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Effects of *Arthrospira platensis* on growth, skin color and digestive enzymes of Koi, *Cyprinus carpio*

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Abstract

This study evaluated the effects of diets containing 0, 2.5, 5, 7.5 and 10% *Arthrospira platensis* on skin pigmentation, growth performance, and digestive enzymes of koi, *Cyprinus carpio*. A completely randomized design was performed with five treatments and three replicates. One hundred and fifty koi fish with average initial weight of 30±1g were assigned to fifteen experimental tanks. The experiment lasted for eight weeks. Koi fish fed with 2.5% *A. platensis* (1.17±0.02) showed a significant lower growth performance than those fed with 7.5 and 10% *A. platensis* (1.39±0.02 and 1.46) (p<0.05). However, carotenoid (astaxanthin) concentrations of skin significantly increased with increased levels of dietary algae (p<0.05). The highest values of a* (red zones) and b* (yellow zones) were observed in fish skin fed with 10% *A. platensis* (4.33±0.15, 5.23±0.15, 5.23±0.15, 5.7±0.36, and 6.33±0.32, respectively). The activities of protease, amylase and lipase in dietary supplementation of 10%*A. platensis* were significantly higher than those of the control group (protease from 5.17±0.76 to 11 , amylase from 22±1.73 to 32.67±1.53 and lipase from 16.33±2.08 to 74.33±1.53. Liver enzymes also decreased significantly by increased dietary supplementation. (ALT: from 192.33±5.5 to 80.67±1.52, AST: from 1741.7±18.92 to 712.33±10.5, ALP: from 452.33±8.14 to 48±1, LDH: from 7287.3±34.64 to 7119.7±17.89). These results revealed that inclusion of 5-10% *A. platensis* in diets has a significant positive effect on growth rate, pigmentation, and improvement of digestive and liver enzymes activities in koi fish.

Keywords: *Arthrospira platensis*, Pigmentation, Growth, Carotenoids, Enzymes activities, Koi

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Introduction

The koi carp is one of the economically important ornamental fish in China. Because of its numerous color combinations, the koi has grown into one of the most popular pet hobbies in the world (Lin *et al*., 2012). More than 100 different types of coloration have been developed for these fish, which are valued as the pet animal. Color is one of the most important quality criteria dictating the market value of koi. Since fish cannot synthesize the appropriate amount of carotenoids de novo, they rely on a dietary supply of these pigments to achieve their natural skin pigmentations. Fish use carotenoids, the most important groups of natural pigments, for pigmentation of their skin and flesh (Sun *et al.*, 2012). There are four main pigment groups that give color to skin and tissues of animals and plants, namely: melanin, pteridium, purin, and carotenoid (Kop and Durmaz, 2008). Carotenoids are responsible for red, orange and yellow colors of fish and crustaceans (Kop and Durmaz, 2008). About 90% of carotenoids found in tissues are located in flesh in the free form, but large amounts are also found in the skin which varies in maturing fish. Skin color is primarily dependent on the presence of chromatophores, which are large, star-shaped, pigment-containing cells located in the skin (Teimouri *et al*., 2013).

Recent efforts have focused on natural compound alternatives to synthetic carotenoids because of health concerns about the use of synthetic additives and their high cost (Storebakken *et al*., 2004). Carotenoids, particularly those that are vitamin A precursors, have received increasing attention in recent years (Krinsky, 1991).

However, research on diet optimization to enhance fish health is still unknown (Abdel-Tawwab *et al*., 2008). Spirulina, *Arthrospira platensis* is a freshwater blue-green filamentous algae, which has received increasing attention for its bioactive components such as vitamins, minerals, polyunsaturated fatty acids, carotenes and other pigments that have antioxidants activity (Lin *et al*., 2007; Wang *et al*., 2007; Teimouri *et al*., 2013). Moreover, *A. platensis* is a rich source of protein (60-70%). Therefore, its application in fish/shrimp feed has received more attention (Lu *et al*., 2002; Lu and Takeuchi, 2004; Palmegiano *et al*., 2005, 2008). *A. platensis* can also be considered as a nutritional supplement with various health benefits in humans and as a feed supplement for animals with some economic benefits (Kahn *et al*., 2005; Watanuki *et al*., 2006; Tongsiri *et al*., 2010). Spirulina also improves the intestinal flora in fish causing breakdown of indigestible feed components to extract more nutrients from the feed together with stimulating the production of enzymes of the alimentary tract (Mustafa *et al*., 1994; Nandeosha *et al*., 2001; Dernekbasi *et al*., 2010; Promya and Chitmanat, 2011;

