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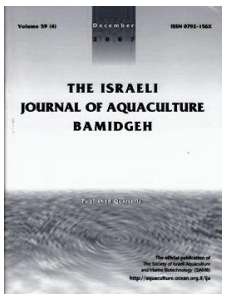

Effects of Dietary Astaxanthin on Innate Immunity and Disease Resistance against *Edwardsiella tarda* in Olive Flounder *Paralichthys olivaceus*

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Key words: olive flounder, astaxanthin, immune responses, *Edwardsiella tarda*

Abstract

We report on the non-specific immune responses and disease resistance against *Edwardsiella tarda* in olive flounder fed diets supplemented by astaxanthin. Four experimental diets were formulated to be isonitrogenous (45% crude protein) and isocaloric (17.1 MJ/kg, dry matter). Astaxanthin was added to the diets at 0 (control), 1%, 2%, and 3%. Three replicate fish groups (30 fish/tank) were fed one of the experimental diets for 15 days. After 15 days, 30 healthy fish per dietary treatment were selected and injected with 1 ml *E. tarda* suspension to evaluate the disease resistance of the fish. Dietary supplementation of astaxanthin resulted in significantly higher non-specific immune responses than in fish fed the control diet. Cumulative mortality in the challenge test with *E. tarda* was significantly lower in fish fed the astaxanthin-supplemented diets than in fish fed the control diet. Results indicate that dietary supplementation of astaxanthin can enhance non-specific immune responses and disease resistance of olive flounder against *E. tarda*, and that a relatively short feeding period of astaxanthin can improve immunity in fish.

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Introduction

The olive flounder, *Paralichthys olivaceus*, is the most important marine cultured species in Korea, Japan, and China. However, one of the current problems in aquaculture is that farmers use a large quantity of antibiotics to protect cultured fish from pathogenic agents such as edwardsiellosis, vibriosis, and streptococcosis, and antibiotic residuals in aquaculture products are causing serious concern to consumers. One promising way to prevent the overuse of antibiotics in aquaculture is to investigate the immunomodulatory effects of vitamin C or E, and the sparing effects of other antioxidant nutrients on the antibiotic. Vitamins have been used as immunostimulants in aquafeeds and considerable research has focused on vitamins C and E as antibiotic substitutes (Eo and Lee, 2008; Galaz et al., 2010). Antioxidants, such as vitamin C (Waagbo et al., 1993), vitamin E (Galaz et al., 2010), and astaxanthin (Christiansen et al., 1995a), have been supplemented in diets to promote health and immunity of finfish.

Astaxanthin, one of the dominant carotenoids in marine animals, showed both a strong quenching effect against singlet oxygen and a strong scavenging effect against free radicals (Miki, 1991). Miki (1991) concluded that astaxanthin has the properties of a "super vitamin E". It is the major carotenoid in Atlantic salmon and rainbow trout (Choubert et al., 2006; Ytrestoyl et al., 2006) and the most common carotenoid used for salmonid pigmentation (Choubert et al., 2009). It also increases egg survival and percentage of fertilized eggs (Christiansen and Torrissen, 1997), protects eggs against harsh environmental conditions (Craik, 1985), and improves growth performance (Torrissen, 1984).

The synergistic effects of astaxanthin with vitamin A have been reported, mainly from the viewpoint of pigmentation, reproduction, and growth in salmonids (Christiansen and Torrissen, 1996), antioxidant status, immunity (Christiansen et al., 1995b), and growth performance during the first feeding of Atlantic salmon (Christiansen et al., 1994; 1995a). Thus, administration of dietary astaxanthin might be a promising alternative to antibiotics in the aquaculture industry. In this study, we examine the vitamin C sparing effect of astaxanthin, its effects on non-specific immune response and disease resistance against pathogenic agents, and the immunostimulant efficiency of its short-term administration in olive flounder.

Materials and Methods

Experimental design and diets. A feeding trial was conducted using a completely randomized design. Four isonitrogenous (45% crude protein) and isocaloric (17.2 MJ/kg) diets were prepared (Table 1). Astaxanthin powder was supplemented in diets at levels of 0 (control), 1%, 2%, and 3%. Dry ingredients were thoroughly mixed with distilled water. The dough was extruded as 3.0 mm diameter threads through a meat chopper (SMC-12, Jeju, Korea) and freeze-dried at -40°C for 24 h. Pellets were crushed into desirable particle sizes (0.4-2.0 mm) and stored at -20°C until use. The gross energy of each diet was determined using values of 16.7 KJ/g protein or carbohydrate and 37.6 KJ/g lipid (Garling and Wilson, 1976).

