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Influence of Dietary Vegetable Crops on Rainbow Trout (*Oncorhynchus mykiss*) Immune System and Growth Performance

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ABSTRACT

***Background*:** Carotenoids such as β-carotene, α-carotene, lycopene, lutein and β-cryptoxanthin are a family of pigmented compounds which are synthesized by many vegetable crops and microorganisms but not animals. In human and murine models, carotenoids are shown to mediate their effects via different mechanisms such as gap junction communication, cell growth regulation, modulating gene expression and immune response. In some fish species, the immunomodulating action of synthetic carotenoids has been the subject of some research. How ever, studies on the effects of carotenoids from natural sources on fish growth performance and immune parameters are lacking. In the current study, a preliminary study with 60 days feeding was conducted to study the influence of different natural sources of carotenoids from some vegetable crops on growth and some immune indices in rainbow trout (*Oncorhynchus mykiss*).

***Materials, Methods & Results*:** Purified isonitrigenous (crude protein: 40.16%) and isocaloric (3660 kcal kg^{-1}) diet with 4.5 g of powdered crops namely tomato (*Solanum lycopersicum*) and sweet pepper (*Capsicum annuum*) per kg feed or control diet without any treatment was prepared. Rainbow trout weighing 150 ± 9 g were distributed equally into 2 groups with 33 fish in each group. Each group contained 11 fish in triplicate reared in individual ponds. In treatment group, fish were fed diet containing 4.5 g of powdered crops for 60 days while in control group, basal diet without any treatments was fed. At the end of the feeding trial, 4 fish per tank were sampled to analyze the growth parameters. Seven fish were also bled from the caudal vein to collect serum sample for immune parameters. During this study no mortality of fish was observed in different groups. Results of this study showed that condition factor and feed intake were similar among the groups while specific growth rate and weight gain showed a significant increase in treatment group compared to control group. Immunological parameters namely peroxidase content, antibacterial activity, α1-antiprotease, total antiprotease activities and total protein did not vary among the groups, even though slight decrease in serum peroxidase content was shown in treatment group. On the other hand, serum lysozyme activity of fish fed treatment diet was significantly higher than control group.

***Discussion*:** Enhanced growth performance in the current study might be attributed to some intermediary metabolism which could enhance nutrient utilization and may ultimately result in improved fish growth. Lysozyme is secreted by leukocytes and is a marker of leukocyte activity, increasing concomitantly with phagocytic activity. Administration of natural carotenoids in fish diet exerts a stabilizing or protective effect against oxidative damage, and enhances the proliferation of these cells, which could result in increased serum lysozyme level. Feeding natural carotenoids might act as a direct scavenger of reactive oxygen species and decrease the body's need for certain antioxidant enzymes. Therefore, slight decrease in serum peroxidase content can be attributed to this point. In conclusion, this study showed that rainbow trout appear to benefit from inclusion of crops in diet in terms of improved growth performance and immune system.

Keywords: rainbow trout, immune system, growth performance, *Solanum lycopersicum*, *Capsicum annuum*.

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INTRODUCTION

Fish respond to infectious agents in both non-specific and specific ways even though they rely mostly on non specific immune systems. Immunostimulants have the ability to augment the resistance to diseases by enhancing nonspecific and specific defense mechanisms [5]. Among immunostimulants, depending on their sources, natural ones are preferable because they are biocompatible, biodegradable, cost effective and safe for the environment [5,15].

Carotenoids are a family of pigmented compounds that are synthesized by many vegetable crops and microorganisms but not animals [8]. Some major dietary carotenoids are β-carotene, α-carotene, lycopene, lutein and β-cryptoxanthin [14]. Recent studies show that carotenoids may mediate their effects via different mechanisms such as gap junction communication, cell growth regulation, modulating gene expression and immune response [8,14]. However, carotenoids such as α- and β-carotens and β-cryptoxanthin have the advantage of being converted to vitamin A and its related role in the development and disease prevention. A few studies in fish have been observed on the influence of dietary synthetic caroteniod sources on immune responses [1,6,7,16,17]. However, study the effects of carotenoids from natural sources in modulating immune responses in fish still lacking. The effects of carotenoids from marine algae and red yeast on rainbow immune system were previously observed [2]. Hence, an investigation aimed to study the effects of some vegetable crops as the sources of carotenoids on the immune mechanisms in rainbow trout was performed.

