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Comparison and investigation of the effects of natural carotenoids and dietary astaxanthin on carcass pigmentation, growth performance and serum lysozym activity of rainbow trout *(Oncorhynchus mykiss)*

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Abstract

In order to investigate and compare nutritutional and physiological effects of natural and synthetic carotenoids, four experimental diets were formulated which were similar in terms of digestible energy and crude protein content. Control diet contained 100mg/kg astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione), diet 1 contained 2.5g/kg natural carotenoids and 75 mg/kg astaxanthin, diet 2 included 5 g/kg natural carotenoids and 50 mg/kg astaxanthin and diet 3 was made up of 7.5 g/kg natural carotenoids and 0 mg/kg astaxanthin, respectively. Experimental fish with average weight of 150 g were fed experimental diets for 10 weeks. The design of the study was completely randomized with four replications of four treatments. The study was conducted in concrete pools in a closed circuit system. As a result, diet 3 led to improved feed conversion ratio and had higher specific growth rate and weight gain compared with other diets. This difference was significant in case of specific growth rate compared with the control diet ($P<0.05$). Spectrophotometry experiments showed that carcass pigmentation decreased with decreasing dietary astaxanthin level and differed significantly in diet 3 compared with the control diet ($P<0.05$). Serum lysozyme level was significantly different in diet 3 compared with diets 1, 2 and the control diet ($P<0.05$). It was concluded that rainbow trout do not have the ability to change carcass color even when high levels of natural carotenoids are available. The most visible findings of the current study were better growth performance and better non-specific immune response of the fish due to the key roles of carotenoids.

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Introduction

Due to market demand increase of fishery products and economic importance of pigments in salmons and Penaeidae shrimp, several studies have been conducted in this area. Generally, the color of farmed trout which are kept jammed is created by adding two synthetic carotenoids, namely cantaxanthin and astaxanthin. This process is often accompanied by heavy costs in the aquaculture feed industries (Gourveia *et al.*, 1997). So, finding a good, cheap alternative instead of expensive carotenoid sources can be a good motivation for the study of carotenoids. Carotenoid molecules are made up of eight isoprenoid chains which are symmetrically arranged around a double bond and make a Tetraterpene. The possibility of using carotenoids other than synthetic astaxanthin and cantaxanthin, i.e. carotenoids with plant and algae origin, as nutritional supplements has been one of the challenges of aquatic organisms' nutrition (Gourveia *et al.*, 1997; Kop and Durmaz, 2008). Studying modification reactions of carotenoids inside the body of an aquatic organism has been one of the main reasons of carotenoid researches. However, carotenoids are responsible for different physiological functions of the body such as light absorption, anti-oxidant properties and strengthening the immune system against various diseases (Wang *et al.*, 2006; Giordano *et al.*, 2000; Amar *et al.*, 2004). Xantophylls with chemical formula of $C_{40}H_{56}O_2$ are oxidixed forms of carotenes which contain hydroxyl groups and are more polar than carotenes. Xantophylls are generally found in yellow to orange plants such as Marigold and yellow pepper. In case of using pigments with natural origin instead of synthetic ones in aquatic food industries, there is the possibility of saving up to 20% of the costs (Baker *et al.*, 2002).

In another study, Yamashita *et al.* (1996) illustrated that xantophyll can change into vitamine A and apocarotenoids in the liver of black bass. Researchers could prove the important role of carotenoids in impovering the non-specific immune system of fish. Nakano *et al.* (1999) observed the positive role of adding carotenoids to diets on increasing rainbow trout resistance against oxidative corruption stress.

Amer *et al.* (2001) worked on the effect of β-carotene on growth performance and immune system of rainbow trout and illustrated that β-carotene and astaxanthin stimulated the non-specific immune system of the fish. They also found that serum lysozyme increased significantly, but the presence of carotenoids had little effect on growth and feed intake. In another study, Amer *et al.* (2012) showed that increasing natural and synthetic dietary carotenoids has a positive effect on inhibiting Infectious Hematopoietic Necrosis Virus (IHNV).

