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Effects of dietary citric acid on growth performance, mineral status and intestinal digestive enzyme activities of large yellow croaker *Larimichthys crocea* (Richardson, 1846) fed high plant protein diets

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ABSTRACT

The positive effects of citric acid (CA) on aquaculture species have been reported. However, extensive application of CA needs a comprehensive understanding of its nutritional functions. A 9-week feeding trial was conducted to determine the effect of dietary CA on growth performance, tissue mineral content, intestinal enzyme activities and oxidative status of large yellow croaker *Larimichthys crocea* fed high plant protein diets. Six isonitrogenous and isolipidic diets were formulated and fed to triplicated groups of fish. A high fish meal diet formulated with 45% fish meal and 11.5% soybean meal was set as the positive control diet, while a high plant protein diet formulated with 31.50% fish meal and 30.63% soybean meal was used as the negative control diet. The other four diets were supplemented with 0.4%, 0.8%, 1.6% and 3.0% of CA into the negative control diet, respectively. The results showed that the specific growth rate, feed efficiency, protein and phosphorus retention, phosphorus and zinc concentrations in whole body and intestine, activities of the leucine-aminopeptidase, alkaline phosphatase and Na^+, K^+-ATPase were significantly reduced after soybean meal replacement and recovered by dietary CA supplementation ($P < 0.05$). Data on oxidative stress and anti-oxidative responses of intestine showed that the content of malondialdehyde was significantly increased in soybean meal-enhanced diets, which decreased with supplementation of CA varying from 0.4% to 1.6% ($P < 0.05$). The total anti-oxidative capacity, activities of total superoxide dismutase and Cu–Zn superoxide dismutase were decreased by soybean meal replacement and increased as CA level increasing from 0.4% to 0.8% ($P < 0.05$). In conclusion, 0.8–1.6% of CA in diet is helpful for large yellow croaker fed high plant protein diets to get better growth performance. The improvement of growth performance could be partly due to the increased mineral bioavailability, enhanced intestinal antioxidant capacity and recovered intestinal function by dietary CA supplementation.
Statement of relevance: This study is not a test of commercial aquaculture.

1. Introduction

Large yellow croaker *Larimichthys crocea* is one of the most successful marine fish culture operations in terms of the number of juveniles produced and commercial size fish production annually in China (Liu et al., 2008). The production of this farmed species was more than 120,000 metric tons in 2014 (China Fishery Statistical Yearbook, 2015). However, chopped or minced trash fish is still the major diet for large yellow croaker, which brought about resource waste and water pollution. Increased demands and limited supply led to the expensiveness of fish meal (Hardy, 2010), which caused higher cost and less market share of formulated feed compared with trash fish. Therefore, replacing some of the fish meal by plant protein ingredients with lower cost could be a feasible way for sustainability of large yellow croaker culture.

Suppressed growth performance has been reported in various carnivorous fish species fed feed containing higher plant protein ingredients (Kaushik et al., 1995; Burrells et al., 1999), including large yellow croaker (Zhang et al., 2008a). The reduced growth performance is partially explained by the presence of anti-nutritional factors in plant protein sources (Francis et al., 2001). Moreover, differences of the nutritional composition and nutritional availability between plant protein ingredients and fish meal, including sulfur amino acid, hydroxyproline, phosphorus (P) and zinc (Zn) (Gatlin and Wilson, 1984; Savolainen and Gatlin, 2010; Vandenberg et al., 2011; Liu et al., 2014), also result in growth reduction. The adverse effects of fish meal replacement on digestive capacity, intestinal morphology and oxidative status have been widely published as well (Burrells et al., 1999; Krogdahl et al., 2003; Dong et al., 2013). Dietary organic acids have been shown to engender positive effects, such as enhanced growth (Pandey and Satoh, 2008;

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Goosen et al., 2011; Zhu et al., 2015), increased dietary nutrition availability and elevated activities of digestive enzymes (Vielma et al., 1999; Sarker et al., 2007; Castillo et al., 2014). In this context, organic acids may be helpful in overcoming some problems brought by fish meal replacement.