MATERIALS AND METHODS

Specimens

Sixty-six fish (mean body weight 150 ± 9 g) of the rainbow trout (*Oncorhynchus mykiss*) were kept in 300 L running water concrete tanks at 14 ± 1ºC and in a fish farm in Tabriz, Iran. Study was started from July 2010 and specimens were exposed to a natural photoperiod (14:10 h light/dark schedule) in the fish farm. Fish were acclimatized to the experimental rearing condition for 14 days. Dissolved oxygen, ammonia, nitrate, nitrite and pH were monitored and remained within limits recommended for rainbow trout.

Carotenoid preparation

Tomato (*Solanum lycopersicum*) and sweet pepper (*Capsicum annuum*) were sundried. The dried crops were finely ground and passed through a fine meshed sieve to ensure homogeneity. Powders were mixed in equal volume and stored at 4ºC for further use.

Dietary intake of carotenoid

Sixty-six fish were divided randomly into two groups, in triplicate, with 11 fish in each tank. Two purified isonitrigenous (crude protein: 40.16%) and isocaloric (3660 kcal kg^{-1}) diets supplemented with vegetable crop powder (4.5 g kg^{-1}) or without carotenoid as control group were prepared as shown in Table 1. Feeding trial was conducted for 60 days. Feed was given at 1.5 % of total biomass, three times daily.

Table1. Composition of the experimental diet (% of dry matter).

Experimental diets	
INGREDIENT	
Fish meal, menhaden[1]	50.00
White wheat	10.00
Soybean meal (48%)	17.27
Soybean oil	7.00
Grain corn	12.48
DL-Methionine	0.5
L-Lysine	0.5
Vitamin mixture[2]	0.5
Mineral mixture[3]	0.5
Vitamin E	0.5
Choline chloride	0.1
Binders	0.15
PROXIMATE ANALYSES (IN DRY MATTER)	
Dry matter (%)	90.26
Crude protein (%)	40.16
Crude lipid (%)	15.54
Crude fiber (%)	3.99
Digestible energy (kcal kg^{-1})	3660

[1]ATA Co. Tabriz, Iran. Crude protein: 64.5%. [2]ATA Co. Tabriz, Iran. Vitamin premix (mg kg^{-1} unless otherwise stated): vitamin A 600 IU; vitamin D_3 300 IU; vitamin E 7; vitamin B_1 8; vitamin B_2 7; vitamin B_3 15; vitamin B_5 22; vitamin B_6 6; vitamin B_9 3; vitamin B_{12} 3; vitamin H_2 4; vitamin C 25. [3]ATA Co. Tabriz, Iran. Mineral premix (mg kg^{-1} diet): Copper 25; Ferrous 75; Zinc 35; Selenium 3; Manganese 20; Magnesium 20.

Growth performance

At the end of feeding period, 4 fish from each individual tank were starved for 24 h, then weighted and factors such as feed intake (g feed/ fish), weight gain, specific growth rate (SGR) and condition factor (CF) were calculated as following:

Weight gain = final weight (g) – initial weight (g);

SGR = 100/ (ln $W_2 - W_1$)/ T; where W_1 and W_2 are the initial and final weight, respectively, and T is the number of days in the feeding period;

CF = W_2/ $L^3 \times 100$, where W_2 is final weight (g), L is total length (cm).

Blood collection

At the end of the feeding trial, 7 fish from each tank were sampled. The fish were anaesthetized with solution containing clove powder (200 mg L^{-1}). Blood samples were collected from the caudal vein and allowed to clot at 4ºC for 5 h. After centrifugation, serum was removed and frozen at -80ºC until use.

