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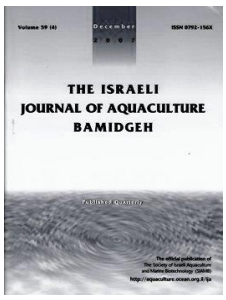

Effects of Dietary *Spirulina pacifica* on Innate Immunity and Disease Resistance against *Edwardsiella tarda* in Olive Flounder *Paralichthys olivaceus*

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Abstract

We tested the effects of short-term dietary supplementation of *Spirulina pacifica* on non-specific responses and disease resistance to *Edwardsiella tarda* in olive flounder, *Paralichthys olivaceus*. Four isonitrogenous (46% crude protein) and isocaloric (17.3 MJ/kg dry mass) experimental diets were formulated to contain 0, 3, 6 or 9% *S. pacifica* (designated SPI0, SPI3, SPI6 and SPI9, respectively). Three replicate fish groups (30 fish/tank) were fed one of the experimental diets for 15 days. After 15 days, healthy fish (30 per dietary treatment) of similar size were injected with 1 mL of *E. tarda* suspension to evaluate disease resistance. Dietary supplementation with spirulina resulted in significantly enhanced non-specific immune responses compared to the control groups. Cumulative mortality in the challenge test with *E. tarda* was significantly lower in fish fed diets containing spirulina compared to that of fish fed the control diet. These results indicate that dietary supplementation of spirulina can enhance non-specific immune responses and disease resistance of olive flounder even over a relatively short feeding period.

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Introduction

Aquaculture farmers currently use large quantities of antibiotics to fight pathogenic agents such as edwardsiellosis, vibriosis, and streptococcosis in commercial fish. The potential effect of residual antibiotics in fish is a concern to consumers. Over the last decade, considerable research has been conducted on the use of microalgae with high levels of antioxidants as immunostimulants to reduce the need for antibiotics (Watanuki et al., 2006; Macias-Sancho et al., 2014).

Spirulina is one of the most frequently used microalgae species in animal feed due to its high protein content and well-balanced amino acid profile compared with other plant protein sources, making it a potentially good additive for aquaculture fish meal (El-Sayed, 1994; James, 2010; Kim et al., 2013b). Spirulina has been shown to enhance fecundity (James et al., 2009), egg quality (Lu and Takeuchi, 2004), pigmentation (Teimouri et al., 2013a; Teimouri et al., 2013b), and immunomodulatory activity, such as phagocytic and natural killer cell activity in various fish species (Qureshi and Ali, 1996). Several studies have investigated the effects of spirulina on growth performance, nutrient utilization, antioxidant capacity, disease resistance, and innate immune responses of various fish species, including olive flounder *Paralichthys olivaceus* (Kim et al., 2006; Bhuvaneswari, 2014; Kim et al., 2013a,b), common carp *Cyprinus carpio* (Nandeesha et al., 1998), tilapia *Oreochromis niloticus* (Takeuchi et al., 2002), Mekong giant catfish *Pangasianodon gigas* (Tongsiri et al., 2010), African sharptooth catfish *Clarias gariepinus* (Promya and Chitmanat, 2011), and shrimp *Litopenaeus vannamei* (Macias-Sancho et al., 2014).

Olive flounder is one of the most important fish species in marine aquaculture in Korea, Japan and China (Pham et al., 2006). Production of this species in Korean aquaculture is high and particularly important, but suffers from edwardsiellosis in culture facilities and surroundings (Galindo-Villegas et al., 2006). Dietary supplementation with spirulina could be a promising alternative to antibiotics in aquaculture of this species. In this study, we examined the effects of short-term supplementation of spirulina on non-specific immune responses and disease resistance of olive flounder to *Edwardsiella tarda*.

Materials and Methods

Experimental design and diets. We formulated four experimental diets containing 0, 3, 6 or 9% spirulina powder (SPI0, SPI3, SPI6 and SPI9). The diets were isonitrogenous and isocaloric in terms of crude protein (46%) and gross energy (17.3 MJ/kg) according to Kim et al. (2002). All dry ingredients were thoroughly mixed with distilled water, then the dough was extruded through a meat chopper (SMC-12, Korea) at 3.0-mm diameter and freeze dried at -40°C for 24 h. The energy value of each diet was estimated based on mammalian physiological fuel values, i.e., 16.7 kJ/g proteins or carbohydrates and 37.6 kJ/g lipid (Lee and Putnam, 1973). The dietary formulation and proximate compositions are presented in Tables 1 and 2. The pellets were crushed to desirable particle sizes (0.4-2.0 mm) and stored at -20°C until use.

