280.00 ± 14.27	276.48 ± 4.01	279.48 ± 8.83	295.10 ± 13.67	0.43	NR	—	—	—
SGR (% day^{-1})[3]	2.31 ± 0.03	2.38 ± 0.07	2.37 ± 0.01	2.38 ± 0.03	2.45 ± 0.04	0.43	NR	—	—	—
FCR[4]	1.30 ± 0.01	1.25 ± 0.07	1.32 ± 0.01	1.33 ± 0.03	1.24 ± 0.04	0.46	NR	—	—	—
FI (% day^{-1})[5]	2.65 ± 0.02	2.60 ± 0.10	2.75 ± 0.01	2.76 ± 0.04	2.63 ± 0.04	0.21	NR	—	—	—
Survival (%)[6]	98.33 ± 1.67	100.00 ± 0.00	100.00 ± 0.00	98.33 ± 1.67	100.00 ± 0.00	0.58	NR	—	—	—

Note: means in the same row with different superscripts are significantly different ($P < 0.05$). [1]Regression: Ln: Linear; Qd: Quadratic; Cu: Cubic; NR: No relationship; Op. level: Optimal level of CGA. [2]Weight gain rate (WGR, %) = (final body weight − initial body weight)/initial body weight × 100. [3]Specific growth rate (SGR, %per day) = (ln final body weight − ln initial body weight)/total duration of the experiment × 100. [4]Feed conversion ratio (FCR) = feed consumed/(final body weight − initial body weight). [5]Feeding intake (FI, %per day) = 100 × feed consumed/[days × (initial body weight + final body weight)/2]. [6]Survival (%) = (final number of fish/initial number of fish) × 100.

Table 4: Body color and total carotenoid of koi fed the experimental diets for ten weeks.

Item	Control	CGA-2	CGA-4	CGA-6	CGA-8	ANOVA	Regression[1]			
						P value	Model	*P* value	R^2	Op. level
Body color										
$L*$	79.39 ± 1.17^{b}	79.54 ± 1.12^{b}	75.68 ± 0.78^{a}	76.25 ± 1.00^{a}	73.74 ± 0.95^{a}	0.00	Ln	0.00	0.36	—
$a*$	5.20 ± 1.20^{a}	5.98 ± 0.68^{ab}	7.09 ± 1.12^{b}	7.30 ± 0.32^{b}	8.55 ± 1.22^{b}	0.05	Ln	0.01	0.21	—
$b*$	42.44 ± 1.65^{a}	49.35 ± 2.63^{ab}	52.77 ± 2.04^{b}	53.57 ± 1.92^{b}	53.48 ± 3.40^{b}	0.03	Ln	0.04	0.14	—
Total carotenoid (mg/kg)										
Muscle	10.37 ± 0.65^{b}	9.43 ± 0.23^{a}	11.79 ± 0.79^{b}	10.93 ± 0.17^{b}	9.57 ± 0.47^{a}	0.02	NR	—	—	—
Epidermis	47.67 ± 4.41	52.43 ± 2.77	51.00 ± 2.57	56.27 ± 3.05	59.20 ± 1.26	0.09	NR	—	—	—
Scale	39.50 ± 3.96^{a}	57.37 ± 1.67^{c}	53.93 ± 0.86^{bc}	48.90 ± 3.43^{b}	40.43 ± 1.32^{a}	0.00	Qd	0.00	0.52	381.84

Note: means in the same row with different superscripts are significantly different ($P < 0.05$). [1]Regression: Ln: Linear; Qd: Quadratic; Cu: Cubic; NR: No relationship; Op. level: Optimal level of CGA.

amplification reaction was completed. Reverse transcriptase negative controls and no-template controls were included on all plates. The qRT-PCR specificity was confirmed by agarose gel electrophoresis and dissociation curve analysis: the presence of a single band of appropriate size obtained on gel and a clear single peak dissociation curve. Each assay was run on an Applied Biosystems 7500 Real-Time PCR system in triplicates. β-Actin gene was used as an endogenous reference to assess relative mRNA transcript levels. Relative gene-expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after obtaining the threshold cycle (Ct) values of each sample [41].

2.11. Statistical Analysis. The data are presented as mean ± standard error of mean and carried out statistical analysis by the SPSS 22.0 statistical program. Polynomial contrast analysis was performed on the growth, body color, antioxidant activity, biochemical, and immunological parameters. The difference was considered significant at $P < 0.05$, and if statistical significance was detected (linear, quadratic or cubic), the data were subjected to regression analysis to fit the best model. The inflammatory gene expression was analyzed by ANOVA, which tested for homogeneity of variance first, and Duncan's multiple comparisons were followed to determine significant differences.