Kalinowsky *et al.* (2005) found that carotenoids have a positive effect on growth performance of *pagarus pagarus*. A similar study by Hu *et al.* (2006), who worked on the effect of dietary vitamine A and β-carotene on growth performance and bio-convirtibility of β-carotene to vitamine A in young hybrid tilapia, was indicative of the fact that dietary β-carotene resulted in an increase in growth performance of the fish. Hernandez *et al.* (2007) observed that vitamine A supplement in *paralichthys olivaceus diets led to an increase in growth performance.*

Carcass quality and color are the most important factors consumers take into account when choosing fishery products (Kalinowsky *et al.*, 2005). Generally, trout cultured in natural environments have desirable red color and high market demand because of using natural and live food such as gammarus. On the other hand, farmed fish in mechanized pools lack these desirable traits because they are fed by hand. Several studies have been conducted regarding the effect of natural and synthetic carotenoids on carcass and skin color of fish, all of which emphasized the positive effect that carotenoids have in this regard.

Torrissen *et al.* (1989) in their study showed that astaxanthin has higher ability than other carotenoids to change the color of various oragans of salmon, especially its skin. It can even be absorbed more than the other carotenoids. Kalinowsky *et al.* (2005) who

worked on the effect of carotenoids on color change and increasing market demand of *Pagrus pagrus*, observed that dietary carotenoids, especially astaxanthin has a key role in changing the color of skin and carcass.

Researches proved that natural carotenoids of *Dunaliella salina* algae not only have antioxidant property, but also increase the color of skin and flesh (Wang *et al.*, 2006). Of course, it should be noted that the source of carotenoids as well as aquatic species are important in carcass and skin pigmentation of the aquatic organism. In industrial scale, adding synthetic astaxanthin with huge costs is possible for fish feed producers. Therefore, the current study tried to investigate the feasibility of using cheap sources of natural carotenoids or obtaining the optimal replacement level instead of synthetic astaxanthin to reduce the feed costs. In general, the main aim of the present study was to compare and investigate the role of natural carotenoids along with astaxanthin on growth performance, serum lysozyme level and carcass color change of rainbow trout *(Oncorhynchus mykiss)*.

Materials and method

In the present study, 960 rainbow trout with average weight of 150 were used (60 in each pond). The Fish were kept in 16 octagon concrete ponds (1×1×1m) with inlet water of 2 lit sec^{-1}. The water temperature was 14° C with the pH level of 7.3 and the dissolved oxygen of water was 7.5 mg/lit. Feeding was three times a day according to NRC (1999) and was based on 2.5% of the body weight. Chemical analysis of the experimental diets was done as follows:

The crude protein of the diets was determined by Kjeldalh (kjeltec Analyzer unit 2300 Foss Model), fat by solvent extraction, ash by placing the samples in a muffle furnace (550°C) for 12 h, fiber by placing the samples remaining in a muffle furnace (600°C) for 6 h after acid and alkali hydrolysis and moisture by drying (105°C) until constant weight has been attained. Nitrogen free extract was calculated by subtracting the values of protein, fat, fiber and ash from the dry matter.

The amino acid profile of the experimental diets was determined using high-performance liquid chromatography (HPLC, Knauer/smatline Model, Germany). All methods are based on those described in the Association of Official Analytical Chemists, (AOAC, 1990) and modified as described in Aksnes *et al.* (2006).

In the present study four isonitrogenous (40% crude protein) and isocaloric (3650 kcal/kg digestible energy) diets which differed in terms of pigment levels were formulated considering the nutrient requirements for cold water fish (NRC, 1999) (Tables 1 & 2). Control diet was based on fish meal and 100 mg/kg astaxanthin, diet 1 contained 2.5 g/kg natural carotenoids and 75 mg/kg astaxanthin, diet 2 included 5 g/kg natural carotenoids and 50 mg/kg astaxanthin and diet 3 was made up of 7.5 g/kg natural carotenoids and 0 mg/kg astaxanthin, respectively. The pellet sizes were 3-3.5 mm. In the present study, the natural carotenoid used was the product of Biogold manufactured by German Biochem Company. The product purity was 20 grams per kilogram.

Growth performance

Average weight gain, specific growth rate, condition factor and feed conversion ratio as the most important factors of growth performance were measured in the current study (Espe *et al.*, 2008).

Weight gain (gr) = final weight – initial weight

$$\text{Specific growth rate} = \frac{(\text{Ln final weight} - \text{Ln initial weight}) \times 100}{\text{Experimental period (days)}}$$

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

Feed conversion ratio = food consumption (gr) / weight gain (gr).