Table 1. Analysis of the major ingredients for the experimental diet fed to olive flounder (% dry mass).

Ingredients	Moisture	Protein	Lipid	Ash
White fish meal	8.7	74.8	9.4	14.0
Casein[1]	4.0	91.0	1.0	5.6
Spirulina powder[2]	6.0	61.3	1.8	9.7
Yeast	5.5	42.2	1.0	5.6

[1] Casein was purchased from USB Co. Ltd., Cleveland, OH, USA.
[2] Spirulina powder was provided by Cyanotech Ltd., Kailua-Kona, Hawaii, USA.

Table 2. Formulation and proximate composition of the experimental diets (% DM).

	Diets			
Ingredients	*SPI0*	*SPI3*	*SPI6*	*SPI9*
White fish meal	45.0	45.0	45.0	45.0
Casein	10.0	8.0	6.0	4.0
Spirulina powder	0.0	3.0	6.0	9.0
Starch	10.0	9.0	8.0	7.0
Wheat flour	23.0	23.0	23.0	23.0
Yeast	2.0	2.0	2.0	2.0
Mineral mixture[1]	1.0	1.0	1.0	1.0
Vitamin mixture[2]	1.0	1.0	1.0	1.0
Squid liver oil	8.0	8.0	8.0	8.0
Proximate composition				
Dry matter, % DM	89.0	89.2	89.8	88.6
Protein, % DM	45.6	46.1	46.6	46.3
Lipid, % DM	8.0	8.3	8.4	8.7
Ash, % DM	9.0	9.2	8.9	8.8
Gross energy	17.3	17.3	17.3	17.2

[1] Mineral premix (g kg^{-1} of 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.

[2] Vitamin premix (g/kg of 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.

Experimental fish and feeding trial. Juvenile olive flounder were transported from a private hatchery (Haesoori Fisheries Co., Jeju Island, Korea) to the Marine and Environmental Research Institute, Jeju National University, Korea. All fish were fed a commercial diet for 2 weeks to allow them to acclimate to the experimental facilities and conditions and recover from the stress of transportation. Three hundred and sixty fish (initial body weight 28.3 ± 0.2 g) were randomly distributed into twelve 150 L tanks in a flow-through system supplied with sand-filtered seawater at a flow rate of 3 L/min. The daily water exchange rate was about 70% of the total volume. Each experimental diet was fed to three groups of fish (30 fish/group) to apparent satiation twice a day (8:00 and 18:00 h) for 15 days. The diurnal cycle was 12 h light/12-h dark. Experimental protocols followed the guidelines approved by the Animal Care and Use Committee of Jeju National University.

Samples and analyses. At the end of the feeding trial, nine fish per tank (27 fish per treatment) were anesthetized in tricaine methanesulfonate (MS-222) solution (100 mg/L) and blood was taken from caudal veins for hematological assay. Phagocytes are cellular components of immunity and their activation in fish is well documented resulting in increased microbicidal activities (Magnadóttir, 2006). The increased neutrophil activities from the dietary spirulina shown by nitro-blue tetrazolium (NBT) indicate that the spirulina played an important role as an immunostimulant in olive flounder. We measured the superoxide anion content produced from blood leukocytes during respiratory burst by NBT assay (NBT; Sigma, USA) described by Anderson and Siwicki (1995) and modified by Kumari and Sahoo (2005). Blood and 0.2% NBT were mixed in equal proportions (1:1), incubated for 30 min at room temperature, and then 50 µL were dispensed into glass tubes. Then, 1 mL of dimethylformamide (Sigma, USA) was added and centrifuged at 2,000×g for 5 min. Finally, the optical density (OD) of the supernatant was measured at 540 nm. Dimethylformamide was used as the blank. Myeloperoxidase (MPO) is an important enzyme for microbicidal activity that utilizes the reactive oxygen radical, H_2O_2, to produce hypochlorous acid which is potent in killing pathogens. MPO activity was measured according to Quade and Roth (1997) with the Kumari and Sahoo (2005) modification. We diluted 20 µL of serum with HBSS (Hanks balanced salt solution without Ca2+ or Mg2+, Sigma, USA) and 5 mM H2O2. The color change reaction was terminated after 2 min by adding 35 µL of 4 M sulfuric acid. Finally, optical density was read at 450 nm. We measured liver superoxide dismutase (SOD) activity with a SOD assay kit (Cayman, Ann Arbor, USA). Fish liver was homogenized in nine volumes of 20 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1% Triton X-100. The