Teimouri *et al*., 2013). Therefore, the aim of this study was to assess the effect of *A. platensis* as a dietary supplement on growth performance, carotenoids skin (astaxanthin) and digestive enzymes of koi carp (*C. carpio*).

Materials and methods

Feed preparation

Five diets were formulated using the microalgae. *A. platensis* (Sinamicroalgae Co., Qeshm, Iran). Basal diet formulation and proximate composition of *A. platensis* analysis (AOAC, 2005) are shown in Tables 1 and 2. The algae were substituted at concentrations of 0, 2.5, 5, 7.5 and 10% of the diet. The basal diet was considered as a control. Dietary feed ingredients were ground using a laboratory grinder and then blended into homogenous dough by adding water prior to adding the *A. platensis*. The pellets were obtained by pressing the mixed materials through a 4-mm die in a grinding machine. The pellets were than stored in plastic containers at 4°C until used (Alishahi *et al*., 2011). All fish were fed with the control diet during the first 7 days after stocking to adapt them to the new feeding regime.

Fish rearing conditions

Growth parameters, weight and length of all fish were measured every 15 days interval and at the end of the trial, individual weights of fish were obtained using a digital balance (1 mg precision) (Wangmi *et al.*, 2009). After an 8-week feeding period, weight gain (g kg-1), specific growth rate (SGR g kg-1/day), feed conversion ratio (FCR), condition factor (CF g cm^{-3}) and survival rate (g kg-1) were calculated according to the following equations:

Weight gain = (final wet weight- initial wet weight)

Feed conversion ratio = (dry feed intake/wet weight gain)

Specific growth rate = (100×[ln final weight - ln initial weight]/trial duration)

Protein efficiency ratio = (wet weight gain/dry protein intake)

Survival = (final number of fish /initial number of fish)×100.

Carotenoid and astaxanthin analysis

The total carotenoid content in feed, skin and algae samples was measured according to the methods of AOAC (1995) with slight modifications. Samples of 1g skin were collected from both lateral sides between the abdominal and dorsal regions of the fish with a careful removal of adhering adipose tissue. The samples were then transferred to 10 mL pre-weighed glass tubes and grounded into 10 mL 98% acetone (Merck, Germany) containing anhydrous sodium sulfate with a homogenizer (Ultra-turrax IKA®T18 basic).

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Table 1: Ingredients and proximate composition of the experimental diets (g $^{kg-1}$).

Ingredients	control
Fish meal	150.0
Soy bean meal	170.0
Soy bean, full fat	80.0
Solvent-extracted cotton seed meal	110.0
Wheat shorts	250.0
Wheat flour	150.0
Attapulgite meal	40.0
Vitamin/minerals premix[a]	100
Soy bean oil	200
Proximate composition(%)	88.3
Dry matter	30.7
Crud protein	5.3
Crud fat	10.4
Ash	88.3

a: Vitamin premix (mg $^{kg-1}$): thiamine-HCl, 8.0; riboflavin, 8.0; niacin mix, 100.0; pyridoxine-HCl, 20.0; cyanocobalamine, 0.1; pantothenate, 20.0; biotin, 1.0; inositol, 100.0; folic acid, 5.0; ascorbic acid, 250.0; Vitamin A, 20.0; Vitamin D, 8.0; Vitamin E, 150.0; Vitamin K, 10.0; BHT,10.0; α-cellulose, 1289.9. Mineral premix (mg $^{kg-1}$): $MgSO_4 \cdot 7H_2O$, 300.0; $FeSO_4 \cdot 7H2O$, 180.0; $ZnSO_4 \cdot 7H_2O$, 120.0; $MnSO_4 \cdot 7H_2O$, 35.0; KI, 0.65; Na_2SeO_3, 0.5; $CoCl \cdot 6H_2O$ (1%), 7.0; $CuSO_4 \cdot 5H_2O$, 5.0; zeolite, 7351.85.

