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Effect of *Spirulina platensis* Meal as a Feed Supplement on Growth Performance and Pigmentation of Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract: This study evaluated the effects of diets containing 0, 2.5, 5, 7.5 and 10% *Spirulina platensis* and 50 mg of synthetic astaxanthin kg^{-1} on skin and fillet pigmentation and growth performance of rainbow trout (*Oncorhynchus mykiss*). A completely randomized experimental design was developed with six treatments (three replicates each). Two hundred and sixteen rainbow trout with average initial weight of 101±8 g were assigned to eighteen experimental tanks. The experiment lasted for ten weeks. Addition of *S. platensis* did not influence growth related parameters ($P>0.05$). However, carotenoid concentrations of skin and fillet significantly increased with increasing the levels of the alga ($P<0.05$). Inclusion of 10% *S. platensis* as a natural pigment source resulted in the highest carotenoid deposition in both tissues. The highest values of a* (redness) and b* (yellowness) were observed in fish fillet fed with10% *S. platensis*. In the skin, however, the highest value was found for fish fed synthetic astaxanthin. In addition, feeding on control diet significantly increased L* (luminosity), C* (chroma) and H° (hue) in both skin and fillet compared to feeding with other diets. The present results demonstrated that *S. platensis* can be introduced as an alternative natural carotenoid source instead of synthetic astaxanthin in rainbow trout diets. Inclusion of 5% *S. platensis* was found to be a suitable dietary level to ensure pigmentation as well as no negative effects on fish growth.

Key words: *Spirulina platensis* · Pigmentation · Growth Performance · Rainbow Trout

INTRODUCTION

There are four main pigment groups that give color to skin and tissues of animals and plants, namely: melanines, purines, pteridiums and carotenoids. Carotenoids are responsible for red, orange and yellow colors of fish and crustaceans [1]. A distinguished feature of salmonids is their ability to deposit ingested carotenoids in their muscle. Nowadays muscle pigmentation imparted by these lipophilic compounds has become an important quality criterion for the consumers [2]. Salmonids like other animals cannot synthesize carotenoids *de novo*; therefore, they depend entirely on dietary supplements to achieve their natural pigmentation [3].

About 90% of carotenoids found in tissues are located in flesh in their free form, but large amounts are also found in the skin and ovaries in maturing fish [4]. Skin color is primarily dependent on the presence of chromatophores, which are large, star-shaped, pigment-containing cells located in the skin. Fish have multiple chromatophores: melanophores, xanthophores, erythrophores, iridophores, leucophores and cyanophores. Colors in the yellow-to-red range are produced by xanthophores and erythrophores [5].

Carotenoids such as astaxanthin and canthaxanthin are commonly used in aquaculture as pigmentation sources [6]. However, recent efforts have focused on natural compounds as alternatives to synthetic carotenoids because of concerns about the use of synthetic additives and their high cost. Natural sources of carotenoids such as *Xanthophyllomyces dendrorhous* [7], *Haematococcus pluvialis* [3] and *Capsicum annuum* [6] have been tested on pigmentation of salmonids; however, low digestion of plant matters by fish may reduce the availability of pigments by digestive tract of fish [3].

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Unlike these organisms, *Spirulina platensis* does not have cellulose cell wall. Its cells have mucopolymer murein that is easily digested by the digestive enzymes secreted by fish [8]. *S. platensis* is a photosynthetic, filamentous, blue-green microalgae and is generally regarded as a rich source of vitamins, essential amino acids, minerals, essential fatty acids (γ-linolenic acid) and antioxidant pigments such as carotenoids and phycocyanin [9]. *S. platensis* can enhance the natural mucous layer of the skin resulting in a shiny appearance of the fins and skin [10]. As a pigmentation additive, *S. platensis* was found to enhance the color of red tilapia [11], Mekong giant catfish [12], goldfish [10] and prawn [13].

As a feed ingredient, there have been several studies on algae meal as a dietary protein source for fish. It has been confirmed that addition of small amounts of algae to fish feed can exert pronounced effects on growth, lipid metabolism, body composition and disease resistance [14]. Feeding *S. platensis* activated protein synthesis and somatic growth in red sea bream [14], guppy [15], African sharptooth catfish [16] and Indian carps [17]. Hence, *S. platensis* may have potential to be used as a natural feed supplement for increasing fish growth and pigmentation.