homogenate was centrifuged at 10,000 rpm to remove debris. The resultant supernatant was used for the SOD assay according to the method of Ukeda et al. (1997). Analyses of moisture and ash in the experimental diets were performed by standard procedures (AOAC, 1990). We measured crude protein with an automatic Kjeltec Analyzer Unit 2300 (FOSS, Sweden) and crude lipid content with a Soxhlet Extraction System C-SH6 (Korea).

Challenge test. At the end of the feeding trial, 10 fish per tank (30 fish/dietary treatment) were randomly collected and stocked into fifteen 40 L tanks for an *E. tarda* challenge test. These fish were injected intraperitoneally with *E. tarda* suspension (3×108 cfu/mL). The same experimental diets were fed during the challenge test and mortality was monitored hourly and recorded for 8 days.

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among the dietary groups, Duncan's multiple range test was used to determine the mean differences. Two-way ANOVA was used for NBT analysis according to time after feeding. 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. Percentage data were arcsine transformed before statistical analysis.

Results

NBT activity in the blood at 3, 6, and 24 h after the last feeding of each experimental diet is shown in Table 3.

Table 3. Nitro Blue Tetrazolium (NBT) activity (optical density, 540 nm) of olive flounder at 3, 6, 12, and 24 h after the last feeding of experimental diets containing 0% (SPI0), 3% (SPI3), 6% (SPI6), or 9% (SPI9) spirulina for 15 day.[1]

	Time (after the last feeding)			
Diets	3 h	6 h	12 h	24 h
SPI0	3.26±0.27[a]	3.20±0.10[a]	3.42±0.24[a]	3.25±0.00[a]
SPI3	3.35±0.36[a]	3.67±0.24[b]	3.79±0.07[a]	3.74±0.22[a]
SPI6	3.38±0.22[a]	3.66±0.39[b]	3.52±0.20[a]	3.49±0.59[a]
SPI9	3.30±0.10[a]	3.59±0.07[b]	3.63±0.38[a]	3.44±0.14[a]
		Two-way ANOVA		
		Level	Time	Level x Time
P value		0.023	0.113	0.904

[1] Values are presented as means ± SD (n=3). Different letters in the same time period column indicate significant different (P<0.05).

NBT activity at 6 h after feeding was found to be significantly higher in fish fed SPI3, SPI6 and SPI9 than that of fish fed the control diet (SPI0) by the one-way ANOVA test. The two-way ANOVA results indicated that NBT was significantly increased by dietary supplementation levels of spirulina (p = 0.023), but not significantly affected by the time after feeding (p = 0.113). Also, there was no significant interaction between the spirulina levels and time after feeding (p = 0.904).

The MPO activity of fish fed the SPI9 diet was slightly but significantly higher than that of fish fed 0%, 3% and 6% diets (Fig. 1).

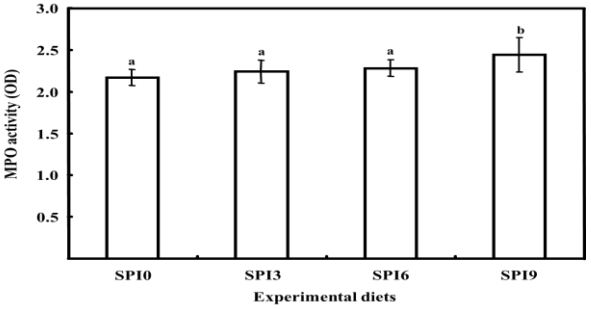

Fig. 1. Myeloperoxidase (MPO) activity of olive flounder after the last feeding of experimental diets containing 0% (SPI0), 3% (SPI3), 6% (SPI6), or 9% (SPI9) spirulina powder for 15 days. Values are presented as means ± SD (n = 3). Different letters above bars indicate significant differences between dietary treatments (P < 0.05).