Table 2: Proximate composition of spirulina and fish meal used in the experiment (%).

Ingredients fiber	Crud protein	Lipids	Ash	Crude
A. platensis	66.93	1.75	8.70	1.77
Fish meal	68.63	11.46	13.63	-

The samples were then stored for one day at 4°C, and extracted with acetone two or three times until no more color could be seen. The solutions were centrifuged at 3500 rpm for 10 min, and optical densities were measured by a spectrophotometer (UnicoS-2150UV) at 450 nm. A similar method was used for total carotenoid analysis of *A. platensis*. Total carotenoid concentration of skin and algae were determined spectrophotometrically in 98% acetone (Sing extinction coefficients; E1% 1CM) 2500)

Astaxanthin was analyzed by high performance liquid chromatography (HPLC), using a Hitachi L-6200 pump, a silica column (Lichrosorb Si-60 5 micro 2504.6 mm column I.D., E. Merck Company), a Hitachi L-4250 UV-VIS detector at 470 nm, and a Hitachi D-2000 Chromato-Integrator. The operational conditions were: mobile phase, 14% acetone in hexane; solvent flow rate, 1.5 mL min^{-1}; injection volume, 100 Al; and pump program, the sequence was 0–20 min Mixture A (acetone:n-hexane, 14:86) and 20.5–40 min Mixture B (100% n-heptane). This system was controlled by a chromatographic data system (Scientific Information Services Corporation), which also integrated the areas under the peaks. The standard was chromatographically pure astaxanthin (Hoffman La Roche Ltd., Basel, Switzerland).

Photograph analysis

At the end the trial, three or five fish from each treatment were randomly caught to evaluate their skin color. As koi carp is an ornamental fish, it is necessary to use a simple, rapid and accurate tool to analyze the color of the live animal. Photographs were taken according to Tlusty and Hyland (2005). Nikon D80 digital SLR camera was used for this purpose. The camera was mounted on a tripod between the two light sides. The camera was set up at 25 cm above the specimens and could capture the whole fish image. Photographs of skin (whole fish) and fillet were taken under these conditions: shutter speed was 10, the aperture was F16 and the zoom was 35. The images were analyzed with Adobe Photoshop CS4 software (version 11). Pictures were opened in RGB mode.

In this study, skin color was assessed with flectance spectroscopy with transformation into color parameters based on the tristimulus values, L*, a*, b* and dE, representing lightness, redness, yellowness and chromatic aberration, respectively. The original adjusted value of the white standard was L*=97.40±0.01, a*=-0.1±0.01, b*=1.92±0.01). The measurements were performed on the largest zone of black, red, L* and b* were measured in the white color zones. L*and a* were measured in the red color zones.

Digestive enzymes analysis

Five fish from each tank were anaesthetized with clove oil in 140 mg $^{L-1}$ after 24 h starvation and the blood samples were obtained from the caudal vein with a 27 gauge needle. The pool blood samples were collected into heparinized Eppendorf tubes and the sera samples were separated by centrifugation at 300 ×g for 10 min at 4°C. The sera samples were frozen at -80°C until they were used (Alishahi *et al*., 2011).

Amylase activity was evaluated using 1% starch solution in 20 mM sodium phosphate buffer at pH 6.9 containing 6.0 mM NaCl as substrate (Worthington, 1993). Lipase activity was assayed based on the measurement of fatty acids release due to enzymatic hydrolysis of triglycerides in a stabilized emulsion of olive oil (Borlongan, 1990). Protease activity was measured using the casein method (Kunitz, 1947) and modified by Walter (1984) using substrate casein (1%) in 50 mM tris/HC1 buffer, pH 9 or universal buffer (Stauffer, 1989).

Liver enzymes analysis

Hepatopancreas enzymes (ALT, AST, LDH) were measured using the kinetic colorimetric method and ALP via kinetic enzymatic method according to Johnson *et al*. (2008).

