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Effects of Dietary *Hizikia fusiformis* on Growth and Immune Responses in Juvenile Olive Flounder (*Paralichthys olivaceus*)

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ABSTRACT : An eight week feeding trial was conducted to investigate the effects of dietary supplementation of hizikia (*Hizikia fusiformis*) on growth performance, immune responses and resistance of juvenile olive flounder (*Paralichthys olivaceus*) to *Streptococcus iniae*. Four experimental diets (designated as Hiz 0, Hiz 2, Hiz 4 and Hiz 6) were formulated to be isonitrogenous (50% crude protein) and isocaloric (17.2 MJ/kg DM). Hizikia powder was added at 0%, 2%, 4% and 6% in diets Hiz 0, Hiz 2, Hiz 4 and Hiz 6, respectively. Three replicates of fish groups (15 fish/tank) were fed one of the experimental diets. At the end of feeding trial, no significant differences were observed in final body weight, specific growth rate, protein efficiency ratio, feed utilization and feed intake among fish groups fed the experimental diets. However, there was clear trend that the growth performances of fish were improved by the increment of dietary hizikia showing a positive growth effects. Mean phagocytes activated with nitro-blue-tetrazolium were significantly increased with the increment of dietary hizikia. The cumulative mortality was significantly ($p<0.05$) lower in the fish groups fed Hiz 6 diet (no mortality) than that in the other fish groups for 15 days of *S. iniae* challenge test. The findings of this study suggest that a dietary supplementation of hizikia could enhance the nonspecific immune response and improve the resistance of juvenile olive flounder to *S. iniae*. (**Key Words** : Olive Flounder, *Hizikia fusiformis*, Immune Response, *Streptococcus iniae*)

INTRODUCTION

Olive flounder (*Paralichthys olivaceus*) is currently the most important marine aquaculture fish species in Korea. Its aquaculture production increased from 1,037mt in 1990 to 36,100mt in 2001 (Ministry of Maritime Affairs and Fisheries, 2002). However, one of the current problems in the culture of the species is the fact that fish farmers use a large quantity of antibiotics to prevent the species from bacterial diseases.

Streptococcus iniae was isolated and described for the first time in a freshwater dolphin, *Inia geoffrensis* (Pier and Madin, 1976). This bacterium has been reported as a causative pathogen and resulted in a large economic loss in many fish species, such as rainbow trout (Eldar and Ghittino, 1999; Lahav et al., 2004), tilapia (Perera et al., 1994; Shoesmaker and Klesius, 1997; Shoesmaker et al., 2000; Shelby et al., 2002), hybrid stripped bass (Stoffregen et al., 1996), gilthead seabream and European sea bass (Zlotkin et al., 1998), barramundi (Bromage and Owens, 2002) and olive flounder (Nakatsugawa 1983; Nguyen and Kanai, 1999; Nguyen et al., 2002). Streptococcus disease has been reported to occur frequently in the aquaculture of olive flounder in Korea during summer season when the water temperature increases and fluctuates. Antibiotics, such as ampicillin, ciprofloxacin, doxycycline, streptomycin and oxytetracycline have been used to treat the infectious bacterial diseases in aquaculture farms. However, the efficacy of these treatments depends upon many factors, such as the drug concentration, infectious intensity and time of treatment (Heo et al., 2001; Chen et al., 2005). In addition, the use of antibiotics can lead to a pollution, a damage into the ecological system in the surrounding environment of fish farms and drug resistances of the fish against pathogenic agents. The residual of these chemicals in aquaculture products has also been reported as a serious concern for consumers. Use of natural compounds to improve nonspecific immune response of fish and to prevent the outbreak of a disease has been considered as a feasible solution to develop a sustainable and antibiotic-free aquaculture system.