Therefore, the main objective of the present study is to investigate the effects of *S. platensis* on growth parameters and also its potential as an alternative natural carotenoid source to synthetic astaxanthin in rainbow trout diets.

MATERIALS AND METHODS

Experimental Diets: Five diets were formulated using the microalgae *S. platensis* (Sinamicroalgae Co., Qeshm, Iran). Since the proximate composition of *S. platensis* and fish meal was nearly similar, the alga was substituted for 0, 2.5, 5, 7.5 and 10% of the dietary fishmeal. Zero percentage was designated as a control diet. The sixth experimental diet was also added for comparing chemical and natural pigments. In this diet, Lucantin® Pink (50 mg kg^{-1}; BASF, Ludwigshafen, Germany) was used as a source of synthetic astaxanthin. The formulation and proximate composition of control diet, *S. platensis* and fish meal are given in Tables 1 and 2, respectively.

Dietary feed ingredients were ground using a laboratory grinder and then blended into a homogenous doughy matter by adding water, which pelleted by pressing through a 4 mm die in a grinding machine. The pellets were then stored in plastic containers at -30°C until use.

Table 1: Feed ingredients and nutrients composition (% dry weight) of the experimental diet

37. Ingredients	38.	%
39. Soybean meal	40.	16
41. fish meal	42.	42
Wheat gluten	4	
Wheat flour	15	
Meat meal	8	
Fish oil	6	
Soybean oil	6	
Mineral premix[a]	0.5	
Vitamin premix[b]	0.5	
Binder	2	
Proximate composition		
Dry matter	90	
Crude protein	42	
Crude lipid	17	
Crude fiber	1.3	
Ash	10	
Energy (kcal g^{-1})	3.8	

[a]Mineral premix consisted of (mg kg^{-1} premix): 2600 mg Mn, 600 mg Cu, 6000 mg Fe, 4600 mg Zn, 50 mg Se, 100 mg Iu, 50 mg Co, 100000 mg cholin chloride, up to 1 kg carrier.

[b]Vitamin premix consisted of (mg kg^{-1} premix): 1200000 IU Vitamin A, 400000 IU Vitamin D3, 3000 IU Vitamin E, 1200 mg K3, 5400 mg C, 200 mg H2, 200 mg B1, 3360 mg B2, 7200 mg B3, 9000 mg B5, 2400 mg B6, 600 mg B9, 4 mg B12. Each value is the mean of three sub-samples

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

	Ingredients	
Proximate composition (%)	Spirulina	Fish meal
Dry matter	94	92
Crude protein	62	60
Crude lipid	8	9.4

All fish were fed the control diet during the first 7 days after stocking to adapt them to feeding and handling practices. After that, the fish were fed with the experimental diets.

Fish Rearing and Sampling: Fish (n= 216; 101±8 g) were randomly divided into eighteen 300L fiberglass tanks in triplicates (12 fish per replication). The whole water of the tanks was exchanged with well water every day. Water quality parameters were checked three times per week after the first feeding. Those parameters were kept within optimal rang for rainbow trout; temperature13±2°C, salinity 0.6±0.1 ppt, pH 7.6±0.2 and dissolved oxygen (DO) 8.6±1mg L^{-1}. All tanks were maintained under a constant photoperiod (12 h dark: 12 h light) created by fluorescent lamps. Fish were fed by hand, twice per day at a rate of 2% of body weight during the experiment.

The experiment lasted for ten weeks. On the last day of the experiment, all fish were weighed individually. Afterward, three fish were randomly selected from each tank and sacrificed using 300 mg L^{-1} clove essence solution. The left fillet was selected for color analysis.