Statistical analysis

All data were expressed as the mean±SD and verified for normality after transformation (ASIN). One-way

ANOVA was used to determine the effects of *A. platnsis* on growth performance and pigmentation and activity of enzymes using SPSS (version 18). Duncan's multiple range test was utilized to compare the mean values among the treatments due to the main effects. The differences were considered as significant at $p<0.05$. Linear regression analyses were also used to find the relationship between carotenoid contractions and color parameters.

Results

A. platensis supplemented diets did change growth parameters in koi fed with 10% *A. platensis* in comparison with the control diet. Final weight, weight gain, and specific growth rate increased significantly ($p<0.05$) with the increase in *A. platensis* levels in fish diets (Table 3). The optimum growth was obtained at the level of 7-10% *A. platensis* whereas the control diet produced the lowest fish growth. No significant differences were observed in fish survival rate among the different treatments ($p>0.05$). Fish fed on control diet consumed less diet giving a higher FCR (2.94%).

Color data showed that the fish fed diets containing pigments turned to pinkish at the end of the experiment (Tables 4 and 5). In contrast, all fish skins in the control group were poorly pigmented (4.33%). a* and b* increased with increasing the level of *A. platensis* in the diets. The fish fed with 10% *A. platensis* displayed more red and yellow dish hue than those of control and 2.5% *A. platensis*. The highest levels of skin pigmentation and carotenoid (astaxanthin) deposition were found in 10% *A. platensis* (6.33). Control diet had the lowest carotenoid (astaxanthin) concentration (4.33). Regression analysis indicated that color parameters were significantly correlated with carotenoid (astaxanthin) concentrations. In the white zones, *A. platensis* diet groups had significantly stronger L* than that of the control group ($p<0.05$). There was no difference in skin astaxanthin content between fish fed with 2.5% and 5% *A. platensis* (5.23%) ($p>0.05$).

At day 60, protease, lipase, amylase enzymes activities were studied. Specificity of amylase was significantly higher ($p<0.05$) in 10% *A. platensis* diet (74.33 U mg^{-1} protein) compared with control (16.33 U mg^{-1} protein) (Table 6) and showed a statistically significant increase in the protease activity in comparison with control diet (5.17 U mg^{-1} protein) and 10% *A. platensis* diet (11 U mg^{-1} protein) ($p<0.05$), but there was no significant difference between 5% and 7% *A. platensis* groups. Similarly, significantly higher lipase activity was recorded in and 10% *A. platensis* diet compared to the other treatments (32.67 U mg^{-1} protein ($p<0.05$).

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Table 3: Growth performance of koi fed on different levels of *Arthrospira platensis* in an 8-week experimental period.

Growth parameters	Control diet	A.p 2.5%	A.p 5%	A.p 7.5%	A.p10%
Final weight (g)	29	29	29	29	29
Initial weight (g)	57.8±0.48 [d]	58.58±0.78 [d]	60.02±0.54 [c]	67.02±1 [b]	69.72±0.29 [a]
Weight gain (g)	99.31±1.66 [d]	102±2.68 [d]	106±1.86 [c]	131±3.45 [b]	140±1 [a]
SGR (%/day)	1.15± 0.01 [d]	1.17±0.02 [d]	1.21±0.01 [c]	1.39±0.02 [b]	1.46 [a]
Survival rate (%)	100	100	100	100	100
FCR	2.94±0.05 [a]	2.87±0/09 [a]	2.73±0.05 [b]	2.22±0.06 [c]	2.07±0.01 [d]
PER	0.76 ±0.01 [d]	0.78±0/02 [d]	0.82±0.01[c]	1±0.02 [b]	1.07±0.01 [a]

Values are means of triplicate groups ±SEM. Means along a row with different letters are significantly different (p<0.05). FCR, food conversion rate; PER, protein efficiency rate; SGR, special growth rate.

Table 4: Color parameters (L*, a*, b* and dE) in koi fed on different levels of *Arthrospira platensis*.