Experimental fish and feeding trial. Juvenile olive flounder were transported from a private hatchery (Haesoori Fisheries Co., Jeju Island, South Korea) to the Marine and Environmental Research Institute at Jeju National University, South Korea, acclimated to the experimental facilities and conditions, and fed a commercial diet for 2 weeks. Three hundred and sixty fish (28.3±0.2 g) were randomly distributed into twelve 150-l tanks (30 fish per tank) in a flow-through system supplied with sand-filtered sea water at a flow of 3 l/min. Diets were fed twice a day (08:00 and 18:00) to apparent satiation to three groups of fish (90 fish per treatment), 7 days a week for 15 days. Experimental protocols followed guidelines approved by the Animal Care and Use Committee of Jeju National University.

Samples and analyses. At the end of 15 days of feeding, three fish per tank (9 fish per treatment) were selected 3, 6, 12, and 24 h after the last feeding and anesthetized in tricaine methanesulfonate (MS-222) solution (100 mg/l). Blood was taken from caudal veins to evaluate non-specific immune response. Superoxide anion content produced by

Table 1. Formulation and proximate composition of astaxanthin-supplemented diets fed to olive flounder for 15 days.

	Diet (% astaxanthin)			
	0	*1%*	*2%*	*3%*
Ingredient (%)				
White fishmeal	45	45	45	45
Wheat flour	23	23	23	23
Soybean meal	15	15	15	15
Squid liver oil	8	8	8	8
Starch	5	4	3	2
Astaxanthin powder[1]	0	1	2	3
Yeast	2	2	2	2
Mineral mixture[2]	1	1	1	1
Vitamin mixture[3]	1	1	1	1
Proximate composition				
Dry matter (%)	4.57	4.20	3.23	3.60
Protein (% DM)	45.3	45.9	45.8	45.1
Lipid (% DM)	12.1	12.7	12.0	11.9
Ash (% DM)	9.0	9.2	8.9	8.8

[1] Astaxanthin powder was kindly provided from Cyanotech Ltd., Kailua-Kona, Hawaii, USA.

[2] Mineral premix (g/kg mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[3] Vitamin premix (g/kg mixture): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

blood leukocytes during respiratory burst was measured by nitro-blue-tetrazolium (NBT) assay as described by Kumari and Sahoo (2005). Liver superoxide dismutase (SOD) activity was determined with an SOD assay kit (Cayman, Ann Arbor, USA). Total ascorbic acid concentrations in liver were measured using the 2,4-dinitrophenylhydrazine (DNPH) colorimetric method described by Dabrowski and Hinterleitner (1989). Samples were homo-genized in a 5% trichloroacetic acid (Sigma, USA) solution containing 250 mM $HClO_4$ (Sigma, USA) and 0.08% EDTA (Sigma, USA). The homogenate was centrifuged at 15,000 rpm for 30 min at 4°C. Twenty-five micro liters of 0.2% dichorophenolindophenol (Sigma, USA) was added to the supernatant (250 ml) and the mixture was incubated at room temperature for 20 min. Then, 250 ml of 2% thiourea (Sigma, USA) in 5% meta-phosphoric acid (Sigma, USA) and 250 ml of 2% DNPH (Sigma, USA) were added and the mixture was incubated at 60°C for 3 h. The absorbance of the mixture was measured at 524 nm after adding 500 ml of ice-cold 18 M sulfuric acid (Fluka, Germany). L-ascorbic acid (Sigma, USA) was used to develop a standard curve. Approximate compositions of the experimental diets were analyzed by methods of AOAC (1995).

Challenge test. At the end of the feeding trial, 10 healthy fish per tank (30 fish per treatment) were randomly selected, intra-peritoneally injected with an *Edwardsiella tarda* suspension (3×10^8 cells/ml), and distributed into fifteen 40-l tanks for a challenge test. The experimental diets were fed during the challenge test and mortality was recorded until day 8, by which time all the fish in the control group were dead.