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Table 1 Formulation and proximate composition of the experimental diets containing different levels of hizikia (g/1,000 g dry matter)

Ingredients	Diets			
	Hiz 0	Hiz 2	Hiz 4	Hiz 6
White fish meal	500.0	500.0	500.0	500.0
Soybean meal	70.0	70.0	70.0	70.0
Corn gluten meal	70.0	70.0	70.0	70.0
Hizikia[1]	0.0	20.0	40.0	60.0
Wheat flour	244.0	224.0	204.0	184.0
Yeast	10.0	10.0	10.0	10.0
Mineral mix [2]	5.0	5.0	5.0	5.0
Vitamin mix [3]	4.6	4.6	4.6	4.6
Myo-inositol	0.4	0.4	0.4	0.4
Vitamin C	4.0	4.0	4.0	4.0
Vitamin C and E	1.0	1.0	1.0	1.0
Choline chloride	1.0	1.0	1.0	1.0
Squid liver oil	80.0	80.0	80.0	80.0
CMC	10.0	10.0	10.0	10.0
Proximate composition				
Dry matter (%)	95.7	96.6	96.8	97.0
Protein (% DM)	50.9	50.6	50.1	51.7
Lipid (% DM)	12.6	12.2	12.7	12.6
Ash (% DM)	8.0	8.6	9.2	9.4
Metabolizable energy (MJ/kg DM) [4]	17.2	17.2	17.2	17.1

[1] Hizikia was kindly provided by Professor Jeon, Y.-J., Faculty of Applied Science, Cheju National University.

[2] Mineral premix (g/kg 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.

[3] 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.

[4] Metabolizable energy was estimated on the basis of mammalian physiological fuel value for dietary ingredients (Lee and Putman, 1973).

Hizikia (*Hizikia fusiformis*), an edible brown algae, is wildly distributed in Korea and Japan. It has been reported to have high antioxidant activity (Siriwardhana et al., 2003a; 2003b; 2004; 2005; Karawita et al., 2004) and enhance immune response and disease resistance in human (Shan et al., 1999) and mice (Liu et al., 1997; Okai et al., 1997; 1998). However, no information on the *in vivo* effects of dietary hizikia on nonspecific immune response and disease resistance in fish is available. Therefore, this study was conducted to investigate the effects of dietary hizikia supplementation on growth performances, immune response and resistance of juvenile olive flounder to *S. iniae*.

MATERIALS AND METHODS

Experimental diets

Four experimental diets (designated as Hiz 0, Hiz 2, Hiz 4 and Hiz 6) were formulated to be isonitrogenous and isocaloric in terms of crude protein (50%) and metabolizable energy (17.2 MJ/kg) according to Kim et al. (2002). The energy value of each diet was estimated on the basis of mammalian physiological fuel value, i.e., 16.7 KJ/g protein or carbohydrate and 37.7 KJ/g lipid (Lee and Putman, 1973). The dietary formulation and proximate compositions are presented in Table 1. Hizikia powder was supplemented in diets Hiz 0, Hiz 2, Hiz 4 and Hiz 6 at the level of 0%, 2%, 4% and 6% (dry matter basis), respectively. The proximate composition of the dietary protein sources used in this study is given in Table 2. All dry ingredients were thoroughly mixed with distilled water. The dough was extruded through the meat chopper (SMC-12, Korea) in 3.0 mm diameter size and freeze dried at -40°C for 24 h. The pellets were crushed into desirable particle sizes (0.4-2.0 mm) and stored at -20°C until use.

Experimental fish and feeding trial

Juvenile olive flounder were transported from a private hatchery (Chang-Hae Fisheries Co., Jeju Island, Korea) to Marine and Environmental Research Institute, Cheju National University, Korea. The transported fish were fed a commercial diet for 2 weeks to be acclimated in the experimental facilities and conditions, and to be recovered from the stress of transportation. One hundred eighty fish (initial body weight 14.3±0.1 g) were randomly distributed into twelve 35 L tanks in a flow through system supplied with sand filtered seawater at a flow rate of 3 L/min. Then one of the experimental diets was fed to three groups of fish (15 fish/group) at a feeding rate of 3.5% body weight, twice a day (8:00 and 18:00 h), 7 days a week for 8 weeks. The growth of fish was measured every two weeks and feeding rate was adjusted accordingly. Feeding was stopped 24 h prior to weighing.