Photograph Analysis: Photographs were taken according to Tlusty and Hyland [18]. 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 speeds were 10, aperture was F16 and zoom was 35. The images were analyzed with Adobe Photoshop CS4 software (version 11). Pictures were opened in RGB mode. Measurements were processed at three locations along the fillet and skin above the lateral line; close to the head, midway between the head and the tail and close to the tail. In each position, five repetitions were recorded and the average value was used in this study. The tristimulus L*a*b* measurement mode was used as it relates to the human eye response to color. The L* variable represents lightness (L* = 0 for black, L* = 100 for white), a* scale represents the red/green, +a* intensity in red and -a* intensity in green. The b* scale represents the yellow/blue, +b* intensity in yellow and -b* intensity in blue. The chroma is an expression of the intensity and clarity of the color and the hue is an angular measurement where 0° indicates a red hue and 90° denotes a yellow hue.

Carotenoid Analysis: The carotenoid content was extracted according to the method of Torrissen and Naevdal [19], with some modification. Samples of 1 g skin were collected from both sides between the abdominal and dorsal regions of the fish, being careful to remove adhering adipose tissue. The sample was then transferred to 10 ml pre-weighed glass tubes and ground in 10 ml acetone (98%, Merck, Germany) containing anhydrous sodium sulphate with a homogenizer (Ultra-turrax IKA® T18 basic). The samples were stored for one day at 4°C and then extracted with acetone two or three times until no more color could be obtained. The solutions were centrifuged at 3500 rpm for 10 min and then absorptions were measured by a spectrophotometer (Unico S-2150UV).

A similar method was used for total carotenoid analysis of fillet and *S. platensis*. Approximately 25 g of fish fillet was minced by a food processor for 5-10 s and then 5 g sub-samples analyzed for carotenoid content. Total carotenoid concentration of skin, muscle and algae was determined spectrophotometrically in acetone using extinction coefficients (E1%, 1 cm) 2500 for carotenoids at 450 nm. Total determined carotenoid content of the *S. platensis* meal was 1.26 mg g^{-1}.

Statistical Analysis: All data were expressed as the mean ± SD. All data were verified for normality after transformation (ASIN). One-way ANOVA was used to determine the effects of *S. platensis* on growth performance and pigmentation using SPSS (version 17). Duncan's multiple range test was used to compare differences between the means at 5% probability. Linear regression analyses were also used to relate carotenoid concentrations and color parameters.

RESULTS

S. platensis supplemented diets did not change growth related parameters in rainbow trout, although a larger weight gain and SGR were observed in rainbow trout fed diet containing 7.5 and 10% *S. platensis* compared to the other treatments (Table 3). FCR was not also influenced by *S. platensis* inclusion ($p>0.05$).

Color data showed that the fish fed diets containing natural and/or synthetic pigments turned to pinkish at the end of the experiment (Table 4). In contrast, all fish fillet in the control group were poorly pigmented. a* and b* increased with increasing the levels of *S. platensis* in the diets. The fish fed 10% *S. platensis* displayed more reddish hue than those of control and 2.5% *S. platensis*. Feeding on control diet increased significantly L*, C* and H° compared to feeding on the other diets ($p < 0.05$).

The highest levels of fillet pigmentation and carotenoid deposition were found in10% *S. platensis* (Fig. 1). However, there were no differences in carotenoid concentration of the trout's fillet fed 2.5, 5% *S. platensis* and astaxanthin diets. Control diet had the lowest carotenoid concentration.

Regression analysis indicated that color parameters are significantly correlated to carotenoid concentrations (Fig. 2).

As shown in Table 5, skin luminosity (L*) was not affected by the treatments. a* and b* increased significantly with increasing *S. platensis* inclusion, but fish fed astaxanthin presented the largest estimates of a* and b* ($P<0.05$). In addition, C* and H° showed an indirect relation with the increasing levels of *S. platensis*. Although both *S. platensis* and synthetic astaxanthin significantly elevated the carotenoid concentration (Fig. 3), maximum carotenoid skin content was observed with 10% *S. platensis*.