Statistical analysis. The diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences between mean group values, the differences were assessed using Duncan's multiple range test. Statistical significance was determined by setting the aggregate type I error at 5% ($p<0.05$) for each set of comparisons. Data are presented as means±SD.

Results

No significant differences were observed in NBT activity among fish groups three hours after the last feeding (Fig. 1). Six hours after the last feeding, NBT activity of fish fed 2% astaxanthin was significantly higher than that of fish fed any other diet. Twelve hours after the last feeding, NBT activity was significantly higher in fish fed the 2% and 3% diets. However, 24 h after the last feeding, NBT activity of fish fed the 1% and 3% diets was dramatically but not significantly higher than of the control. Liver SOD activity (Fig. 2) and vitamin C concentration (Fig. 3) increased with the increment of dietary astaxanthin in a dose-dependent manner. Cumulative mortality during the challenge test with *E. tarda* is presented in Fig. 4. Dramatic fish mortalities occurred after day 4 post-

challenge. Fish fed the astaxanthin-containing diets seemed to have higher resistance to *E. tarda* than fish fed the control diet.

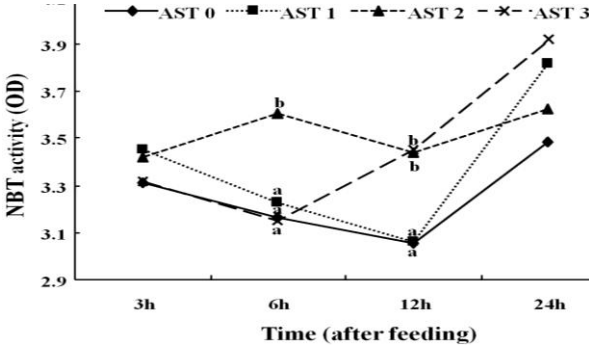

Fig. 1. Nitro-blue-tetrazolium (NBT) activity of olive flounder 3, 6, 12, and 24 h after the last feeding of experimental diets containing 0 (AST0), 1% (AST1), 2% (AST2), or 3% (AST3) astaxanthin for 15 days (means±SD, n = 3). Different letters in the same time period indicate significant differences (p<0.05).

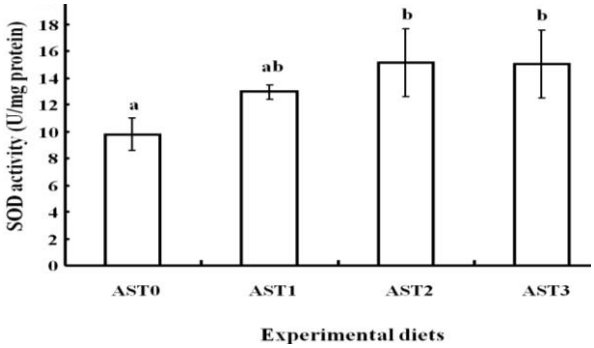

Fig. 2. Liver superoxide dismutase (SOD) activity of olive flounder after the last feeding of experimental diets containing 0 (AST0), 1% (AST1), 2% (AST2), or 3% (AST3) astaxanthin for 15 days (means±SD, n = 3). Different letters above bars indicate significant differences between treatments (p<0.05).

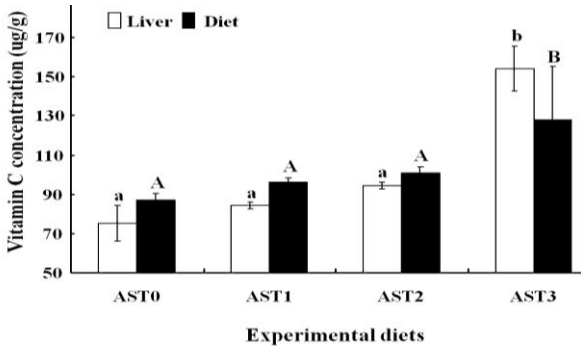

Fig. 3. Vitamin C concentration in the liver of olive flounder after the last feeding of experimental diets containing 0 (AST0), 1% (AST1), 2% (AST2), or 3% (AST3) astaxanthin for 15 days (means±SD, n = 3). Different lowercase and capital letters indicate significant differences between liver contents and diets, respectively (p<0.05).