3. Results

3.1. Growth Performance. After 10-week feeding period, no significant differences ($P > 0.05$) were observed in WGR, SGR, FCR, and FI of koi among the groups (Table 3). Similarly, survival was not clearly affected by the CGA diets ($P > 0.05$). No relationship was found between growth performance and the CGA level by polynomial contrast analysis.

3.2. Body Color. Results of body color and total carotenoid in koi are presented in Table 4. Comparing with control group, dietary with CGA decreased the $L*$ value of koi and increased the CF, $a*$ and $b*$ values of koi ($P < 0.05$). Except for the control and CGA-8 diets, other CGA adding improved the total carotenoid in the scale of koi ($P < 0.05$). The total carotenoid of muscle in koi of the CGA-2 and CGA-8 diets was

lower than that of the control group ($P < 0.05$). No significant differences were observed in the total carotenoid of epidermis in all experimental groups ($P > 0.05$).

The relationship between dietary CGA levels and the values of $a*$, $b*$, and $L*$ in koi was best explicated by linear order polynomial (Ln) models. A quadratic response (Qd) was found for the relationship between the total carotenoid in scale and dietary CGA levels, which indicated that the optimal level of CGA was estimated to be 381.84 mg/kg. No relationship was found between the CGA levels and the total carotenoid in muscle and epidermis.

3.3. Antioxidant Activity. Table 5 shows the effect of dietary CGA on antioxidant activity in serum of koi. The SOD activities of koi from CGA-4, CGA-6, and CGA-8 groups were higher than that of control and CGA-2 groups ($P < 0.05$). The highest and lowest CAT activities were observed in CGA-4 and CGA-2, respectively ($P < 0.05$). Meanwhile, CAT activity of CGA-4 was obviously higher than the control group ($P < 0.05$). Compared with the control group, the activities of GSH-Px in CGA-4 and CGA-8 were clearly increased, while that in CGA-2 was significantly decreased ($P < 0.05$). Except for CGA-8, GSH activities of CGA-2, CGA-4, and CGA-6 were obviously higher than that of the control group ($P < 0.05$). In addition, CGA diets were distinctly reduced the MDA level in serum of koi compared with the control diet ($P < 0.05$).

The linear order polynomial (Ln) models was best explicated the relationship between the SOD activity with dietary CGA levels in koi. Similar result was also found in the relationship between the CAT activity with dietary CGA levels in koi. In the relationship between the GSH-Px, GSH activity with dietary CGA levels in koi was well explicated by the cubic order polynomial (Cu) models and the quadratic relationship polynomial (Qd) models, respectively. No relationship was found for MDA with dietary CGA levels. The optimal level of CGA was estimated to be 231.74-782.66 and 550.78 mg/kg for GSH-Px and GSH, respectively.

3.4. Serum Biochemical Parameters. Compared with the control diet, serum ALT of koi was obviously decreased by the different CGA level diets ($P < 0.05$, Table 5). There was no significant difference in serum AST and ALP activity among all the groups ($P > 0.05$). The relationship between dietary CGA levels and serum ALT was best explicated by quadratic relationship polynomial (Qd) models. The optimal level of CGA was estimated to be 522.78 mg/kg for ALT.

3.5. Immune Response

3.5.1. Blood Parameters. The immunological parameters in serum of koi fed the different CGA levels diets are shown in Table 5. Levels of C_3 were significantly enhanced in CGA-4, CGA-6, and CGA-8 groups when compared with the control and CGA-2 groups ($P < 0.05$). CGA-4 group showed the highest complement C_4 level than other four groups ($P < 0.05$). Besides, no significant differences were observed in LZM level between the control and CGA groups, excepting for CGA-2 group ($P > 0.05$). Of the immunological parameters, a cubic response (Cu) was found for the relationship between LZM and dietary CGA levels, while no relationship was found for C_3 and C_4 with dietary CGA levels. The optimal level of CGA was estimated to be 383.89-975.73 mg/kg for LZM.

3.5.2. Inflammatory Factors Expression. TGF-β expression was clearly increased in the CGA-6 group compared with CGA-2, CGA-4, and control diets ($P < 0.05$, Figure 1). No significant differences in IL-1β, IL-8, and TNF-α expression were observed among groups ($P > 0.05$).