Table 2. The proximate composition of major protein ingredients used in the experimental diets (% DM)

Ingredients	Moisture	Protein	Lipid	NFE[1]	Ash
White fish meal	8.72	68.33	8.56	0.32	14.07
Soybean meal	11.68	46.91	2.52	36.44	6.54
Corn gluten meal	9.50	61.70	1.03	26.59	1.18
Yeast	5.49	42.15	0.49	46.25	5.62
Hizikia[2]	8.91	17.07	0.58	57.92	15.52

[1] Nitrogen Free Extracts = 100-(% moisture−% protein+% lipid−% ash).

[2] Hizikia was kindly provided by Professor Jeon, Y.-J., Faculty of Applied Science, Cheju National University.

Table 3. Growth performances and blood cell counts of juvenile olive flounder fed the experimental diets containing different levels of hizikia for 8 weeks*

Diets	Hiz 0	Hiz 2	Hiz 4	Hiz 6
Initial body weight (g)	14.35±0.17	14.32±0.06	14.44±0.09	14.33±0.18
Final body weight (g)	69.77±6.87	68.35±3.06	71.67±8.22	75.36±5.17
Weight gain (WG)[1]	386.1±47.3	377.4±23.0	400.3±60.7	422.1±37.8
Specific growth rate (SGR)[2]	1.22±0.07	1.21±0.04	1.25±0.09	1.28±0.06
Protein efficiency ratio (PER)[3]	1.35±0.15	1.66±0.21	1.36±0.42	1.74±0.09
Feed conversion ratio (FCR)[4]	1.47±0.15	1.20±0.14	1.56±0.46	1.11±0.06
Feed intake (g/g BW)[5]	0.98±0.08	0.87±0.09	0.98±0.20	0.82±0.03
Survival (%)	98.33±3.33	90.63±4.27	95.56±3.85	93.33±6.67
Red blood cell (million cell/mm^3)	4.21±0.20	3.51±0.28	3.80±0.08	4.03±0.73
White blood cell (million cell/mm^3)	0.15±0.01	0.15±0.01	0.14±0.00	0.14±0.00

* Values are presented as mean±SD. Values in the same row having different superscripts are significantly different ($p<0.05$).

[1] WG (%) = 100×(final mean body weight-initial mean body weight)/initial mean body weight.

[2] SGR (%) = [($\log_e$ final body weight-$\log_e$ initial body weight)/days]×100.

[3] PER = wet weight gain/total protein given.

[4] FCR = dry feed fed/wet weight gain.

[5] FI (g/g BW) = dry feed consumed (g)/BW (g).

Sample collection and analysis

At the beginning and the end of feeding trial, all fish were weighed to calculate weight gain, feed conversion ratio, protein efficiency ratio and specific growth rate. Analyses of moisture and ash in the experimental diets were performed by the standard procedures (AOAC, 1995). Crude protein was measured by automatic Kjeltec Analyzer Unit 2300 (FOSS, Sweden). Crude lipid was determined by Soxhlet Extraction System C-SH6 (Korea). Four fish per tank (12 fish per treatment) were anesthetized in tricaine methanesulfonate (MS-222) solution (100 mg/L) and blood was taken from caudal veins for red blood and white blood cell counts. The blood cell counts were determined by hematocytometer.

Polyphenol analysis

Total polyphenol concentration in the experimental diets was determined by a colorimetric method with some modifications (Skerget et al., 2005). Briefly, 1 g of each diet was extracted with 250 ml methanol for 2 h at 40°C. The extracted solution was cooled and filtered through a 0.45 μm syringe filter (Whatman Inc., Clifton, NJ). Folin-Ciocalteu reagent (0.2 N, Sigma) of 2.5 ml was added into filtered solution and kept for 5 min at room temperature, and then 2 ml of Na_2CO_3 solution (75 g/L) was added. The mixture was incubated for 5 min at 50°C and cooled. The absorbance was read at 760 nm using a spectrophotometer (Genesys 10 UV, Rochester, NY, USA). The results were expressed in gram of Gallic acid per kg dry diet.