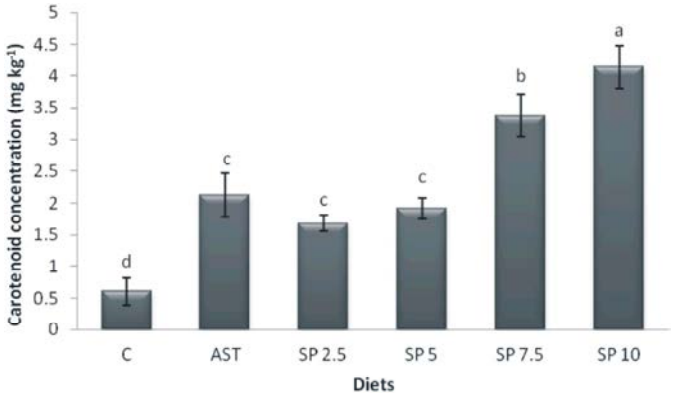

Fig. 1: Fillet carotenoid concentration (mg kg^{-1}) in rainbow trout feeding on different levels of *S. platensis* and synthetic astaxanthin over 10 weeks experimental period
All values are means of three replicates (tanks)/treatment ± standard deviation

Table 3: Growth performance in rainbow trout feeding on different levels of *S. platensis* and synthetic astaxanthin over 10 weeks experimental period

	Diets					
Growth parameters	Control	AST	SP 2.5	SP 5	Sp 7.5	SP 10
Initial weight (g)	101.9 ±1.5	101.4 ± 1.8	101.7 ± 0.6	102.1 ± 0.8	101.5 ± 0.7	102.1 ± 0.9
Final weight (g)	224.9^{ab} ± 4.2	218.5^{b} ± 7.5	227.3^{ab} ± 6	217.7^{b} ± 6.3	234.9^{a} ± 9.2	235.8^{a} ± 3.5
Weight gain (%)	120.7^{ab} ± 7.5	115.5^{ab} ±11	123.4^{ab} ± 4.7	113.1^{b} ± 4.8	131.4^{a} ± 7.7	130.7^{a} ± 1.1
SGR (%/day)	1.31^{ab} ± 0.05	1.27^{ab} ± 0.08	1.33^{ab} ± 0.03	1.26^{b} ± 0.03	1.39^{a} ± 0.05	1.39^{a} ± 0.08
FCR	1.13 ± 0.02	1.1 ± 0.06	1.1 ± 0.04	1.1 ± 0.01	1.04 ± 0.03	1.03 ± 0.1

Values are means of triplicate groups ± SD. Means with the different superscript letters are significantly different (P<0.05)

Table 4: Color parameters of fillet in rainbow trout feeding on different levels of *S. platensis* and synthetic astaxanthin over 10 weeks experimental period

	Diets					
Color parameters	Control	AST	SP 2.5	SP 5	Sp 7.5	SP 10
Luminosity (L*)	33.1^{a} ± 1.6	30.5^{b} ± 1.3	31.1^{ab} ± 1	29.6^{bc} ± 1.7	27.6^{cd} ± 0.7	25.5^{d} ± 1
Redness (a*)	4.3^{c} ± 1.2	11^{b} ± 1.3	4.5^{c} ± 0.0	9.6^{b} ± 1.5	11.1^{b} ± 1.2	14.3^{a} ± 1.6
Yellowness (b*)	3.1^{c} ± 1.2	7^{b} ± 1.5	4^{c} ± 1	9.1^{b} ± 1.7	15.8^{a} ± 1.8	14.1^{a} ± 1.8
Chroma (C*)	59^{a} ± 1.5	51.1^{b} ± 0.5	58.6^{a} ± 0.7	51^{b} ± 1.7	47.1^{c} ± 1.2	46^{c} ± 1.3
Hue (H°)	25.5^{a} ± 1.5	15.1^{c} ± 1.7	13.8^{c} ± 1.8	15.8^{c} ± 1.5	20.6^{b} ± 2.3	15.8^{c} ± 2.2

Values are means of triplicate groups ± SD. Means with the different superscript letters are significantly different (P<0.05)

Table 5: Color parameters of skin in rainbow trout feeding on different levels of *S. platensis* and synthetic astaxanthin over 10 weeks experimental period