4. Discussion

In the present study, CGA supplementation had no significant effects on the growth and feed efficiency in koi. Similar results were reported by Sun et al. [24], who found that the supplementation of 200-800 mg/kg CGA had no significant effects on the growth of grass carp (*Ctenopharyngodon idella*). Besides, dietary CGA had no significant effects on the growth performance of white shrimp (*Litopenaeus vannamei*) cultured under normal conditions for 56 days [42]. However, some studies showed the positive effects of dietary CGA on the growth performance of Chinese soft-shelled turtle (*Trionyx sinensis*) [43], Jian carp (*Cyprinus carpio* Jian) [44], and juvenile grass carp (*C. idella*) [45]. The inconsistent reports above may be due to the dose of CGA and different feeding periods of the trial, because Zhang and Wen [44] found that adding the high dose of CGA for a long period of time can inhibit the growth of fish. But such information is insufficiently comprehensive to explore for the mechanism of CGA on the growth of koi. So, further research is necessary for exploring this issue.

Ornamental quality is an important attribute to reflect the quality and value of koi. The main factors affecting the ornamental quality include skin pigmentation, shape of fin, fish size, and so on [46]. Brightness ($L*$), redness ($a*$), and yellowness ($b*$) can represent the condition of skin pigmentation in koi [47]. In this study, dietary CGA supplementation linearly increased $a*$ and $b*$ of koi, decreasing $L*$, which indicated that CGA has potential to improve skin pigmentation in koi. Especially, the increased $b*$ is beneficial for Yamabaki ogon, which means that the fish is more yellow in color. Moreover, dietary CGA improved the total carotenoid in the scale of koi at present study, which further indicated that CGA diets yielded better ornamental quality for koi by affecting pigmentation. This result is similar to the previous result that CGA and green bamboo extract as feed additives improved the body coloration of koi (*C. carpio* L.) [48]. The reasons might be as follows to explain CGA diets improve body pigmentation of koi in this study. Firstly, it may be related to the fact that CGA, as an antioxidant, reduces the consumption of pigments (carotenoids and pteridines pigments) by free radicals and increases the deposition of pigments in body [49, 50]. Besides, it is also possible that CGA promotes the normal migration, differentiation, and development of xanthophore and erythrophore to increase the number of mature pigment cells by improving the antioxidant effect of liver [49, 50]. In fact, this study found that CGA could really improve the antioxidant

Table 5: Antioxidant activities and immunological and biochemical parameters in serum of koi fed the experimental diets for 10 weeks.

Item	Control	CGA-2	CGA-4	CGA-6	CGA-8	ANOVA *P* value	Regression[1] Model	*P* value	R^2	Op. level
Antioxidant activities										
SOD (U/mL)	53.34 ± 2.19^{a}	47.02 ± 3.12^{a}	76.28 ± 5.13^{b}	71.14 ± 3.06^{b}	69.77 ± 2.09^{b}	0.00	Ln	0.00	0.38	—
CAT (U/mL)	52.75 ± 2.53^{ab}	47.09 ± 6.98^{a}	68.84 ± 4.81^{c}	62.60 ± 2.33^{bc}	63.36 ± 3.62^{bc}	0.02	Ln	0.05	0.13	—
GSH-Px (U/mL)	841.88 ± 42.85^{b}	654.48 ± 17.28^{a}	1256.27 ± 114.64^{d}	1021.79 ± 46.84^{bc}	1050.17 ± 64.30^{c}	0.00	Cu	0.02	0.32	231.74-782.66
GSH (umol/L)	5.37 ± 0.15^{a}	7.30 ± 0.80^{b}	7.24 ± 0.14^{b}	10.08 ± 0.38^{c}	6.38 ± 0.31^{ab}	0.00	Qd	0.00	0.38	550.78
MDA (nmol/L)	5.14 ± 0.40^{b}	3.66 ± 0.14^{a}	3.50 ± 0.21^{a}	3.95 ± 0.20^{a}	4.20 ± 0.28^{a}	0.00	NR	—	—	—
Immunological parameters										
C_3 (g/L)	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.07 ± 0.00^{b}	0.07 ± 0.01^{b}	0.07 ± 0.01^{b}	0.00	NR	—	—	—
C_4 (g/L)	0.03 ± 0.00^{ab}	0.03 ± 0.00^{a}	0.05 ± 0.01^{c}	0.04 ± 0.00^{b}	0.04 ± 0.00^{b}	0.00	NR	—	—	—
LZM (U/mL)	159.40 ± 5.32^{b}	108.65 ± 4.56^{a}	169.30 ± 17.79^{b}	174.26 ± 6.51^{b}	167.22 ± 5.87^{b}	0.01	Cu	0.00	0.52	383.89-975.73
Biochemical parameters										
AST (U/L)	144.50 ± 10.94	138.80 ± 9.28	112.60 ± 14.82	153.83 ± 6.01	127.40 ± 5.90	0.00	NR	—	—	—
ALT (U/L)	5.00 ± 0.73^{b}	1.80 ± 0.80^{a}	1.33 ± 0.21^{a}	1.50 ± 0.50^{a}	2.00 ± 0.45^{a}	0.00	Qd	0.00	0.50	522.78
ALP (U/L)	13.00 ± 1.10	13.83 ± 1.14	15.17 ± 0.87	15.67 ± 0.92	16.17 ± 0.91	0.01	NR	—	—	—