Respiratory burst assay

The respiratory burst of phagocytic cells based on the superoxide anion (O_2^-) production was measured by nitro blue tetrazolium (NBT) assay with some modification (Anderson et al., 1992; Stasiak and Bauman, 1996). A drop of whole blood was placed on cover slip and incubated in humidified chamber at room temperature for 30 min. Then the cover slips were thoroughly rinsed with 0.85% saline solution and inverted onto a slide with drops of 0.2% NBT solution. The slides were continuously incubated for another 30 min and counted using a light microscope. Four random fields of positive, dark blue stained cells (activated neutrophils) were observed for each cover slip at 400 X.

Bacterial challenge

S.iniae isolated from an outbreak of streptococcus disease in Jeju Island, Korea was used for the challenge test. Seven healthy fish of similar sizes (70 g/fish) per each tank (21 fish per dietary treatment) were selected after the feeding trial and intraperitoneally injected with 1 ml of *S. iniae* suspension (10^6 colony forming units (CFU)/ml). Before the bacterial challenge test, an optimum concentration (10^6 CFU/ml) of bacterium was determined with similar sizes of extra olive flounder 2 weeks before termination of the feeding trial. After the bacterial injection, the fish groups were transferred to twelve 30 L tanks and fed with their respective diets for 15 days. The filtered seawater was supplied at a flow rate of 3 L/min. The mortality was observed twice daily for 15 days. The dead fish with bacterial infection were re-inspected by the clinical symptoms.

Statistical analysis

Data were subjected to one-way ANOVA in SPSS version 11.0. Significances between group means were compared using Duncan's multiple test. Data presented are mean±SD. Regression analysis was adopted to explain a linear relationship between dietary hizikia and growth. The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis.

Table 4. Mean days to the first mortality and cumulative mortality of olive flounder after 15 days of post-challenge with *Streptococcus iniae*[*]

Diets	Days to first mortality	Cumulative mortality** (%)
Hiz 0	7.0	33.33±8.25
Hiz 2	8.0	33.33±8.25
Hiz 4	8.0	28.57±20.22
Hiz 6	***	***

* Values are presented as mean±SD. Values in the same column having different superscripts are significantly different ($p<0.05$).
** Values are mean of triplicate groups.
*** No mortality was observed during the challenge test.

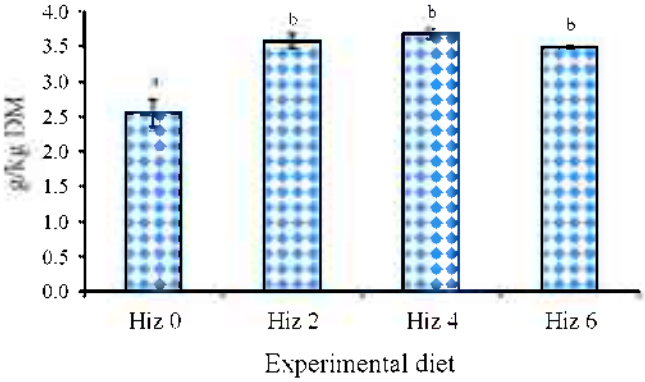

Figure 1. Total polyphenolic compounds in the experimental diets supplemented with 0%, 2%, 4% and 6% hizikia. Values are mean of triplicate groups. Bars with different letters are significantly different ($p<0.05$).

Differences were considered significant at $p<0.05$.

RESULTS

The growth performances of juvenile olive flounder (initial body weight 14.3±0.1 g) fed the experimental diets containing different levels of hizikia are presented in Table 3. At the end of 8 week feeding trial, no significant differences were observed in final body weight, specific growth rate, protein efficiency ratio, feed utilization and feed intake among fish groups fed the experimental diets. However, there was a trend that the growth of fish increased with the increment of dietary hizikia showing a positive growth effect of its dietary supplementation ($y = 13.1x + 364$, $r^2 = 0.75$). Survival of all fish groups was over 90% during the feeding trial. Red blood and white blood cell counts of the fish fed the diets containing hizikia were comparable to that of the control diet (Hiz 0) and there was no significant difference.