SOD activity in the liver was increased by higher levels of dietary spirulina (SPI9) in a dose-dependent manner (Fig. 2). However, there were no significant differences in SOD activity among fish fed SPI0, SPI3 and SPI6.

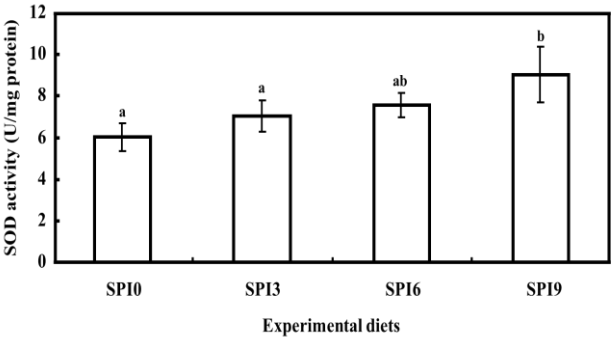

Fig. 2. Liver superoxide dismutase (SOD) activity of olive flounder after the last feeding of experimental diets containing 0% (SPI0), 3% (SPI3), 6% (SPI6), or 9% (SPI9) spirulina powder for 15 days. Values are presented as means ± SD (n = 3). Different letters above bars indicate significant differences among dietary treatments ($P < 0.05$).

A dramatic increase in mortality of olive flounder infected with *E. tarda* occurred 4 to 5 days post-challenge (Fig. 3). The fish fed diets supplemented with spirulina had higher resistance to *E. tarda* than the fish fed the control diet (SPI0). After 7.5 days, cumulative mortality for the SPI9 group was 40%, whereas mortality of the control group was 80%. This seems to be the most dramatic result as opposed to those of NBT and MPO which are negligible.

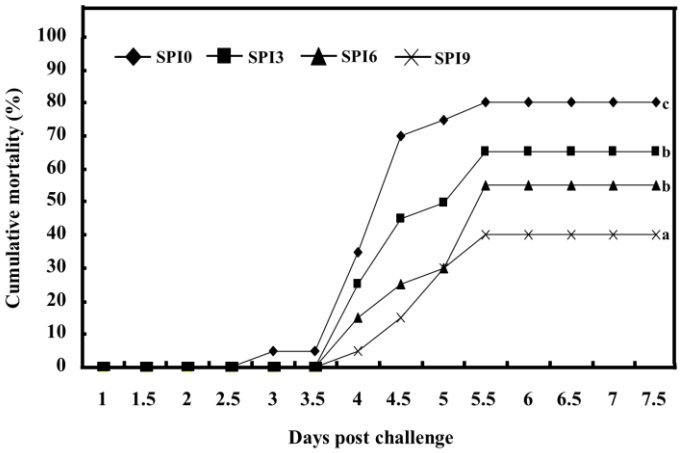

Fig. 3. Mean cumulative mortality after challenge with *Edwardsiella tarda* by intraperitoneal injection in olive flounder fed experimental diets containing 0% (SPI0), 3% (SPI3), 6% (SPI6), or 9% (SPI9) spirulina for 15 days (n = 3). Different letters indicate significant differences among dietary treatments ($P < 0.05$).

Discussion

This study showed that olive flounder fed diets containing spirulina had significantly higher neutrophil counts than fish fed the control diet 6 h after feeding (Table 3). The MPO activity of fish fed the SPI9 diet was significantly higher than that of fish fed the control diet (Fig 1). This suggests that spirulina supplementation in diets can enhance the non-specific immune responses of the fish. Spirulina and other microalgae function as antioxidants. Several antioxidants, such as vitamins C and E (Eo and Lee, 2008; Galaz et al., 2010) and spirulina (Duncan and Klesius, 1996; Watanuki et al., 2006; Macias-Sancho et al., 2014) have been added to diets for fish and crustaceans to promote their health and innate immunity by increasing antioxidant effects. Similarly, significant effects were reported on NBT activity in olive flounder (Kim et al., 2013 a, b). NBT activity of olive flounder (~13 g size) was significantly increased when fed diets supplemented with spirulina up to 3.4% inclusion in the diet, but not significant with levels higher than 6.8% inclusion which even resulted in a decreasing tendency as shown in the present study (Kim et al., 2013a).