Immunological parameters

- Lysozyme activity

Briefly, 25 µL of individual serum was mixed with 175 µL *Micrococcus lysodiekticus*[1] suspension at 750 µg mL^{-1} in 0.1 M phosphate citrate buffer, pH 5.8. The change in turbidity was measured after 4 and 9 min at 450 nm at approximately 20ºC using a microplate reader. A unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001 per min and expressed as U mL^{-1} of serum [19].

- Serum bactericidal assay

Briefly, 33 µL of serum in triplicate was diluted with 100 µL of 10^8 mL^{-1} bacterial suspension of *Salmonella typhimurium* (ATCC 1370) in Tryptone soy broth (TSB)[2]. After incubation for 6 h at 18ºC, plate was shaken slightly for 10 min and supernatant was discarded. 100 µL of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT)[3] (0.5 mg ml^{-1}) was added to each well [20]. After 15 min in the dark, the optical density (600 nm) of the viable bacteria was measured.

- Serum α1-antiprotease assay

In tubes 10 µL of serum was diluted with 20 µg of trypsin dissolved in 100 µL of Tris-HCl (50 mM, pH 8.2). In the serum blank, trypsin was replaced with 100 µL of Tris-HCl, and in the positive control no serum was added to trypsin. All tubes were made up to 200 µL with Tris-HCl and incubated at room temperature for 1 h. Then, 2 mL of 0.1 mM substrate, BAPNA (Na-benzoyl-DL-arginine-p-nitroanilide HCl)[4] dissolved in Tris-HCl (containing 20 mM calcium chloride), was added to all tubes and incubated for a further 15 min [13]. Finally the color-change was stopped by adding 500 µl of 30% acetic acid and the optical density was read at 410 nm in a UV-visible spectrophotometer[5]. The OD of the positive control, i.e. the activity of trypsin without the addition of any serum was taken as 100% activity. The OD values of the serum blank were deducted from trypsin plus serum, and the percentage trypsin inhibition by the serum was calculated [13].

- Serum total antiproteases assay

In tubes 10 µL of serum was diluted with 20 µg of trypsin dissolved in 100 µL of PBS (pH 7.4). All tubes were incubated at room temperature for 30 min. Then, 1 mL of casein dissolved in PBS (2.5 mg/ mL), was added to all tubes and incubated for a further 15 min. Finally the color-change was stopped by adding 500 µL of 10% trichloroacetic acid. All tubes were centrifuged at 800 g for 10 min to remove the precipitate. The optical density was read at 280 nm in a Biophotometer[6] [13].

- Serum peroxidase content

Serum samples (15 µL) were placed in each well of a 96 well plate. 135 mL of HBSS without Ca^{+2} or Mg^{+2} was then added to each well. Finally, 50 mL of 20 mM 3,30,5,50-tetramethylbenzidine hydrochloride (TMB)[7] and 5 mM H_2O_2 was added. The color-change reaction was stopped after 2 min by adding 50 mL of 2 M sulfuric acid and the optical density was read at 450 nm by ELISA reader [3].

Total protein content

Serum total protein was determined following the method of Bradford [9].

Statistical analysis

The results were subjected to Independent-Sample T-Test to compare different treatments using the SPSS 15. Correlation coefficients were significant with $P < 0.05$.

RESULTS

During this study no mortality of fish was observed in different groups. Significant differences

($P < 0.05$) in SGR and weight gain were recorded between dietary treatments while feed intake and CF were not significantly affected by treatment diet (Table 2). Immune parameters of two groups are presented in Table 3. Serum peroxidase content, antibacterial activity, α1-antiprotease and total antiprotease values did not show any significant changes. Serum total protein was not also enhanced in treatment group. Serum lysozyme activity of fish fed treatment diet was significantly higher than control group.

Table2. Growth performance of rainbow trout fed diets with experimental diets (initial average weight 150 ± 9 g). Values are means ± SE of three replicates. Means in one row with different letters are significantly different ($P < 0.05$).