Citric acid (CA), as one kind of organic acid, has been reported to increase the availability of dietary minerals in aquaculture species when used as feed additive (Sugiura et al., 1998; Sarker et al., 2005), in some cases, supplementation of CA could trigger beneficial effects on growth performance (Sarker et al., 2007; Castillo et al., 2014). Based on an analysis of published data, the growth-promoting effect of CA is partly due to the gastrointestinal acidification and antimicrobial effects in pig (Partanen and Mroz, 1999). It is generally considered that lower gastric pH induced by CA result in increased activity of digestive enzymes, which lead to higher nutrients availability (Castillo et al., 2014; Márquez et al., 2012). In addition, the chelation and complex formation of the minerals can be affected by CA, resulting in increased bioavailability of dietary minerals (Khajepour and Hosseini, 2012a; Zhu et al., 2015). Many studies on effects of CA on growth, immune responses and mineral utilization in aquatic animals had been published (e.g., Baruah et al., 2005; Baruah et al., 2007; Ng and Koh, 2011). However, successful application of CA in aquafeeds requires further understanding of its mode of action.

Minerals are essential for growth and health in fish. Some kinds of mineral serve as a cofactor or activator for enzyme systems (NRC, 2011), such as Mn superoxide dismutase (Mn SOD) and Cu–Zn SOD, which are both capable of ameliorating oxidative stress and show positive correlation with mineral status respectively (De Rosa et al., 1980; Okado-Matsumoto and Fridovich, 2001; Ma et al., 2014; Zhang et al., 2016). Considering that replacement of fish meal could suppress growth through dysfunction of intestinal digestion and absorption which was in part attributed to oxidative damage (Zhang et al., 2013), alleviated intestinal oxidative damage brought by increased mineral status may be a mode of action of CA for promoting growth. However, the effects of CA on intestinal health and function have not been investigated in detail.

Thus, the purpose of the present study was to investigate the effect of CA supplementation in diet on mineral status, intestinal oxidative status and intestinal absorption in large yellow croaker fed high plant protein diet. The study would contribute to dietary fish meal replacement also.

Table 1
Composition and proximate analysis of the experimental diets (% dry matter).

Ingredient	FM	SBM	0.4% CA	0.8% CA	1.6% CA	3.0% CA
Fish meal[a]	45.0	31.5	31.5	31.5	31.5	31.5
Soybean meal[a]	11.50	30.63	30.63	30.63	30.63	30.63
Wheat meal[a]	24.0	24.0	24.0	24.0	24.0	24.0
Fish oil[a]	2.7	3.7	3.7	3.7	3.7	3.7
Lecithin[a]	2.5	2.5	2.5	2.5	2.5	2.5
Mineral premix[b]	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin premix[c]	2.0	2.0	2.0	2.0	2.0	2.0
Attractant[d]	0.3	0.3	0.3	0.3	0.3	0.3
Ethoxyquin	0.05	0.05	0.05	0.05	0.05	0.05
Mold inhibitor[e]	0.1	0.1	0.1	0.1	0.1	0.1
Citric acid (CA)	0.0	0.0	0.4	0.8	1.6	3.0
Microcrystalline cellulose	9.85	3.22	2.82	2.42	1.62	0.22
Proximate composition						
Moisture (%)	6.5	6.6	6.7	6.7	6.7	6.7
Crude protein (%)	47.5	47.9	47.6	47.6	47.5	47.7
Crude lipid (%)	8.8	8.5	8.4	8.8	8.9	9.0
Ash (%)	9.1	8.2	8.2	8.4	8.3	8.3
P (g/kg diet)[f]	10.0	9.0	9.5	8.9	10.1	10.1
Available P (g/kg diet)[g]	7.7	6.2	6.2	6.2	6.2	6.2
Mn (mg/kg diet)	37.1	38.4	39.1	38.9	39.9	40.6
Cu (mg/kg diet)	13.2	14.2	11.6	14.3	12.8	12.0
Zn (mg/kg diet)	102.1	101.2	97.6	103.1	104.0	110.3
pH	6.24	6.26	6.02	5.85	5.53	5.22

[a] Those ingredients were supplied by Qingdao Great-Seven Bio-Tech, Co., Ltd. Shandong Province, China.
[b] Vitamin premix (mg/kg or g/kg diet): thiamin, 25 mg; riboflavin, 45 mg; pyridoxine·HCl, 20 mg; vitamin B12, 0.1 mg; vitamin K3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin, 1.20 mg; retinol acetate, 32 mg; cholecalciferol, 5 mg; 50% alpha-tocopheryl acetate, 240 mg; 35% ascorbic acid polyphosphate, 2000 mg; choline chloride, 2500 mg; ethoxyquin, 150 mg; microcrystalline cellulose, 13.892 g. All those ingredients were supplied by Qingdao Master Bio-Tech, Co., Ltd. Shandong Province, China.
[c] Mineral premix (mg/kg or g/kg diet): NaF, 2 mg; KI, 0.8 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $FeSO_4 \cdot H_2O$, 80 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $MnSO_4 \cdot H_2O$, 60 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; Ca $(H_2PO_4)_2 \cdot H_2O$, 3000 mg; NaCl, 100 mg; microcrystalline cellulose, 15.447 g. All those ingredients were supplied by Qingdao Master Bio-Tech, Co., Ltd. Shandong Province, China.
[d] Attractant: contained 50% glycine and 50% betaine. All those ingredients were supplied by Qingdao Master Bio-Tech, Co., Ltd. Shandong Province, China.
[e] Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid. All those ingredients were supplied by Qingdao Master Bio-Tech, Co., Ltd. Shandong Province, China.
[f] Analyzed data.
[g] Calculated data.