Color parameters	Control diet	A.p 2.5%	A.p 5%	A.p 7.5%	A.p 10%
L* of black zone	30.42±5.50[a]	41.70±6.82[ab]	46.35±4.90[ab]	53.10±2.11[b]	55.60±5.90[d]
dE of black zone	19.40±1.90[a]	24.15±2.85[b]	32.13±2.80[b]	46.25±0.45[c]	50.45±4.75[d]
L* of red zone	54.12±3.75[a]	60.25±10.50[b]	64.20±2.80[a]	70.90±2.61[ab]	74.17±2.86[c]
a* of red zone	14.80±2.30a	20.15±0.05[d]	26.80±0.70[c]	33.33±1.17[ab]	38.10±6.40[b]
dE of red zone	20.45±1.33[a]	25.78±5.70[b]	32.90±6.20[c]	37.77±4.03[c]	40.28±5.69[d]
L* of white zone	73.25±2.05[a]	80.80±5.50[b]	89.10±3.40[b]	93.40±3.25[c]	95.34±2.32 [d]
b* of white zone	4.45±1.15[a]	8.30±1.44[b]	12.10±1.80[ab]	14.30±3.29[ab]	17.57±1.30[c]

Values are means of triplicate groups ±SEM. Means along a row with different letters are significantly different (p<0.05).

Table 5: Astaxanthin content in koi fed on different levels of *Arthrospira platensis*.

	Control diet	A.p 2.5%	A.p 5%	A.p 7.5%	A.p 10%
Astaxanthin	4.33 ± 0.15 [d]	5.23 ± 0.15 [c]	5.23 ± 0.15 [c]	5.7 ± 0.36 [b]	6.33 ± 0.32 [a]

Values are means of triplicate groups ±SEM. Means along a row with different letters are significantly different (p<0.05).

Table 6: Protease, lipase and amylase activities (U mg^{-1} protein) in koi fed on different levels of *Arthrospira platensis.*

Parameters	Control diet	A.p 2.5%	A.p 5%	A.p 7.5%	A.p 10%
Protease	5.17 ± 0.76 [d]	7 ± 1 [c]	10 ± 1 [ab]	9.07 ± 0/4 [b]	11 ± 1 [a]
Lipase	22 ± 1.73 [c]	24.33 ± 1.53 [bc]	25.67 ± 1.53 [b]	25 ± 1 [b]	32.67 ± 1.53 [a]
Amylase	16.33 ± 2.08 [e]	22 ± 1.73 [c]	24.33 ± 1.53 [bc]	25 ± 1 [b]	74.33 ± 1.53 [a]

Each value is a mean ±SD of three replicate, within each row means with different superscripts letters are statistically significant. p<0.05 (one way ANOVA and subsequently post hoc multiple comparisons with DMRT).

While no significant difference was noted between 5% and 7% *A. platensis* diets (p>0.05) (Table 6).

Liver enzymes (ALT, AST and LDH) decreased with an increase in *A. platensis* levels in fish diets (Table 7, Fig. 1). The highest activities of ALT, AST, ALP and LDH were obtained in control group (192.33, 1741.7, 452.33 and 7287.3 $\mu kat.^{L-1}$), respectively. The lowest liver enzymes activities were obtained in fish fed with 7-10% *A. platensis.*