Blood sampling

At the end of the experiment, 7 fish per tank wereeuthanized and bled from the caudal vein. Blood

samples were transferred into Eppendrof tubes and allowed to clot at room temperature for 1 h. Then samples were kept at 4 ºC for 5 h. The sera were separated by centrifugation (1500 × g for 5 min at 4 ^{0C}). The sera samples were stored at -80 ^{C} until required for analysis of immune parameters.

Immune parameters

In order to evaluate immune parameters the method described by Cuesta *et al.* (2005) was used. 135 µl of HBSS without Ca^{+2} or Mg^{+2} was added to 15 µl of serum sample in each well plate. Finally 50 µl of 20 mM 3, 30, 5, 50-tetramethylbenzidine hydrochloride (TMB) (Sigma) and 5 mM H_2O_2 was added. The color-change reaction was stopped after 2 min by adding 50 µl of 2 M sulfuric acid and the optical density values were read at 450 nm by ELISA reader.

Serum lysozyme activity

Serum lysozyme activity was measured using a turbidometric microtitre plate technique according to Tukmechi *et al.* (2011) with slight modification. Briefly, a standard suspension of *Micrococcus lysodeikticus* (75 µg ml^{-1}) was prepared with 0.1 M phosphate citrate buffer, pH 5.8. Rainbow trout serum (25 µl) was added to 75 µl of *Micrococcus lysodeikticus* suspension and the decrease in absorbance after 4 and 9 min at 450 nm. One unit of lysozyme activity was defined as reduction in absorbance of 0.001 per min.

Fish tissue carotenoids

To measure the total amount of tissue pigments, the method proposed by Choubert and storebakken (1989) was used. In so doing, homogenized samples were prepared using 10 g of muscle tissue and then these samples were centrifuged for 10 min at 3500 rpm. After precipitation, the light absorption of acetone containing tissue carotenoids was measured using spectrophotometer at 475 nm.

Statistical analyses

The effects of experimental diets on growth performance, tissue pigments and non-specific immune parameters of Rainbow trout were studied using a completely randomized design with four replications of four treatments. The obtained data were analyzed using GLM procedure of SAS software (9.1) and mean comparison was performed using Duncan test.

Results

According to table 3, specific growth rate of diet 3 was significantly different from the control diet and diet 2 ($P<0.05$). Condition factor, weight gain and feed conversion ratio improved with increasing dietary natural carotenoids, but no significant differences were observed among treatments and the control diet. Tissue pigment concentrations decreased with reducing synthetic astaxanthin level and increasing natural dietary carotenoids and a significant difference was observed in diet 3 compared with the control diet ($P<0.05$). Serum lysozyme level, as one of the most important indicators of non-specific immune parameters of experimental fish, increased with increasing natural carotenoid levels and showed a significant difference in diet 3 compared with the control diet and diets 1 and 2 ($P<0.05$).

Table 1. Composition of experimental fish diets.

	Experimental diets			
Ingredients	(Control)	1	2	3
Fish Meal	50	50	50	50
Astaxanthin(mg/kg)	100	75	25	0
Natural Carotenoids (g/kg)	0	2.5	5	7.5
Corn	12.48	12.48	12.48	12.23
Wheat	10	9.75	9.5	9.5
Soybean Meal	17.27	17.27	17.27	17.27
Fish Oil	7	7	7	7
Mineral Premix[1]	0.5	0.5	0.5	0.5
Vitamin Premix[2]	0.5	0.5	0.5	0.5

D-Methionine	0.5	0.5	0.5	0.5
L- Lysine	0.5	0.5	0.5	0.5
Anti Oxidant [3]	0.1	0.1	0.1	0.1
Colin Chloride	0.15	0.15	0.15	0.15
Binder [4]	1	1	1	1
Amino acid composition				
Arginine (%)	2.58	2.51	2.4	2.38
Histidine (%)	1.02	1.02	1	0.8
Lysine (%)	3.2	3.1	3.02	3.02
Leucine (%)	3.08	3	3	3
Isoleucine (%)	1.87	1.87	1.86	1.86
Cysteine and Methionine (%)	1.95	1.92	1.93	1.9
Phenylalanine (%)	1.74	1.7	1.6	1.61
Tryptophan (%)	0.93	0.91	0.92	0.9
Valine (%)	2.03	2.01	2.02	2

[1] Mineral Premix (g/kg): zinc, 12.5 g; iron, 26 g; manganese, 15.8 g; copper, 4.2 g; cobalt, 0.48 g; selenium, 2 g; iodine, 1 g.