Note: means in the same row with different superscripts are significantly different ($P < 0.05$). [1]Regression: Ln: Linear; Qd: Quadratic; Cu: Cubic; NR: No relationship; Op. level: Optimal level of CGA.

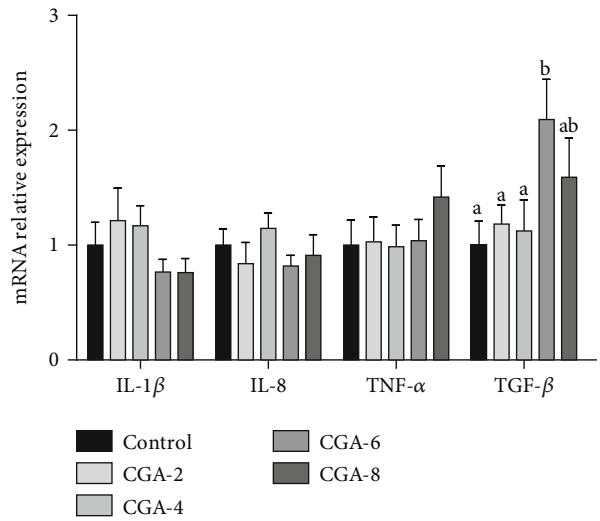

Figure 1: Effect of dietary CGA on relative expression of IL-1β, IL-8, TNF-α, and TGF-β genes in the head-kidney ($n = 6$ per treatment) of koi. Data are expressed as means ± standard error of mean. [ab]Means with different superscripts are significantly different ($P < 0.05$). Control, CGA-2, CGA-4 CGA-6, and CGA-8 expressed that chlorogenic acid (CGA) was supplemented at a level of 0, 200, 400, 600, or 800 mg/kg of basal diet, respectively.

capacity and liver function of koi (Table 5), which provided good evidence for the above inference. Combined with the results of ANOVA and polynomial contrast analysis in body color and total carotenoid, 400-600 mg/kg CGA was the optimal level for gaining the better body color.

The present study demonstrated that dietary CGA supplementation improved antioxidant activities of koi, evidenced by higher SOD, CAT, GSH-Px, GSH, and lower MDA. Yousef, Farsani, Ghafarifarsani, Hoseinifar, & Van Doan [51] indicated that the antioxidant activity of fish protected living cells against free radicals, inflammation, and apoptosis to maintain the health. Our result is consistent with previous studies in Jian carp (*C. carpio* var. Jian) [52]. Yang [53] also found that dietary with 400 mg/kg CGA can improve the antioxidant capacity of grass carp (*C. idellus*) by increasing SOD, CAT, and GSH-Px and decreasing MDA in the serum and liver. Previous studies showed that CGA can activate the Nrf2 signaling pathway to reduce ROS level and play antioxidant effect [54]. Nrf2 is an important transcription factor that regulates the cellular oxidative stress responses and maintains the intracellular redox homeostasis [55]. The activated Nrf2 can inductively regulate its downstream various antioxidant factors (such as SOD, CAT, and GSH-Px) and glutathione redox system to show antioxidant capacity. Moreover, Nrf2, as a master regulator of defensive mechanisms, plays an important role in regulation of innate immune responses under both physiological state and upon oxidative stress [56]. This view may better explain our results of CGA enhancing the immune response of koi. At the same time, it can also better combine the antioxidant with the capacity of immunity in koi fed with CGA. Based on the quadratic (Qd) and cubic (Cu) relationship polynomial models, it was determined that the optimal level of CGA to be 550.78 mg/kg and 231.74-782.66 mg/kg for GSH and GSH-Px, respectively, while SOD and CAT were linearly increased with CGA supplementation. These parameters reflected the better antioxidant activities in koi fed with 550.78 mg/kg CGA in the diet.