2. Materials and methods

2.1. Diet preparation

The formulation and proximate composition of the six experimental diets are shown in Table 1. Fish meal and soybean meal were used as the dietary protein sources. Fish oil and lecithin were used as the lipid sources. The positive control diet (FM) consisted of 45% fish meal and 11.5% soybean meal. The negative control diet (SBM) was formulated to have 31.50% of fish meal and 30.63% of soybean meal. Citric acid (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) with 99.5% purity was added into the SBM diet to formulate experimental diets with 4 graded levels of CA (0.4%, 0.8%, 1.6% and 3.0%, respectively). In addition, all six diets were supplemented with 0.05% of fumaric acid and 0.05% of calcium propionate as mold inhibitors. As shown in Table 1, the pH values of the experimental diets were decreased by the addition of CA.

2.2. Feeding trial

Large yellow croaker juveniles were obtained from Fufa hatchery (Ningde, Fujian Province, China). Juveniles were stocked in a large sea cage (4 × 4 × 4 m) to acclimate to the experimental condition and fed the FM diet for 2 weeks. Prior to the start of the feeding trial, the fish were not fed for 24 h, and then weighed after being anesthetized with eugenol (1:10,000) (Sinopharm Chemical Reagent Co., Ltd., SCR, Shanghai, China). Fish with similar size (initial mean weight 7.71 ± 0.02 g) were distributed to 18 sea cages (1.5 × 1.5 × 2.0 m) at density of 70 fish per cage. Each diet was hand-fed to triplicate groups of fish twice daily (05:00 and 17:00) to apparent satiation for 9 weeks. During the feeding trial, the water temperature ranged from 27.4 to 33.2 °C, salinity varied between 30 and 33‰ and dissolved oxygen was higher than 7 mg/l. Fish were reared under natural light conditions (about 14 L:10D) throughout the feeding trial. And the total amount of feed offered to each replicate (cage) was 1.2 kg during the feeding trial.

2.3. Sample collection and analysis

At the beginning of the feeding trial, twenty fish were sampled and stored frozen (−20 °C) for the analysis of the initial whole-body composition. At the end of the feeding trial, animals were not fed for 24 h and anesthetized with eugenol. They were counted and weighted to calculate the survival rate, specific growth rate (SGR) and feed efficiency (FE). Five fish were randomly selected from each cage to measure the weight and length individually and stored frozen (−20 °C) to determine the whole-body proximate composition and mineral composition. Another five fish were randomly selected from each cage and dissected to obtain intestine for intestine somatic index calculation, meanwhile, intestines were stored frozen (−20 °C) for mineral analysis. Nine fish

Table 2
Effects of dietary citric acid (CA) on growth performance and feed utilization of large yellow croaker.

Treatments	FBW (g)	SGR (%·d-1)	FE	Protein retention (%)	P retention (%)	Survival (%)
FM	28.11 ± 0.74[a]	2.05 ± 0.05[a]	1.09 ± 0.03[a]	35.76 ± 0.88[a]	47.37 ± 2.24[a]	84.29 ± 1.43
SBM	25.49 ± 0.17[c]	1.90 ± 0.01[c]	0.91 ± 0.01[d]	28.96 ± 0.20[d]	27.89 ± 2.80[c]	82.86 ± 2.86
0.4% CA	26.11 ± 0.26[bc]	1.94 ± 0.02[bc]	0.97 ± 0.03[c]	31.25 ± 1.74[cd]	30.72 ± 1.19[bc]	84.76 ± 3.59
0.8% CA	26.53 ± 0.11[b]	1.96 ± 0.01[b]	1.00 ± 0.01[bc]	32.50 ± 0.86[bc]	34.78 ± 5.29[bc]	81.91 ± 2.98
1.6% CA	27.69 ± 0.09[a]	2.03 ± 0.01[a]	1.06 ± 0.02[a]	34.81 ± 0.63[ab]	36.97 ± 2.23[b]	83.81 ± 5.41
3.0% CA	27.81 ± 0.38[a]	2.04 ± 0.02[a]	1.05 ± 0.02[ab]	33.79 ± 1.03[abc]	36.80 ± 2.92[b]	83.81 ± 2.18
ANOVA						
F value	25.201	23.171	33.981	18.480	14.571	0.291
P value	0.000	0.000	0.000	0.000	0.000	0.909

FBW, final body weight; SGR, specific growth rate; FE, feed efficiency.
Values (means ± S.E.) in the same column sharing a common superscript letter were not significantly different.

per cage were randomly chosen to collect intestines, and the intestine samples were stored frozen (−80 °C) for analysis of enzyme activities.