[2] Vitamin Premix: (mg or IU/kg of diet) Vitamin A (as acetate) 1600000 IU; vitamin D3, 400000 IU; choline chloride.12000; niacin, 4000; riboflavin, 8000; pyridoxine, 4000; folic acid, 2000; vitamin B12, 8000; biotin, 1; inositol, 20000; vitamin C, 60000; vitamin H2, 2.4; vitamin B2, 8000; vitamin K3, 2000; vitamin E,40000.

[3] Butil Hydroxi Anisol

[4] Lignosulfate.

Discussion

With increasing dietary natural carotenoids, significant increase was observed in specific growth rate and improvements became visible in weight gain, condition factor and feed conversion ratio ($P<0.05$). The results of the current study regarding growth performance of the experimental trout were in accordance with the findings of Hu *et al.* (2006) and Kalinowsky *et al.* (2005). These researchers also believed that increasing natural carotenoid levels results in improvement in fish growth performance. Carotenoids have a key role in empowering fish immune system and their antioxidant activities have proved to inhibit oxidative stress and reduce hepatocyte damage.

Table 2. Nutritional composition of experimental diets**.**

	Experimental diets			
Ingredients	control	1	2	3
Dry Matter (%)	90.96	90.98	90.98	91.52
Crude Protein(%)	40.3	40.2	40	40
Crude Fat (%)	16.54	16.98	17.73	20.6
Crude Fiber (%)	2.3	2.98	3.48	3.85
Nitrogen Free extract (%)	24.82	23.52	21.27	17.77
Crude Ash (%)	7	7.3	8.5	9.3
Digestible Energy (Kcal/Kg)	3650	3650	3650	3650

Reduction of metabolites resulted from fat oxidation corruption and increased efficiency of the liver in fat metabolism imply the important role of carotenoids in increasing the efficiency of using dietary fat (Nakano *et al.*, 1999). Based on the aforementioned reasons, the results of the present study regarding specific growth rate, feed conversion ratio, weight gain and condition factor indicated that increasing the level of natural dietary carotenoids can result in

better growth performance compared with synthetic ones.

Reduction of tissue pigments concurrent with increasing natural carotenoid levels and decreasing dietary astaxanthin level revealed that astaxanthin has greater ability than natural carotenoids in changing tissue color. In other words, experimental trout showed lower tissue uptake of dietary carotenoids comaperd with dietary astaxanthin.

Table 3. Specific growth rate, condition factor, weight gain, feed conversion ratio, Tissue pigmentation and serum lysozyme of rainbow trout fed experimental diets.

Traits	Experimental Diets			
	Control	1	2	3
Specific growth Rate (%)	1.265±0.047^{b*}	1.325±0.07^{b}	1.492±0.08^{a}	1.557±0.04^{a}
Condition Factor (%)	1.385±0.07^{a}	1.40±0.07^{a}	1.420±0.192^{a}	1.53±0.13^{a}
Weight Gain (gr)	217.08±7.36^{a}	231.94±13.71^{a}	234.94±9.85^{a}	242.11±6.26^{a}
Feed Conversion Ratio	1.28±0.04^{a}	1.13±0.03^{a}	1.055±0.063^{a}	0.97±0.04^{a}
Tissue Pigmentation (mg/kg)	4.91±0.31a	4.69±0.22ab	4.48±0.18ab	3.9±0.11^{b}
Serum Lysozyme (U ml^{-1})	2.944±0.526b	3.111±0.504^{b}	3±0.370^{b}	5.153±1.509^{a}

* In each row, the means with different letters have significant differences (P<0.05). Values are means± SE for three replications.

The findings of the present study suggested the impossibility of bio-converting natural carotenoids to astaxanthin. This was in line with the findings of Torrissen *et al.* (1989). Lysozyme is a mucolytic enzyme with leukocyte origin which defends against microbial invasion. In the current study, 7.5 g/kg of natural carotenoids caused a significant increase in the stimulation of non-specific immune system of fish (P<0.05).

Dietary carotenoids increase the activity and the total number of leukocytes and phagocytic cells. Thereby, they increase the lysozyme enzyme, which is the metabolite of leukocytes, and stimulate the immune system and increase the defensive power against different pathogens. The findings of the current study were in accordance with the findings of Nakano *et al.* (1999) and Amer *et al.* (2004).

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