	Diets					
Color parameters	Control	AST	SP 2.5	SP 5	Sp 7.5	SP 10
Luminosity (L*)	38.5 ± 3.6	31.3 ± 1.8	37.3 ± 2.4	35.3 ± 3.6	34.5 ± 3.1	35.4 ± 4.8
Redness (a*)	3.5^{d} ± 1.1	15.5^{a} ± 1.3	6.3^{c} ± 1	6.3^{c} ± 1.3	8.5^{bc} ± 0.5	10.3^{b} ± 1.5
Yellowness (b*)	8.2^{c} ± 1.3	15.3^{a} ± 0.6	9.8^{c} ± 2.1	8.7^{c} ± 1.1	13.6^{ab} ± 1.2	12.3^{b} ± 0.8
Chroma (C*)	53.8^{a} ± 1.9	43.1^{d} ± 0.8	51.8^{ab} ± 2	52.7^{a} ± 1.6	48.7^{bc} ± 0.7	47^{c} ± 2.5
Hue (H°)	27.5^{a} ± 3.9	15.6^{c} ± 1.1	23^{ab} ± 3.4	21^{abc} ± 5.1	23.7^{ab} ± 2.5	18.3^{bc} ±3.2

Values are means of triplicate groups ± SD. Means with the different superscript letters are significantly different (P<0.05)