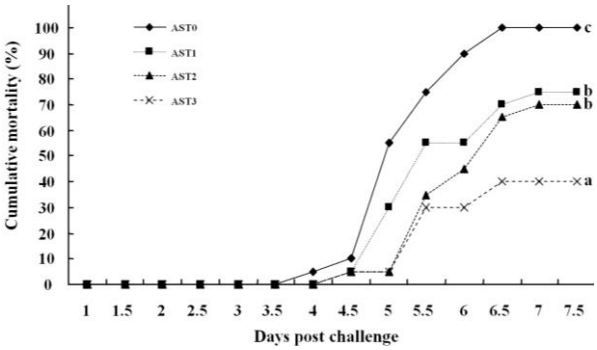

Fig. 4. Mean cumulative mortality after challenge with *Edwardsiella tarda* by intraperitoneal injection in olive flounder fed experimental diets containing 0 (AST0), 1% (AST1), 2% (AST2), and 3% (AST3) astaxanthin for 15 days (n = 3). Different letters indicate significant differences between treatments (p<0.05).

Discussion

Phagocytes are cellular components of immunity and their activation in fish is well documented by an increase of microbicidal activities (Magnadoʼttir, 2006). The increased neutrophil activation shown by NBT results from fish fed the 2% and 3% diets indicates that astaxanthin can play an important role as an immunostimulant in olive flounder.

SOD, an antioxidant enzyme, removes damaging reactive oxygen species by catalyzing dismutation of two superoxide radicals to hydrogen peroxide and oxygen (Fattman et al., 2003). SOD was first related to immunity in a crustacean by Holmblad and Söderhäll (1999) and SOD activity can be used as a criterion for innate immunity of fish (Eo and Lee, 2008). The higher SOD activity in liver of fish fed the 2% and 3% diets seems to be related to the higher dietary vitamin C concentration resulting from the astaxanthin supplementation. The positive correlation between dietary vitamin C and SOD activity was also observed in tiger puffer (Eo and Lee, 2008).

The higher vitamin C concentrations of the 3% diet and liver of fish fed this diet is thought to be due to the vitamin C sparing effect of astaxanthin. Vitamin C is a strong

antioxidant that scavenges reactive oxygen species and can regenerate antioxidant molecules such as α-tocopherol (Lee and Dabrowski, 2004). Sparing effects and/or interactions between vitamins C and E are well documented for fish (Shiau and Hsu, 2002). Vitamin C spares vitamin E by regenerating it from tocopheroxyl radicals (Packer et al., 1979). Astaxanthin is also a strong antioxidant and acts as a free radical scavenger (Krinsky, 1993). The antioxidant activity of astaxanthin is approximately ten times stronger than that of α-tocopherol and other carotenoids such as zexanthin, lutein, tunaxanthin, canthaxanthin, and β-carotein (Miki, 1991).

Antioxidant activity is a defense mechanism in animals as it removes reactive oxygen species. Therefore, it seems that astaxanthin spares vitamin C by regenerating it from dehydro-ascorbic acid that is an oxidized ascorbic acid form with no antioxidant capability. It took eight days after challenge for fish in the 3% group to reach 40% cumulative mortality, whereas the control fish reached 100% mortality during the same period. This demonstrates that dietary supplementation of astaxanthin can have positive effects on the survival of fish in cases of disease outbreaks, by preventing or reducing disease susceptibility. Similarly, injected astaxanthin had significant positive effects on the survival of giant freshwater prawns with a survival improvement of 150% at 4 days and 700% at 6 and 7 days post-bacterial challenge, compared to the challenged control (Angeles et al., 2009). Similar results were reported for black tiger prawn (Supamattaya et al., 2005) and white shrimps (Chang, 2007). Therefore, we attribute the increased disease resistance of olive flounder against *E. tarda* in our study to the dietary supplementation of astaxanthin.

In conclusion, dietary supplementation of astaxanthin can enhance non-specific immune responses and improve disease resistance of olive flounder against *E. tarda*. However, dietary supplementation of astaxanthin for fish probably depends on the age and size of the fish and the period of administration. Further studies need to focus on growth performance and feed utilization with longer periods of administration and related to innate immunity and disease resistance.

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