Parameter	Control group	Treatment group
Specific growth rate	0.932 ± 0.029^a	1.680 ± 0.017^b
Condition factor	1.530 ± 0.035	1.420 ± 0.046
Weight gain	45.775 ± 0.501^a	79.188 ± 1.014^b
Feed intake	73.388 ± 1.006	63.705 ± 0.919

Table 3. Immune parameters of rainbow trout fed with experimental diets (initial average weight 150 ± 9 g). Values are means ± SE of three replicates. Means in one row with different letters are significantly different ($P < 0.05$).

Parameter	Control group	Treatment group
Peroxidase content (450 nm)	0.268 ± 0.004	0.258 ± 0.005
Anti Bacterial activity (600 nm)	0.727 ± 0.039	0.752 ± 0.042
α_1-antiprotease (410 nm)	0.046 ± 0.005	0.051 ± 0.005
Total antiprotease (280 nm)	2.484 ± 0.015	2.406 ± 0.018
Total protein (mg mL^{-1})	48.344 ± 1.55	47.566 ± 0.77
Lysozyme (U mL^{-1})	2.944 ± 0.152^a	5.153 ± 0.05^b

DISCUSSION

Pepper is an important agricultural crop for the nutritional value, mainly due to natural colors and antioxidant compounds. Lycopene, a powerful natural caroteniod in pepper, and β-carotene are also present which act as chain-breaking antioxidants and as scavenger and quencher of singlet oxygen [12]. Tomato is also lycopene-rich food with other carotenoids, namely lutein and β-carotene which are present in much smaller amounts [10].

In the current study, administration of vegetable crops influenced the specific growth rate and weight gain in rainbow trout during the term of the experiment. This result is consistent with previous study, where higher growth rate and reduced feed conversion was observed [18]. The positive role of these products can be attributed to some intermediary metabolism which could enhance nutrient utilization and may ultimately result in improved fish growth.

In this study serum lysozyme activity also increased with the addition of vegetable crops. A similar pattern in fish lysozyme activity following the administration of carotenoids was previously shown [1,2]. It is believed that lysozyme is secreted by leuko-

cytes and is a marker of leukocyte activity, increasing concomitantly with phagocytic activity. Incorporation of carotenoids into the leukocyte plasma membrane lipids exerts a stabilizing or protective effect against oxidative damage, and enhances the proliferation of these cells, which could result in increased serum lysozyme levels [1].

When fish encounter pathogens, myeloperoxidase and eosinophil peroxidase in granules of phagocytic cells can be released by degranulation when properly activated. They use H2O2 and halide ions to form chlorides and chloramines to help in the fight [4]. In the present study, no changes in serum peroxidase content compared to the control diet occurred even though slight decrease was shown in treatment group. Similarly, decrease in leukocyte superoxide dismutase activity and serum glutathione peroxidase concentration following administration of beta-carotene in healthy adults was previously shown [11]. It was previously assumed that supplemental carotenoids may act as a direct scavenger of reactive oxygen species and decrease the body's need for certain antioxidant enzymes [11].

In general, our present study indicates the potential protective role for carotenoids from some vegetable crops on rainbow trout growth performance and lysozyme activity.

SOURCES AND MANUFACTURERS

[1]Sigma-Aldrich, Milwaukee, WI, USA.

[2]Merck, Darmstadt, Germany.

[3]Sigma-Aldrich, Milwaukee, WI, USA.

[4]Sigma-Aldrich, Milwaukee, WI, USA.

[5]Shimadzu-120, Tokyo, Japan.

[6]Eppendorf, Hamburg, Germany.

[7]Sigma-Aldrich, Milwaukee, WI, USA.

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REFERENCES

1 **Amar E.C., Kiron. V., Satoh S. & Watanabe T. 2001.** Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*. 32(Suppl 1): 162-173.

2 **Amar E.C., Kiron V., Satoh S. & Watanabe T. 2004.** Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. *Fish and Shellfish Immunology*. 16(4): 527-537.

3 **Cuesta A., Rodriguez A., Esteban M.A. & Meseguer J. 2005.** *In vivo* effects of propolis, a honeybee product, on gilthead seabream innate immune responses. *Fish and Shellfish Immunology*. 18(1): 71-80.