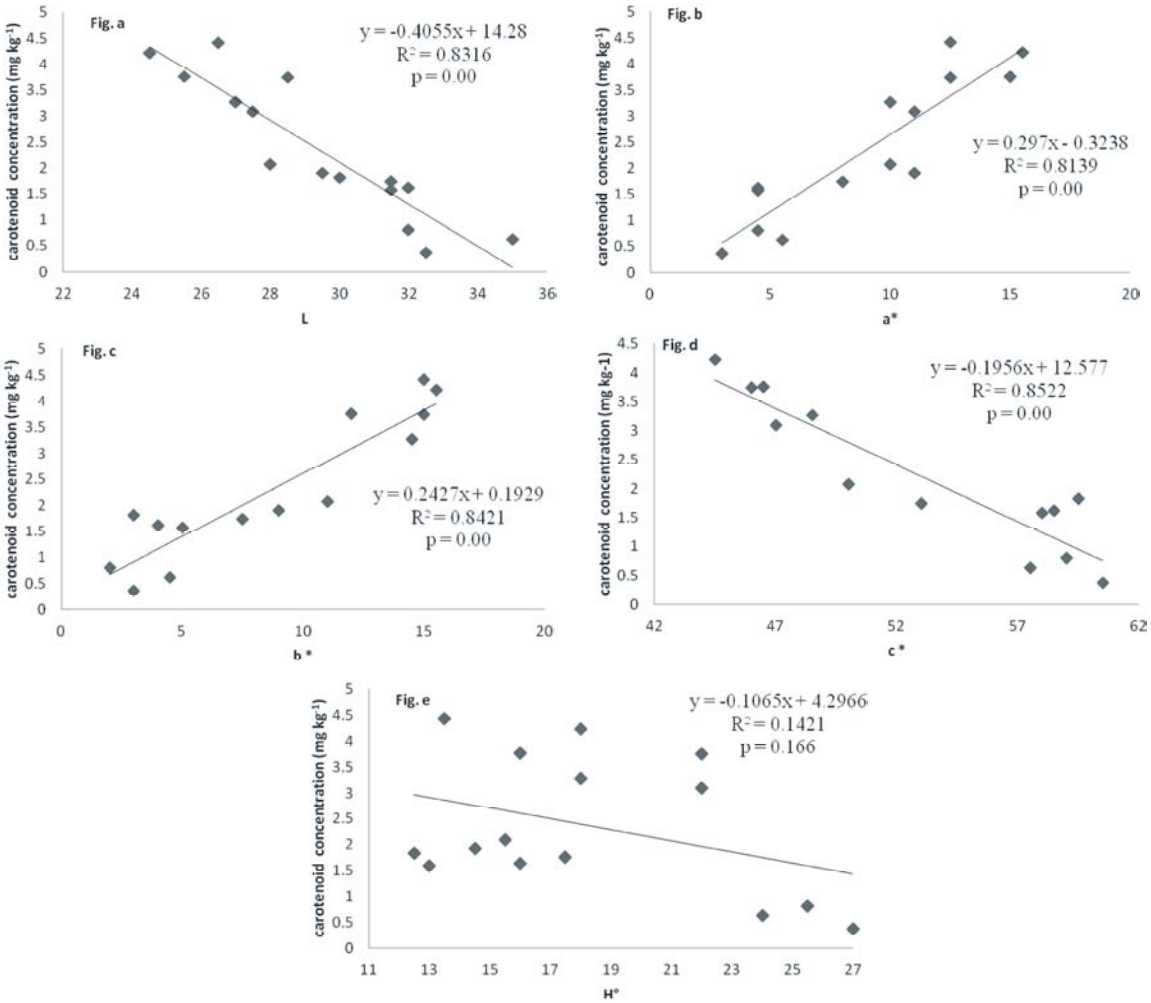

Fig. 2: A comparison between fillet carotenoid concentration and (a) L*, (b) a*, (c) b*, (d) C* and (e) H° in rainbow trout feeding on different levels of *S. platensis* over 10 weeks experimental period

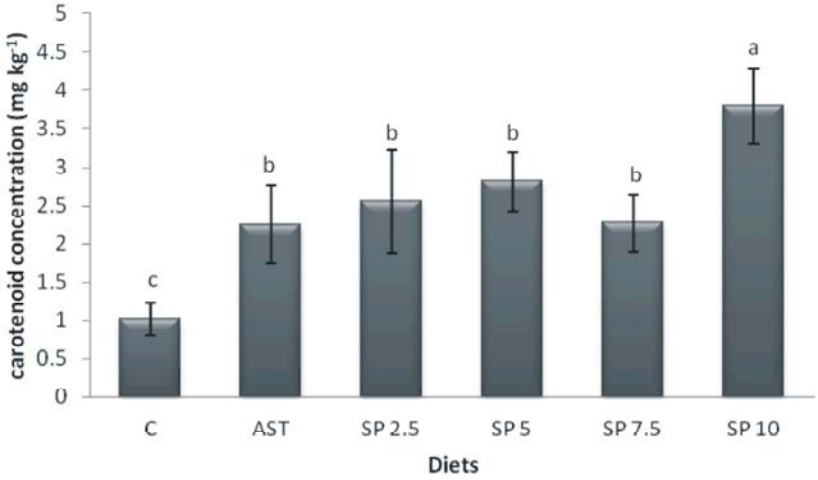

Fig. 3: Skin carotenoid concentration (mg kg^{-1}) in rainbow trout feeding on different levels of *S. platensis* and synthetic astaxanthin over 10 weeks experimental period
All values are means of three replicates (tanks)/treatment ± standard deviation