Samples of diets and whole fish body were dried to constant weight at 105 °C to determine moisture. Crude protein was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method (2300-Auto-analyzer, FOSS, Hillerød, Denmark), crude lipid by ether extraction using Soxhlet method (36680-analyzer, BUCHI, Flawil, Switzerland) and ash by combustion using a muffle furnace at 550 °C (AOAC, 1995). P, Mn, Cu and Zn concentrations in the diet, whole-body and intestine were analyzed using the inductively coupled plasma-atomic emission spectrophotometer (ICP-OES; VISTA-MPX, VARIAN, USA) (Tan and Mai, 2001). Duplicate analyses were conducted for each sample.

The intestine samples were rinsed with phosphate-buffered saline (PBS) to remove intestine contents and scraped for mucosal scrapings. The scrapings were homogenized in 9 volumes (*v*/*w*) of ice-cold (0 °C) normal saline and not centrifuged after homogenization. Aliquots of homogenates were stored at −80 °C before analysis. Activity of intestinal enzymes related to digestion and absorption were analyzed as follows. Briefly, activity of leucine-aminopeptidase (LAP) was measured as described by Appel (1974) using L-leucine *p*-nitroanilide as substrate. Activity of alkaline phosphatase (AKP) was measured colorimetrically with a commercial kit using 4-nitrophenylphosphate as substrate according to Barrett (1972). Activity of Na^+, K^+-ATPase was determined by colorimetric method with a kit using ATP as substrate according to Wang et al. (2015). Lipid peroxidation, protein oxidation and antioxidant status were assayed as below. Briefly, the content of malondialdehyde (MDA) in intestine was determined through measuring the pink color produced by the reaction of thiobarbituric acid (TBA) with MDA at 90–100 °C (Esterbauer and Cheeseman, 1990) using commercial kits. The content of protein carbonyl (PC) in intestine was evaluated by 2, 4-dinitrophenylhydrazine (DNPH) method (Lund et al., 2007) using commercial kits. Activities of the intestinal T-SOD and Cu–Zn SOD were analyzed based on the enzymes' ability to inhibit the oxidation of hydroxylamine catalyzed by the xanthine–xanthine oxidase system using commercial kits (Das et al., 2000). Activity of Mn-SOD was calculated by T-SOD activity minus Cu–Zn SOD activity. Protein contents in the homogenates were determined by Coomassie brilliant blue method (Bradford, 1976) using commercial kit. Total anti-oxidative capacity (T-AOC) in intestine was determined by colorimetric method as described by Miller et al. (1993) using commercial kits too. All the commercial kits mentioned above were provided by Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The parameters mentioned above were assayed by colorimetric method and absorbance was measured with the UV spectrophotometer (UV-2401PC, Shimadzu, Kyoto, Japan). Duplicate analyses were conducted for each sample.

2.4. Calculations and statistical analysis

The survival, growth, feed utilization and body indices were calculated by the following formulae:

$$\text{Survival rate}\ (\%) = 100 \times \text{final number of fish/initial number of fish}$$

$$\text{Specific growth rate}\ \left(\text{SGR}, \% \cdot \text{d}^{-1}\right) = 100 \times (\text{Ln final weight} - \text{Ln initial weight})/\text{days}$$

$$\text{Feed efficiency}\ (\text{FE}) = \text{weight gain/feed fed}$$

$$\text{Protein retention}\ (\%) = 100 \times (\text{final body protein} - \text{initial body protein})/\text{protein fed}$$

$$\text{P retention}\ (\%) = 100 \times (\text{final body P} - \text{initial body P})/\text{P fed}$$

$$\text{Condition factor}\ (\text{CF}) = 100 \times \text{fish weight}\ (\text{g})/\text{fish length}\ (\text{cm})^3$$

$$\text{Intestine somatic index}\ (\text{ISI}) = 100 \times \text{intestine weight/fish weight}.$$

Table 3
Effects of dietary citric acid (CA) on body condition indices and the whole-body composition of large yellow croaker.