Mean neutrophil count activated by nitro blue tetrazolium (NBT) of fish fed the diets Hiz 2 and Hiz 4 was significantly higher than that of fish fed the control diet (Figure 2). Mean neutrophils stained by NBT in fish fed

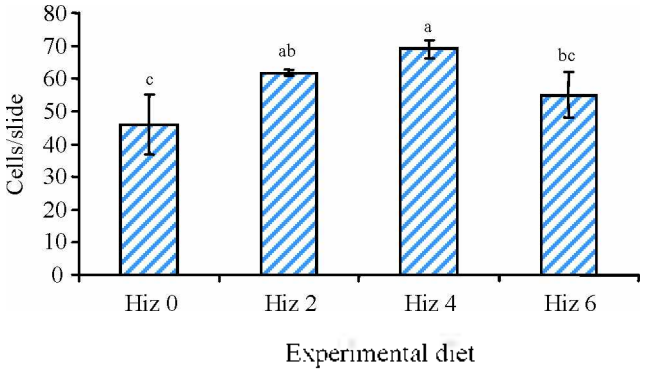

Figure 2. Mean counts of activated neutrophils by nitroblue tetrazolium in fish fed diets Hiz 0, Hiz 2, Hiz 4 and Hiz 6 containing 0%, 2%, 4% and 6% hizikia, respectively. Values are mean of triplicate groups. Bars with different letters are significantly different ($p<0.05$).

diet Hiz 6 was comparable to that of fish fed the diet Hiz 2.

During 15 days of bacterial challenge test (Table 4), the first mortality by *S. iniae* injection was observed in the fish groups fed the control diet (Hiz 0) which was recorded at day 7 after the injection, one day earlier than that of fish groups fed Hiz 2 and Hiz 4 diets. The cumulative mortality was significantly ($p<0.05$) lower in the fish groups fed Hiz 6 diet (no mortality) than that in the other fish groups. At the day 12, the survived fish in hizikia fed groups began to eat and were fully recovered after 3 days.

DISCUSSION

In the present study, the crude protein (50% DM) and energy content (17.2 MJ/kg DM) of diets were formulated based on the protein and energy requirement of juvenile olive flounder (Kim et al., 2002; Choi et al., 2004). Growth performance and feed efficiency of fish fed the experimental diets tended to have positive relationship with the dietary inclusion levels of hizikia (Table 3). The results of growth performance and feed utilization in the present study are significant because, to our knowledge, it is the first hizikia supplemented formulation of diets for olive flounder that resulted in comparable or better growth rate to the control diet (no supplementation of hizikia). Furthermore, the fish fed the control diet grew at a rate comparable to other study (Choi et al., 2004).

Phagocytes (neutrophils and macrophages) are cellular components of immunity and their activation in mammals (Hose et al., 2005; Hume, 2006; Tosi, 2006) and fish (Nikoskelainen et al., 2005; Magnado´ttir, 2006) is well documented with an increase of microbicidal activities. The microbicidal activity is led by a production of reactive oxygen species due to an abrupt rise in oxygen consumption of organisms. In this study, mean neutrophil counts

activated with nitro blue tetrazolium (NBT) in juvenile olive flounder fed diet Hiz 2 and Hiz 4 were significantly higher than that in fish fed the control diet (Figure 2). The number of neutrophils activated by NBT in fish fed diet Hiz 6 was comparable to that in fish fed diet Hiz 2. This finding suggests that some immunostimulants in the hizikia diets might enhance the immune responses of juvenile olive flounder. The higher content of the immunostimulants, such as polyphenol compounds in diets (Figure 1; Hiz 2, 4 and 6) containing hizikia, compared to that of the control diet might be the reason for the enhanced nonspecific immune response of the fish (Figure 2).