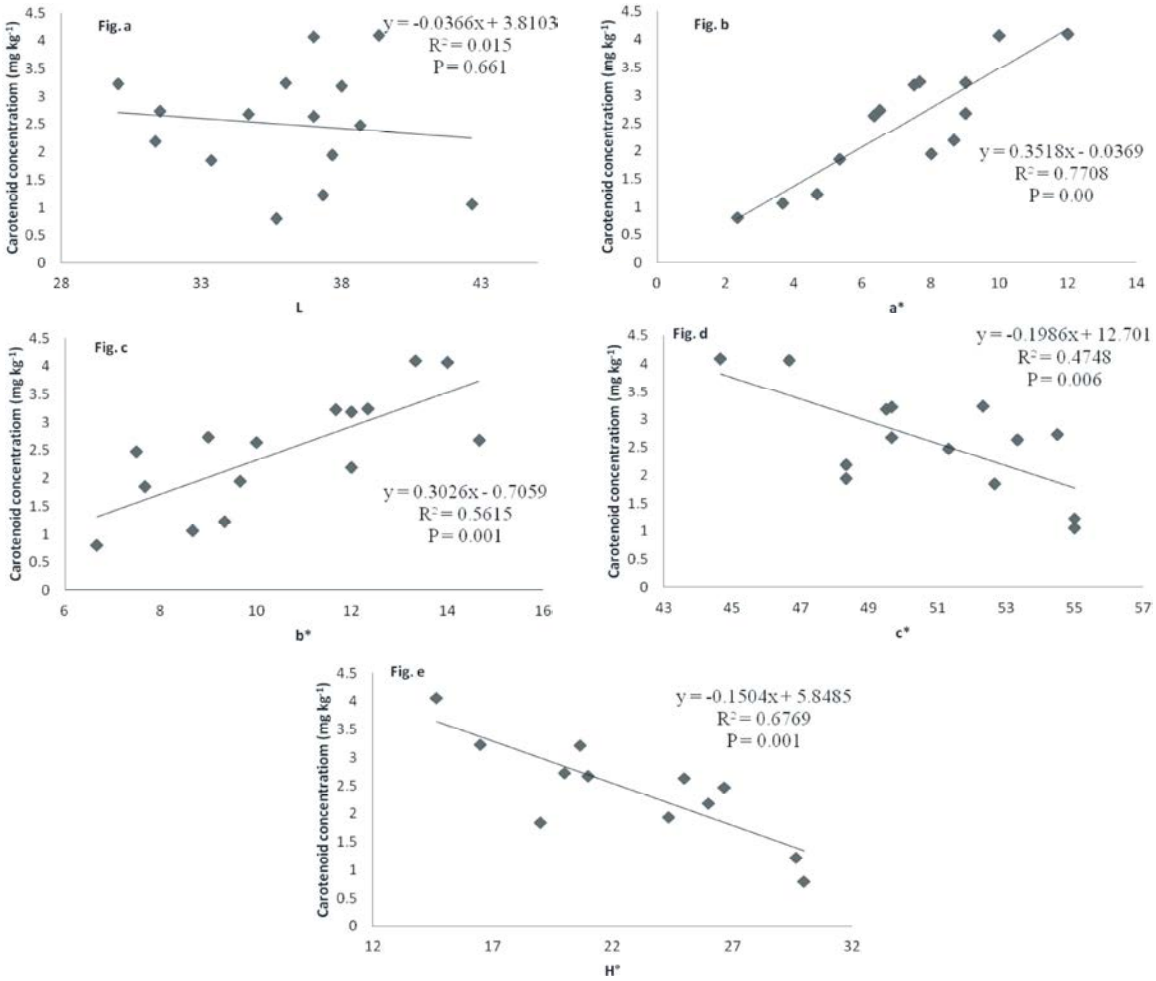

Fig. 4: A comparison between fillet carotenoid concentration and (a) L*, (b) a*, (c) b*, (d) C* and (e) H° in rainbow trout feeding on different levels of *S. platensis* over 10 weeks experimental period

Except L*, other color parameters were correlated to skin carotenoid concentration (Fig. 4), showing that the addition of *S. platensis* had an impact on skin color.

DISCUSSION

The results showed that replacement of fishmeal with *S. platensis* up to 10% did not have negative impacts on growth performance in rainbow trout. This finding is similar to that reported by Nandeesha *et al*. [17] who found that replacement of fishmeal with *S. platensis* up to 100% did not decline growth rate in Indian carps (*Catla catla* and *Labeo rohita*). Similarly, Olvera-Novoa *et al*. [20] and Dernekbasi *et al*. [15] observed that replacing fishmeal with *S. platensis* up to 40% did not change growth rate in tilapia and guppy. A tendency toward better growth performance at 7.5 and 10% *S. platensis* observed in the current study suggests that, unlike plant ingredients, inclusion of *S. platensis* as a feed additive may improve feed efficiency by increasing gut bacterial colonization. Although the action mechanism of *S. platensis* is not clearly demonstrated, James *et al*. [21] suggested that *S. platensis* improves the intestinal flora in fish rendering break down of indigestible feed components to extract more nutrients from the feed; this also stimulates the production of enzymes that transport fats within the fish for metabolism instead of storage.

The current study showed that feeding dietary *S. platensis* increased pigmentation in rainbow trout. Similar results were obtained by Mori *et al*. [22], Okada *et al*. [23] and Promya and Chitmanat [16], who observed that *S. platensis* addition to the diets improved pigmentation in African sharptooth catfish, striped jack and sweet smelt. Moreover, Tongsiri *et al*. [12] observed that feeding *S. platensis* improved texture together with pigment enhancement in Mekong giant catfish.