Treatments	Intestine somatic index (%)	Condition factor (%)	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
FM	0.95 ± 0.01	1.16 ± 0.03	76.43 ± 0.14	16.15 ± 0.06	4.45 ± 0.28	3.60 ± 0.11[b]
SBM	0.94 ± 0.02	1.16 ± 0.06	75.77 ± 0.94	16.00 ± 0.04	4.27 ± 0.19	3.76 ± 0.06[ab]
0.4% CA	0.96 ± 0.03	1.16 ± 0.02	76.08 ± 0.61	16.07 ± 0.73	4.92 ± 0.35	3.83 ± 0.20[ab]
0.8% CA	0.96 ± 0.03	1.18 ± 0.01	76.57 ± 0.93	16.14 ± 0.37	4.26 ± 0.32	4.07 ± 0.14[a]
1.6% CA	0.99 ± 0.06	1.18 ± 0.03	76.55 ± 0.09	16.21 ± 0.10	4.37 ± 0.19	3.69 ± 0.06[ab]
3.0% CA	0.98 ± 0.05	1.14 ± 0.08	76.49 ± 1.39	16.00 ± 0.41	4.30 ± 0.50	3.88 ± 0.24[ab]
ANOVA						
F value	0.761	0.480	0.457	0.159	1.779	3.475
P value	0.595	0.784	0.800	0.973	0.192	0.036

ISI, intestine somatic index; CF, condition factor.
Values (means ± S.E.) in the same column sharing a common superscript letter were not significantly different.

Table 4
Effects of dietary citric acid (CA) on mineral concentrations in the whole body and intestine of large yellow croaker.

Treatments	Whole body				Intestine			
	P (g/kg)	Mn (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	P (g/kg)	Mn (mg/kg)	Cu (mg/kg)	Zn (mg/kg)
FM	16.50 ± 0.44^{a}	10.68 ± 0.48^{abc}	5.68 ± 0.52	171.45 ± 10.55^{a}	22.75 ± 1.75^{a}	32.38 ± 1.18	9.50 ± 0.70	328.95 ± 11.26^{a}
SBM	11.43 ± 0.33^{d}	10.52 ± 0.50^{bc}	5.46 ± 0.37	103.47 ± 6.19^{d}	18.00 ± 1.31^{c}	32.13 ± 1.03	9.33 ± 0.47	265.38 ± 6.90^{c}
0.4% CA	12.34 ± 0.16^{cd}	10.04 ± 0.35^{c}	5.40 ± 0.59	120.43 ± 8.51^{cd}	19.67 ± 0.96^{bc}	30.85 ± 0.62	9.22 ± 0.58	278.82 ± 5.17^{c}
0.8% CA	12.79 ± 0.90^{bc}	10.26 ± 0.26^{bc}	5.12 ± 0.29	140.29 ± 7.94^{bc}	18.62 ± 0.74^{bc}	31.27 ± 2.00	8.30 ± 0.38	276.72 ± 5.24^{c}
1.6% CA	14.08 ± 0.51^{b}	12.09 ± 0.77^{a}	5.63 ± 0.19	154.05 ± 10.50^{ab}	20.99 ± 0.13^{ab}	32.51 ± 1.15	9.23 ± 0.31	308.31 ± 1.55^{b}
3.0% CA	14.07 ± 0.21^{b}	11.51 ± 0.63^{ab}	5.20 ± 0.33	154.44 ± 8.88^{ab}	21.11 ± 0.26^{ab}	31.82 ± 0.46	8.83 ± 0.35	308.36 ± 3.40^{b}
ANOVA								
F value	39.383	6.790	0.904	23.601	8.832	0.912	2.397	44.256
P value	0.000	0.003	0.510	0.000	0.001	0.505	0.100	0.000

Values (means ± S.E.) in the same column sharing a common superscript letter were not significantly different.

Data from each treatment were subjected to one-way analysis of variance (ANOVA). When overall differences were significant, Tukey's test was used to compare the mean values among the treatments by SPSS 16.0 (SPSS Inc., Chicago, USA). The relationships between different indices were examined in Pearson correlation using SPSS16.0.

3. Results

3.1. Growth performance and feed utilization

The data on growth and feed utilization are presented in Table 2. The final body weight (FBW), specific growth rate (SGR), feed efficiency (FE), protein retention and P retention of fish fed SBM diet were significantly lower than those in fish fed with FM diet ($P < 0.05$). However, the parameters mentioned above increased significantly ($P < 0.05$) as inclusion of CA increasing from 0.4% to 1.6%, then leveled off with the further increasing of dietary CA from 1.6% to 3.0%. As shown in Table 2, survival rate ranging from 81.9 to 84.8% was not affected by CA.