SOD is an antioxidant enzyme that removes damaging reactive oxygen derivatives by catalyzing dismutation of two superoxide radicals to hydrogen peroxide and oxygen (Fattman et al., 2003). In this study, SOD activity in the liver of olive flounder was increased with higher levels of dietary spirulina in a dose-dependent manner (Fig 2). SOD activity was used as a criterion for non-specific immunity in crustaceans (Holmblad and Soderhall, 1999). Activity of SOD was suggested as a good indicator of non-specific

immune responses in puffer fish (Eo and Lee, 2008). In the present study, higher SOD activity in the livers of fish fed the SPI9 diet is likely to be related to higher levels of antioxidants in the liver and diets with spirulina supplementation. This positive correlation between dietary spirulina and SOD activity in fish tissue was also observed in olive flounder (Kim et al., 2013a, b). Oral administration (intubation) of spirulina can increase innate immunity of carp by activating leucocyte functions, such as phagocytosis and production of superoxide (Watanuki et al., 2006). Spirulina enhanced innate immunity of channel catfish and thereby increased chemotaxis to *E. tarda* and phagocytosis to zymosan (Duncan and Klesius, 1996).

A dramatic increase in mortality of olive flounder infected with *E. tarda* occurred 4 to 5 days post-challenge (Fig 3). The fish fed diets supplemented with spirulina had higher resistance to *E. tarda* than fish fed the control diet (SPI0). After 7.5 days, cumulative mortality for the SPI9 group was 40%, whereas mortality of the control group was 80%. Thus, dietary supplementation of spirulina significantly reduced the mortality of olive flounder infected with *E. tarda*. After spirulina administration, Nile tilapia, rohu, and carp, when challenged, showed increased disease resistance to *Aeromonas hydrophila* (Watanuki et al., 2006; Abdel-Tawwab and Ahmad, 2009; Andrews et al., 2011). Dietary supplementation of spirulina had positive effects on the non-specific immune responses and thereby delayed or reduced fish mortality (Kim et al., 2013a). Similar results have been reported in Asian tiger shrimp, *Penaeus monodon* (Supamattaya et al., 2005), and white shrimp (Chang, 2007). The non-specific immune system is very important for disease resistance in fish or crustaceans. Several studies have suggested that dietary spirulina can enhance non-specific immune systems in fish or shrimp and thereby increase their resistance to the pathogens. When fed spirulina, the granular hemocyte count in white shrimp increased significantly (10%) (Macias-Sancho et al., 2014) and hemocyte counts and phagocytic acitivity significantly increased when they were immersed in spirulina extracts (Tayag et al., 2010).

In addition, spirulina contains numerous bioactive materials including phytopigments, such as phycobilins, phycocyanine, allophycocyanin and xanthophylls, making it a potential natural dietary source of antioxidants (Miranda et al., 1998; Bhat and Madyastha, 2000; Wang et al., 2007; Bermejo et al., 2008). In a previous study, we investigated the antioxidant effect of experimental diets supplemented with different levels of spirulina. Dietary supplementation of spirulina resulted in slightly higher dietary antioxidant capacity and total polyphenolic compounds concentration (Kim et al., 2013a,b). Since dietary antioxidant capacity is enhanced by spirulina, its supplementation in diets is likely to protect fish from tissue damage by inhibiting formation of reactive oxygen derivatives.

This study confirms previous results related to the non-specific immune responses in fish. With increased innate immunity, olive flounder could enhance disease resistance against the pathogen *E. tarda*. However, the effects of dietary supplementation of spirulina may depend upon the age or size of the fish and the duration or dose of intake. We did not investigate its effects on growth performance or utilization of feed. Further studies are required to examine these effects.

Acknowledgements

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