It has been found that astaxanthin to be the most effective pigment to enhance pigmentation in salmonids [24]. Although the major carotenoid sources of *S. platensis* are β-carotene and zeaxanthin [13], our results showed that the addition of *S. platensis* to the rainbow trout diet led to pigment deposition in fillet. There is some evidence showing that zeaxanthin is also deposited as a pigment in tissue. Schiedt *et al*. [25] observed that astaxanthin deposition efficiency is larger than other carotenoids followed by adonirubin, canthaxanthin, zeaxanthin, lutein and finally β-carotene. Katsuyama *et al*. [26] found that dietary astaxanthin was mostly absorbed, accumulated and partially metabolized to zeaxanthin in rainbow trout. It, therefore, appears that pigmentation in fish muscle fed *S. platensis* in the current study is also caused by deposition of zeaxanthin as major carotenoids in *S. platensis*.

Fillet pigmentation at 7.5 and 10% *S. platensis* was significantly higher than the other groups. This result was expected due to an increase in diet carotenoid concentration. Regarding the two carotenoid sources, there were no differences in the muscle pigmentation between fish fed 5% *S. platensis* and those fed synthetic astaxanthin having almost similar pigment doses. Despite dietary astaxanthin is more effective than zeaxanthin in terms of deposition, feeding on *S. platensis* supplemented diets may have enhanced carotenoid bioavailability. Whereas the efficiency of utilization of dietary astaxanthin for flesh pigmentation of Atlantic salmon and rainbow trout rarely exceeds 10-15% [27] easy breakage of *S. platensis* cell wall may explain the similar levels of carotenoid deposition in the fish fillet compared to the astaxanthin treatment. *S. platensis* has a thin cell wall comprised of 80% pectin and 20% cellulose [28], thus its carotenoid content can be easily assimilated by digestive tract. Pigmentation of rainbow trout fed disrupted cells (cell wall cracked) of *H. pluvialis* was better than rainbow trout fed diets containing intact cells from *H. pluvialis* [29]. Furthermore, since carotenoids are fat-soluble pigments, highly unsaturated fatty acid content of *S. platensis* may increase the pigments absorption [30]. In addition, the *S. platensis* carotenoid might be more easily dissolved in the dietary lipids, whereas commercial products, at least partly, consist of nanoparticles and aggregates [31], which need to be dissolved prior to absorption.

In contrast to fillet, skin pigmentation was higher for astaxanthin diet even though at highest carotenoid concentration (10% *S. platensis*). This condition may have been caused by characteristics of skin cells. Skin xanthophores that contain carotenoid droplets [32] may be easier saturated with astaxanthin. This case remains to be further investigated.

The results also showed that coloration and concentration of carotenoids in rainbow trout are linearly related. Several mathematical models that describe the relationship between instrumentally assessed coloration and carotenoid concentration showed that instrumental color analysis under standard conditions can be used to predict carotenoid levels accurately [7]. A change in fish color parameters and also linear correlations observed in the current study is in agreement with Wathne *et al*. [33], Storebakken *et al*. [7] and Mora *et al*. [6]. The increment in the average values of a* and b* and a decrement of H° with increasing dietary carotenoid concentration is in agreement with Mora *et al*. [6] and Choubert *et al*. [34, 35]. However, *S. platensis* addition decreased C* value in contrast to Mora *et al*. [6] and Choubert *et al*. [34, 35]. The decrease in L* values may be related to the fillet fat content. Nickell and Bromage [36] reported that L* was significantly correlated to flesh lipid content, thus changes in fat content of fillet may have caused this decrement in L*. Watanabe and Vassallo-Agius [27] also reported that the *Spirulina* supplemented diets improved the flesh quality of cultured striped jack mainly by depressing the accumulation of lipids.

CONCLUSION

The results of present study demonstrate the potential of *S. platensis* as a feed additive to induce rainbow trout pigmentation, which could affect the market quality and acceptability of the fish. *S. platensis* can also be supplemented in diet as an effective natural carotenoid source instead of synthetic astaxanthin for fillet pigmentation of rainbow trout. However, pigmentation is not equal in both skin and fillet. Moreover, this study showed that *S. platensis* can be used as a fishmeal replacement in rainbow trout diets due to its high protein content and no negative impacts on fish performance.

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