3.2. Body indices and body composition

The results of body indices and body composition are shown in Table 3. Intestine somatic index and condition factor were not affected by different experimental diets. There were also no significant differences in contents of moisture, crude protein and crude lipid among all treatments, while fish fed 0.8% CA had significantly highest ash contents ($P < 0.05$).

3.3. Mineral status

The mineral concentrations in the whole body and intestine are listed in Table 4. Replacement of fish meal by soybean meal significantly reduced P and Zn concentrations in the whole body and intestine of fish, while supplementation of CA significantly improved concentrations of P and Zn in both tissues as CA level increasing from 0.4% to 1.6% ($P < 0.05$). No further increase was found when CA level increased to 3.0%. Fish fed diet with 1.6% CA showed significantly higher Mn concentration compared to fish fed SBM diet ($P < 0.05$). Intestinal Mn and Cu concentrations were not significantly affected by dietary treatments ($P > 0.05$).

3.4. Digestive enzyme activities

The activities of intestinal digestive enzymes are presented in Table 5. Replacement of fish meal by soybean meal reduced activities of the LAP, AKP and Na^+, K^+-ATPase ($P < 0.05$). The activity of LAP increased significantly as inclusion of CA increasing from 0.4% to 0.8% ($P < 0.05$). There was no further increase as CA level increasing from 0.8% to 3.0%. The activity of AKP increased as CA level increasing from 0.4% to 1.6%, while there was no significant difference between fish fed the SBM diet and that fed the diet supplemented with 1.6% CA. The activity of Na^+, K^+-ATPase increased significantly as inclusion of CA increasing from 0.4% to 1.6% ($P < 0.05$).

3.5. Oxidative stress and anti-oxidative responses

The data on oxidative stress and anti-oxidative responses in intestine are shown in Table 6. Contents of MDA were increased by fish meal replacement, while supplementation of CA from 0.4% to 1.6% reduced contents of MDA. Content of PC was not significantly affected by diet treatments ($P > 0.05$). Fish meal replacement significantly decreased T-AOC, which was significantly increased as CA level increasing from 0.4% to 0.8% and reached a plateau thereafter ($P < 0.05$). Activities of T-SOD and Cu–Zn SOD displayed the same changing pattern as T-AOC. Activity of Mn SOD was not significantly affected by dietary treatments ($P > 0.05$).

3.6. The correlation analysis

As shown in Table 7, activities of LAP, AKP and Na^+, K^+-ATPase were significantly and positively correlated with intestinal P, Zn concentrations, T-AOC, T-SOD and Cu–Zn SOD activities. There were significant negative correlations between activities of LAP, AKP and Na^+, K^+-ATPase and contents of MDA in intestine.

4. Discussion

The present study showed that the partial replacement of fish meal with soybean meal significantly decreased the growth performance, which is in agreement with previous findings in rainbow trout (Kaushik et al., 1995), Atlantic salmon (Krogdahl et al., 2003) and large yellow croaker (Zhang et al., 2008a). Furthermore, addition of 1.6% CA increased the SGR and FE in the present study, which is similar to other findings observed in red sea bream (Sarker et al., 2005), beluga (Khajepour and Hosseini, 2012a) and yellow catfish (Zhu et al., 2015). In

Table 5
Effects of dietary citric acid (CA) on activities of intestinal digestive enzyme of large yellow croaker.

Treatments	LAP	AKP	Na^+, K^+-ATPase
	(mU/mg protein)	(U/g protein)	(U/mg protein)
FM	65.49 ± 2.76^{a}	40.27 ± 4.43^{a}	1.62 ± 0.06^{a}
SBM	41.60 ± 1.98^{c}	28.56 ± 4.02^{b}	1.03 ± 0.08^{c}
0.4% CA	46.71 ± 2.61^{c}	29.96 ± 3.68^{b}	1.15 ± 0.11^{bc}
0.8% CA	55.34 ± 3.53^{b}	29.54 ± 1.74^{b}	1.16 ± 0.09^{bc}
1.6% CA	55.61 ± 2.06^{b}	34.64 ± 0.94^{ab}	1.32 ± 0.10^{b}
3.0% CA	57.93 ± 3.64^{ab}	36.18 ± 2.76^{ab}	1.35 ± 0.10^{b}
ANOVA			
F value	26.714	6.279	16.152
P value	0.000	0.004	0.000

LAP, leucine-aminopeptidase; AKP, alkaline phosphatase.
Values (means ± S.E.) in the same column sharing a common superscript letter were not significantly different.