4 **Cuesta A., Rodriguez A., Salinas I., Meseguer J. & Esteban M.A. 2007.** Early local and systemic innate immune responses in the teleost gilthead seabream after intraperitoneal injection of whole yeast cells. *Fish and Shellfish Immunology*. 22(3): 242-251.

5 **Divyagnaneswari M., Christybapita D. & Michael R.D. 2007.** Enhancement of nonspecific immunity and disease resistance in *Oreochromis mossambicus* by *Solanum trilobatum* leaf fractions. *Fish and Shellfish Immunology*. 23(2): 249-259.

6 **Grether G.F., Kasahara S., Kolluru G.R. & Cooper E.L. 2004.** Sex-specific effects of carotenoid intake on the immunological response to allografts in guppies (*Poecilia reticulata*). *Proceeding of the Royal Society Lond B*. 271(1534): 45-49.

7 **Jha A.K., Pal A.K., Sahu N.P., Kumar S. & Mukherjee S.C. 2007.** Haemato-immunological responses to dietary yeast RNA, u-3 fatty acid and b-carotene in *Catla catla* juveniles. Fish and Shellfish Immunology. 23(5): 917-927.

8 **Kopsell D.A. & Kopsell D.E. 2006.** Accumulation and bioavailability of dietary carotenoids in vegetable crops. *Trends in Plant Science*. 11(10): 499-507.

9 **Kruger N.J. 1999.** The Bradford method for protein quantitation. In: Walker J.M. (Ed). *The Protein Protocols Handbook*. Totowa: Humana Press, pp.11-15.

10 **Lin C.H. & Chen B.H. 2005.** Stability of carotenoids in tomato juice during storage. *Food Chemistry*. 90(3-4): 837-846.

11 **McGill C.R., Green N.R., Meadows M.C. & Gropper S.S. 2003.** Beta-carotene supplementation decreases leukocyte superoxide dismutase activity and serum glutathione peroxidase concentration in humans. *Journal of Nutritional Biochemistry*. 14(11): 656-662.

12 **Navarro J.M., Flores P., Garrido C. & Martinez V. 2006.** Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. *Food Chemistry*. 96(1): 66-73.

13 **Rao V. & Chakrabarti R. 2005.** Stimulation of immunity in Indian major carp *Catla catla* with herbal feed ingredients. *Fish and Shellfish Immunology*. 18(4): 327-334.

14 **Roa A.V. & Roa L.G. 2007.** Carotenoids and human health. *Pharmacological Research*. 55(3): 207-216.

15 **Sakai M. 1999.** Current research status of fish immunostimulants. *Aquaculture*. 172(1-2): 63-92.

16 **Tachibana K., Yagi M., Hara K., Mishima T. & Tsuchimoto M. 1997.** Effects of feeding β-carotene supplemented rotifers on survival and lymphocyte proliferation reaction of fish larvae of Japanese parrotfish (*Oplegnathus fasciatus*) and Spotted parrotfish (*Oplegnathus punctatus*): preliminary trials. *Hydrobiologia*. 358(1-3): 313-316.

17 **Thompson I., Choubert G., Houlihan D.F. & Secombes C.J. 1995.** The effect of dietary vitamin A and astaxanthin on the immunocompetence of rainbow trout. *Aquaculture*. 133(2): 91-102.

18 **Torrissen O.J. 1984.** Pigmentation of salmonids. Effects of carotenoids in eggs and start-feeding diet on survival and growth rate. *Aquaculture*. 43(1-3): 185-193.

19 **Tukmechi A., Rahmati Andani H.R., Manaffar R. & Sheikhzadeh N. 2011.** Dietary administration of Beta-mercapto-ethanol treated *Saccharomyces cerevisiae* enhanced the growth, innate immune response and disease resistance of the rainbow trout, *Oncorhynchus mykiss*. *Fish and Shellfish Immunology*. 30(3): 923-928.

20 **Villamil L., Figueras A. & Novoa B. 2003.** Immunomodulatory effects of nisin in turbot (*Scophthalmus maximus* L.). *Fish and Shellfish Immunology*. 14(2): 157-169.