A significant immunomodulating activity by an *in vitro* test was reported in the water extract of the brown seaweed, *H fusiformis* for mice (Okai et al., 1998). Okai et al. (1998) mentioned that the immunomodulating activity was related to two fractions, polysaccharides and non-polysaccharides. Castro et al. (2004) also reported that water soluble seaweed extracts produced an increase in the respiratory burst activity of turbot (*Psetta maxima* L.) phagocytes in an *in vitro* test. An administration of a hot water extract of another brown algae, *Sargassum duplicatum* through immersion or injection increased the immune response of white shrimp, *Litopenaeus vannamei* by increasing respiratory burst and resistance against to *Vibrio alginolyticus* (Yeh et al., 2006). The studies (Okai et al., 1998; Castro et al., 2004; Yeh et al., 2006) concluded that polysaccharides in water-extracts of seaweeds was the main fraction that could activate the nonspecific immune responses in both teleosts and shrimps. In the present study, however, it was not clearly demonstrated that polysaccharides in hizikia were the main reason for the increased immune response in the fish. The dietary proportion of wheat meal in this study (Table 1) was replaced by the same amount of hizikia and the wheat meal also contains a large quantity of polysaccharides. Therefore, the immunostimulatory effects of hizikia might be contributed by the polyphenol compounds in hizikia. Water extract of hizikia showed relatively high scavenging activities on hydrogen peroxide and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by its high polyphenolic content (Skerget et al., 2005) leading to the subsequent enhancement of immune response.

Aquaculture operations create many unfavorable environments, where many stressors for the cultured fish species could be occurred by transporting, handling, overcrowding and unknown pathogens (Li et al., 2004). A large volume of antibiotics have been used in fish farms to treat diseases caused by stressors and/or pathogens and prevent the cultured fish from the diseases. Therefore, an alternative way to reduce or cease the use of many types of synthetic antibiotics has been proposed. Recently, several studies showed that immune responses in fish were activated by dietary supplementation of β-glucan (Couso et al., 2003; Selvaraj et al., 2005), A3α-peptidoglycan (Zhou et al., 2006), oligonucleotides (Li et al., 2004) and probiotic bacteria (Panigrahi et al., 2005) as well as antioxidant vitamins, such as ascorbic acid (Lin and Shiau, 2005a) and α-tocopherol (Lin and Shiau, 2005b). Information was extremely limited to date for fish on the dietary supplementation of seaweeds as an immunostimulating agent.

The infection of *Streptococcus iniae* has been reported to cause a mass mortality in the culture of olive flounder (Heo et al., 2001) as well as other fish species (Eldar and Ghittino, 1999; Bromage and Owens, 2002; Lahav et al., 2004). In the present study, at the end of the feeding trial, 7 fish of similar size per each tank (21 fish per dietary treatment) were intraperitoneally injected with 1 ml of *S. iniae* suspension (10^6 CFU/ml). The *S. iniae* challenge concentration used in the present study was 1.43×10^6 CFU/g body weight. This was LD_{50} value of the bacteria for juvenile olive flounder that was determined during two weeks of our preliminary study before the termination of the feeding trial. The mortality of 33% was observed in the unsupplemented and 2% hizikia supplemented groups (Table 4) indicating that the challenge dose of *S. iniae* was appropriate. Also, the challenge dose injected into fish was similar to that reported by Peres et al. (2004). The challenged fish by *S. iniae* injection in the present study showed typical clinical signs, such as dark pigmentation, emaciation, skin lesion and bacterial exophthalmia that were described in rainbow trout and other fish species (Eldar and Ghittino, 1999). The relation between nonspecific immune responses and *S. iniae* has been well documented (Li et al., 2004). Li et al. (2004) demonstrated that dietary oligonucleotides from yeast positively influenced immune responses and resistance of juvenile hybrid stripped bass against *S. iniae* infection. Therefore, it is apparent that the fish fed hizikia containing diets possessed a high resistance to streptococcus disease, particularly in the fish groups fed Hiz 6 diet. The reason for the increased immune response and disease resistance of fish (Figure 2, Table 4) might be attributed to the increased polyphenol concentrations (Figure 1) by dietary inclusion of hizikia.

It is concluded that dietary inclusion of hizikia, a brown seaweed, enhanced the nonspecific immune responses and disease resistance of juvenile olive flounder to *S. iniae*. However, it is assumed that the appropriate dietary inclusion of hizikia for fish would depend upon the age/size-related response and time of intake. Further studies should be focused on these subjects and which polyphenol compounds in hizikia contributed to the enhanced nonspecific immune response of the fish.

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