Table 6
Effects of dietary citric acid (CA) on oxidative stress and anti-oxidative responses in intestine of large yellow croaker.

Treatments	MDA	PC	T-AOC	T-SOD	Cu–Zn SOD	Mn SOD
	(nmol/mg protein)	(nmol/mg protein)	(U/mg protein)	(U/mg protein)	(U/mg protein)	(U/mg protein)
FM	2.59 ± 0.33^{c}	9.49 ± 1.23	4.92 ± 0.43^{a}	137.52 ± 8.33^{a}	91.30 ± 8.27^{a}	46.22 ± 0.29
SBM	3.85 ± 0.11^{a}	8.97 ± 1.23	2.97 ± 0.24^{c}	87.26 ± 4.54^{d}	43.54 ± 4.49^{d}	43.72 ± 5.37
0.4% CA	3.27 ± 0.07^{b}	9.06 ± 0.75	4.10 ± 0.14^{b}	97.37 ± 9.75^{cd}	52.87 ± 5.13^{cd}	44.51 ± 4.62
0.8% CA	3.07 ± 0.22^{bc}	8.87 ± 0.74	4.84 ± 0.18^{a}	114.18 ± 7.03^{bc}	63.78 ± 7.64^{bc}	50.40 ± 1.67
1.6% CA	2.63 ± 0.15^{c}	9.30 ± 1.16	5.33 ± 0.22^{a}	115.89 ± 7.45^{bc}	74.88 ± 2.61^{ab}	41.01 ± 7.02
3.0% CA	2.82 ± 0.03^{bc}	9.69 ± 1.05	4.72 ± 0.30^{ab}	119.89 ± 4.97^{ab}	76.91 ± 6.10^{ab}	42.99 ± 2.77
ANOVA						
F value	20.436	0.275	29.218	17.844	25.022	1.711
P value	0.000	0.918	0.000	0.000	0.000	0.207

MDA, malondialdehyde; PC, protein carbonyl; T-AOC, total anti-oxidative capacity; T-SOD, total superoxide dismutase.
Values (means ± S.E.) in the same column sharing a common superscript letter were not significantly different.

contrast, it has been demonstrated that organic acid did not affect growth performance in rainbow trout (Vielma et al., 1999; Gao et al., 2011) and tilapia (Ng et al., 2009). The difference of growth performance could be attributed to fish species, organic acid type and dose used (Lückstädt, 2008). The growth improvement effects of CA might result from the increased mineral availability, recovered digestive function and alleviated intestinal oxidation.

Based on data of ingredient digestibility and mineral requirements, available P and Zn concentrations in the negative control diet were suboptimal to dietary requirement of large yellow croaker (Mai et al., 2006; Li et al., 2007; Zhang et al., 2008b). The increased P and Zn contents in the whole body composition brought by CA addition also confirmed that these two kinds of minerals were inadequate in the negative control diet. Considering that P and Zn are essential minerals for growth, the better growth could be in part ascribed to the improved bioavailability of minerals in diet. As mentioned above, contents of P and Zn in the whole body and intestine were elevated by CA addition. Different studies in fish also found that availability of minerals was regulated by dietary CA. For example, increased P or Zn concentrations had been observed in rainbow trout (Sugiura et al., 1998), red sea bream (Sarker et al., 2005) and beluga (Khajepour and Hosseini, 2012b). Inclusion of soybean meal in the diet has been reported to reduce the bioavailability of minerals due to the inhibitory effects of phytate (Gatlin and Wilson, 1984; Savolainen and Gatlin, 2010). The underlying mechanism is that phytic acid can bind trace elements such as zinc and render them unavailable to the animal, which is a fact well documented in aquatic species (Gatlin and Wilson, 1984; Gatlin and Phillips, 1989). Citric acid has been reported to intensify phytate dephosphorylation *in vitro* (Zyla et al., 1995). Lower pH can result in a higher dissociation of mineral compounds and formation of chelated mineral complexes that can be easily absorbed. In this study, lower pH values were recorded in the diets supplemented with CA. Thus, the improved mineral utilization in the current study might be in part due to the fact that CA liberated the minerals bounded by phytate in soybean meal, making more available minerals for absorption. The increased utilization of minerals in the present study is in contrast with that reported in yellow catfish. Zhu et al. (2015) reported that addition of 2 g/kg CA did not influence the mineral utilization of yellow catfish. The useful dose of CA used in the present study was 16 g/kg diet, which was similar to that used in rainbow trout and beluga (Sugiura et al., 1998; Khajepour and Hosseini, 2012a) and far larger than that used in yellow catfish. Therefore, the different results are likely due to the dose of citric acid supplemented.