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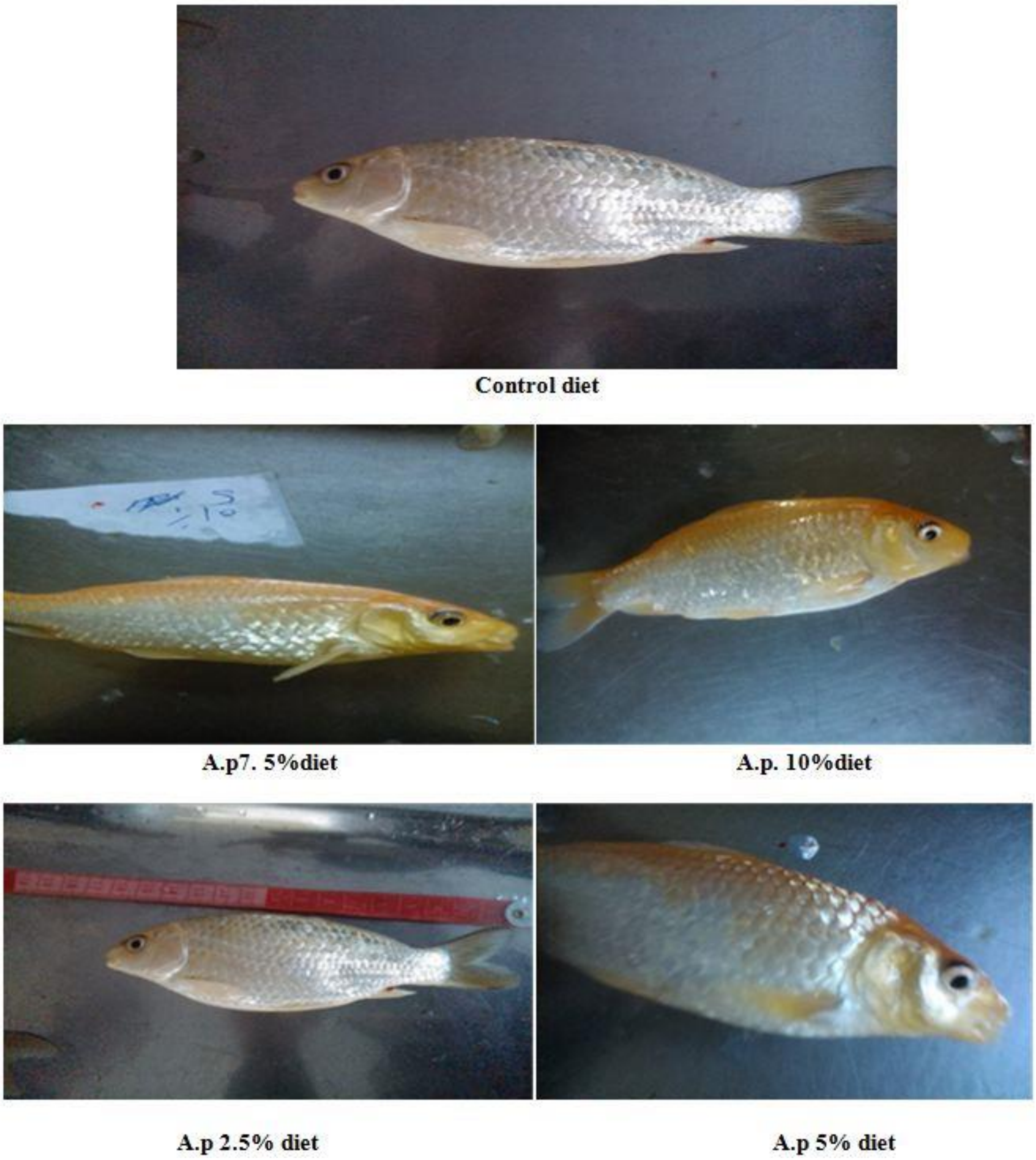
Table 7: The liver enzymes analysis (µkat.$^{L1)}$ in koi fed on different levels of *Arthrospira platensis.*

Parameters	Control diet	A.p 2.5%	A.p 5%	A.p 7.5%	A.p 10%
ALT	192.33 ± 5.5 ^{a}	130.33 ± 1.52 ^{b}	97.67 ± 1.52	88.33 ± 1.52 ^{d}	80.67 ± 1.52 ^{e}
AST	1741.7 ± 18.92 ^{a}	971 ± 3 ^{b}	843.33 ± 4.72 ^{c}	796.33 ± 2.08 ^{d}	712.33 ± 10.5 ^{e}
ALP	452.33 ± 8.14 ^{a}	113 ± 2.64 ^{b}	95.67 ± 1.53 ^{c}	90.67 ± 2.08 ^{c}	48 ± 1 ^{d}
LDH	7287.3 ± 34.64 ^{a}	7123± 102.63 ^{a}	7122.7 ± 74.27 ^{a}	7244.3 ± 38.42 ^{a}	7119.7 ± 17.89 ^{a}

Values are means of triplicate groups ±SEM. Means along a row with different letters are significantly different ($p<0.05$). LDH, Lactate dehydrogenase; ALP, Alkaline phosphatase; AST, Aspartate Amino transferase; ALT, Alanin Amino transferase.