Previous studies showed that hepatocytes release cytokines and reactive oxygen species (ROS) when the liver is damaged, which lead to peroxidation of plasma and mitochondrial membranes, and finally necrosis or apoptosis [57, 58]. And the abundant ALT releases into the blood mostly during liver cell damage [31]. So, serum ALT level is always a common index to monitor the health condition of the liver [31]. The present result showed that ALT significantly decreased in serum of koi when those fed with CGA diets. Similarly, Lai, Chen, Lu, Ma, & Tang [59] also found that dietary CGA clearly reduced the serum ALT level of weaned piglets. The above results indicated that CGA has a great potential to protect the liver of animals. The mechanism may be that CGA inhibited inflammatory response and enhanced antioxidant defense system by regulating the MAPK, TLR3/4, and NF-κB signaling pathways in animals [60–62]. Just enough, the improved antioxidant capacity by CGA in the present study provides support for this deduction. And based on the quadratic (Qd) relationship polynomial models, the lowest level of ALT was gained in koi fed with 522.78 mg/kg CGA in the diet.

Nonspecific immunity, as the first line of defense, plays a crucial role against pathogen invasion [8, 63]. C_3, C_4, and LZM are important nonspecific immune factors to prevent microbial infection [64]. In the present study, serum C_3 and C_4 levels were obviously increased in koi fed diets supplemented CGA with 400 mg/kg to 800 mg/kg. Similarly, dietary 800 mg/kg CGA increased serum C_3 level in grass carp (*C. idellus*) [65]. The supplementation of 200 or 400 mg/kg CGA in the diet could significantly increase C_4 level in serum of Chinese soft-shelled turtle [66]. In addition, adding CGA in diets also clearly improved serum C_3 and C_4 levels of black carp (*Mylopharyngodon Piceus* Richardson) [67] and Jian carp (*C. carpio* var. Jian) [52]. Moreover, based on the cubic relationship between LZM and dietary CGA levels, the optimal level of CGA was estimated to be 383.89-975.73 mg/kg for LZM. Combined the above results with the reference reports mentioned above, it suggested that the 400-800 mg/kg CGA in the diet of koi improved the nonspecific immunity. At present, it is undoubted that CGA has the effect of enhancing immunity, but the mechanism is very complicated and is not clear [68]. Some studies considered that CGA can improve immunity of animal through their antioxidant properties, which included inhibition of lipid peroxidation and DNA damage [69]. Coincidently, our result of CGA in antioxidant activity was also confirmed this inference.

Not just that, our result showed that dietary 600 mg/kg CGA significantly improved the TGF-β expression in the head-kidney of koi. This result indicated that CGA maybe has great potential to play a role of preventing the inflammation occurrence and anti-inflammatory for koi, even other fish, which is consistent with previous reports that CGA has good anti-inflammatory activity [54, 70]. Besides, TGF-β also plays many important roles in the regulation of innate

immunity in aquatic animal [71]. And it has been shown a strong effect to regulate the activation states of monocytes, macrophages, and other immune cells [71]. Interestingly, CGA obviously increased the C_3 and C_4 of koi serum in this study, which further deduced that CGA can regulate innate immunity of fish by affecting TGF-β expression. Combined the immunological parameters in serum (400-800 mg/kg) with the TGF-β expression in the head-kidney (600 mg/kg), it suggested that the dietary with 600 mg/kg can improve the immune response of koi.

According to the polynomial contrast analysis result, the optimal level of CGA was estimated to be 400-600, 550.78, 522.78, and 600 mg/kg for body color, antioxidant activity, biochemical parameters, and immune response, respectively. Combined with the above results, it suggested that the 522.78-600 mg/kg CGA in the diet is beneficial for a healthy aquaculture of koi.

5. Conclusions

Dietary CGA improved the body color, antioxidant activity, biochemical parameters, and immune response of koi without significant effect on the growth of koi. Based on the model observed, the recommended CGA supplementation for koi was 522.78-600 mg/kg diet.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

The research protocols were approved by the animal care and use committee of Beijing Fisheries Research Institute, China.

Conflicts of Interest

The authors have no conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgments

This work was financially supported by the Beijing Innovation Consortium of Agriculture Research System (BAIC0320200204).

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