Table 7
The Pearson correlation analysis between different indices.

Prameter 1	Prameter 2	R1	P1
LAP	Zn	0.826	0.000
	MDA	−0.816	0.000
	T-AOC	0.794	0.000
	Cu–Zn SOD	0.895	0.000
AKP	Zn	0.800	0.000
	MDA	−0.712	0.001
	T-AOC	0.516	0.028
	Cu–Zn SOD	0.860	0.000
Na^+, K^+-ATPase	Zn	0.907	0.000
	MDA	−0.722	0.001
	T-AOC	0.529	0.024
	Cu–Zn SOD	0.845	0.000

R is the Pearson correlation coefficient, *P* is the significance.
LAP, leucine-aminopeptidase; AKP, alkaline phosphatase.
MDA, malondialdehyde; T-AOC, total anti-oxidative capacity; Cu–Zn SOD, Cu–Zn superoxide dismutase.
Positive R and $P < 0.05$ indicate the positive correlation between the two variables, while negative R and $P < 0.05$ indicate the negative correlation between the two variables.

In the present study, replacement of fish meal by soybean meal attenuated activities of the LAP, AKP and Na^+, K^+-ATPase, while the activities were increased by CA supplementation. When it comes to fish meal replacement, similar findings were observed that replacement of fish meal by soybean meal decreased the activity of digestive enzyme activities in Atlantic salmon (Krogdahl et al., 2003; Bakke-McKellep et al., 2007), tilapia (Lin and Luo, 2011) and large yellow croaker (Zhang et al., 2012). The attenuated intestinal digestive enzyme activities caused by soybean meal might result from the destruction of intestinal integrity. Numerous studies observed that fish meal replacement caused intestine histological damages. Bakke-McKellep et al. (2007) reported that the decreased activities of brush border digestive enzymes were concomitant with intestinal pathological changes. Supplementation of CA recovered intestinal digestive function in the present study, similar results have been found in red drum (Castillo et al., 2014) and tilapia (Li et al., 2009). The interpretations could not be attributed to the decreased pH of digesta directly, because the enzymes analyzed here were sampled from intestinal brush border. Castillo et al. (2014) ascribed it to the enhanced number of beneficial bacteria. As mentioned earlier, the recovered digestive function is related to the recovered intestinal integrity also. However, intestinal structure was not analyzed in this study. Further study on intestine histology in response to the supplementation of dietary CA is needed. Accordingly, these results indicated that dietary CA exerted a mitigating role against the dysfunction of intestine caused by soybean meal inclusion.

Although histological damage of intestine was not recorded in the present study, parameters related to intestinal oxidative damage were analyzed. Previous studies showed that the reactive oxygen species (ROS) produced as by-products of metabolism could oxidize cell constituents such as lipids, protein and DNA, and thus pose a threat to cell structure and function when generation rate exceeds their removal (Lebovitz et al., 1996). The content of MDA, as one of the end products of lipid peroxidation, was significantly higher in fish fed the plant based diet, indicating that fish meal replacement induced intestinal oxidative damage. In contrast, supplementation of CA increased T-AOC, T-SOD and Cu–Zn SOD activities and decreased contents of MDA. The alleviated oxidative damage caused by CA could be interpreted in terms of

increased mineral availability. As discussed above, available Zn in the plant based diet were inadequate, while CA supplementation alleviated Zn deficiency in the present study. Zinc plays an important role in enhancing antioxidant status and decreasing lipid peroxidation (Anderson et al., 2001). Previous studies also reported that T-SOD activity increased while MDA decreased as dietary Zn increased (Kucukbay et al., 2006; Ma et al., 2014). Therefore, alleviated oxidative damage could be in part due to increased Zn availability caused by dietary CA.

The results of Pearson correlation analysis suggested that the intestinal digestive function was negatively correlated with intestinal oxidation, but positively correlated with the anti-oxidative capacity. In this context, the potential action pathway of the improvement of growth performance of large yellow croaker by dietary CA could be that dietary CA supplementation in soybean meal based diet can increase mineral availability and alleviate intestinal oxidative damage, eliminate damage of intestine, subsequently recover the function of digestive capacity, and finally displayed the growth-promoting effect.

5. Conclusion

Based on the growth performance, in the present study, the following two conclusions can be drawn. The one is that dietary CA at 0.8–1.6% dose is beneficial for the growth of large yellow croaker fed high plant protein diets. The other one is that the significantly better growth performance brought by dietary CA supplementation could be due to the increased mineral availability, alleviated intestinal oxidation and recovered digestive function.

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