Control diet

A.p7. 5%diet

A.p. 10%diet

A.p 2.5% diet

A.p 5% diet

Figure 1 : The differences in coloration from different treatments.

Significant differences were found for ALT, AST and ALP values in control fish compared to the other treatments ($p<0.05$), while no significant difference was noted among control fish and 10% *A. platensis* diet for LDH (p>0.05).

Discussion

These results showed that feed supplement of *A. platensis* up to 10% did not have negative impacts on growth performance in koi fish. This finding is similar to that reported by Teimouri *et al.* (2013) who found that the replacement of fish meal with *A. platensis* up to 10% did not reduce the growth rate in rainbow trout (*Oncorhynchus mykiss*). A tendency toward better growth performance at 7.5 and 10% *A. platensis* observed in the current study suggests that, unlike plant ingredients, the inclusion of *A. platensis* as a feed additive may improve feed efficiency by increasing gut bacterial colonization. suggested that *A. platensis* improves the intestinal flora in fish causing the breakdown of indigestible feed components to extract more nutrients from the feed; this also stimulates the production of enzymes that transport fast within the fish for metabolism instead of storage. Improvement in the growth of fish by dietary inclusion of Spirulina has been reported earlier in a number of studies (Nakazoe *et al.,* 1986; Mustafa *et al*., 1994; Tongsiri *et al*., 2010; Lin *et al*., 2012., Sun *et al.*, 2012). For instance, use of Spirulina as a protein source in ratios of 25, 50, 75 and 100% improved some growth parameters of Catla and the Rohu (Nandeesha *et al*., 2001).

The results of our study showed that feeding dietary *A. platensis* increased pigmentation in koi fish. Similar results were obtained by Teimouri *et al.* (2013) and Promya and Chitmanat (2011) who observed that *A. platensis* addition to the diets improved pigmentation in rainbow trout and sweet smelt. Moreover, Tongsiri *et al.* (2010) observed that feeding Mekong giant catfish with *A. platensis* improved texture together pigment enhancement especially at 7.5 and 10% concentrations. Colors are the characteristics that attract people to keep the hobby ornamental fish. Microalgal biomass may contribute to enhance the image and quality of koi carp, especially using natural carotenoids. *A. Spirulina* has been also used as a coloring agent in aquaculture (Gomes *et al*., 2002).

Digestion and absorption of food particles and molecules generally take place along the brush border of the columnar epithelial cells, where numerous digestive and absorptive enzymes are localized (Teimouri *et al*., 2013). Examples of such enzymes include maltase, dipeptise doses, lipase and alkaline phosphatase . In the teleost fish, these enzymes are variously distributed along the length of the intestine. The results of the present study showed that specific activities of total protease, lipase and amylase were enhanced in fish. The dietary manipulation was found to affect the digestive activities in the present study, as was also observed by other studies

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with gilthead sea bream administrated plant ingredients (Santigosa *et al*., 2008; Silva *et al*., 2010). AST and ALT are considered as important diagnostic indices. Often their values are used in estimating the health and condition of target animals. The results of the present study showed that Spirulina could decrease levels of these enzymes in koi carp which might be beneficial to avoid fatty liver pathological changes. These results are in agreement with the results reported by Li (2015). Dantagnan *et al*. (2009) also reported that inclusion of macroalgae meal in diets of juvenile rainbow trout enhanced lipid utilization.

The results of the present study demonstrated the potential of *A. platensis* as a feed additive to induce koi pigmentation, which could affect the market quality and acceptability of the fish. Ten percent of *A. platensis* could increase the highest carotenoid (astaxanthin) content and pigment in the skin. Moreover, *A. platensis* can positively improve growth performance and feed efficiency of koi due to its high protein content and improved protease, lipase and amylase enzymes and liver enzymes activities whose values are used in estimating the health and condition of fish. In addition, this study found that the optimum rate of Spirulina in the fish practical diet is 7–10%.

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