Effects of a Probiotic and Herbal Supplemented Diet on Growth, Blood Biochemistry, and Innate Immune Response of Olive Flounder and Parrot Fish

Man-Chul Kim†, Ramasamy Harikrishnan†, Moon-Soo Heo*

Marine Applied Microbes and Aquatic Organism Disease Control Lab, Aquatic Biomedical Sciences, School of Marine Biomedical Sciences, College of Ocean Science & Marine and Environmental Research Institute, Jeju National University, Jeju 690 756, South Korea

(Received 28.3.11, Accepted 1.6.11)

Key words: Oplegnathus fasciatus, Paralichthys olivaceus, probiotics, herbal medicine, innate immune response

Abstract

Olive flounder (Paralichthys olivaceus) and parrot fish (Oplegnathus fasciatus) were challenged with the pathogenic Edwardsiella tarda, then fed an unsupplemented control diet or a diet containing probiotics and herbal extracts for 12 weeks. The effects of supplementation on blood biochemistry, innate immune response, and disease resistance were determined at weeks 1, 2, 4, 6, 8, 10, and 12 post-challenge. Final weights were significantly higher in fish fed the supplemented diet. Activity of serum glutamic pyruvic transaminase (SGPT) and serum glucose (GLU) were significantly higher in fish fed the supplemented diet throughout the trial. Values were significantly higher in fish fed the supplemented diet for serum glutamic oxaloacetic transaminase (SGOT) from week 6 to 12 and total protein in weeks 10-12. Respiratory burst activity and serum lysozyme activity were significantly higher in fish fed the supplemented diet in 4-12. Cumulative mortality in fish fed the supplemented diet was 10% for olive flounder and 5% for parrot fish, while for olive flounder and parrot fish fed the unsupplemented control, mortality averaged 50% and 45%, respectively. Results suggest that diets containing probiotics and herbal extracts enhance growth, blood constituents, and innate immunity in both olive flounder and parrot fish.

* Corresponding author. E-mail: msheo@cheju.ac.kr
† The authors contributed equally to this work.
Introduction

The mariculture industry in South Korea is rapidly expanding. According to statistics of the National Fisheries Research and Development Institute (NFRDI), total fish mariculture production was 48,073 tons in 2001 and production almost doubled within five years to 91,125 tons in 2006. Among the mariculture commodities, olive flounder (*Paralichthys olivaceus*) and parrot fish (*Oplegnathus fasciatus*) are major commercially-important mariculture species, with more than 43,000 tons cultured in Jeju Island, South Korea. But, the number of bacterial, viral, and parasitic diseases in aquaculture are on the rise.

Farmers use large quantities of antibiotics and chemicals to contain diseases. However, residual effects of antibiotics and chemicals in aquaculture products are a serious concern of consumers. Vaccines prevent disease in laboratory conditions but are commercially unviable because of prohibitive cost, insufficient protection, or lack of safety (Ninawe, 2006). Alternative disease control strategies involve improved husbandry, better nutrition, improved water quality, lower stocking densities, beneficial micro-organisms (probiotics), and herbal immunostimulants (Ganguly et al., 2010; Harikrishnan et al., 2010a). Biological and synthetic immunostimulant substances such as levamisole, peptidoglycan, β-glucan, chitin, chitosan, yeast, and vitamins enhance fish innate defense systems (Sakai, 1999). Probiotics and herals are increasingly used in disease control against bacterial fish pathogens especially in Asia (Bai et al., 2009) and South America. Supplementation of feeds with probiotics and herbal extracts can replace antibiotics (Bhuvaneswari and Balasundaram, 2006; Kim and Austin, 2006; Bai et al., 2009; Harikrishnan et al., 2010a,b,c,d,e). We examined the effects of dietary supplementation with probiotics and herbal extracts on growth performance, feed utilization, blood biochemistry, innate immune response, and disease resistance in olive flounder and parrot fish.

Materials and Methods

*Fish*. Olive flounder (26.4±1.8 g) and parrot fish (27.2±1.6 g) were obtained from a local fish farm in Jeju Island, South Korea, in March 2008. Fish were transported in plastic bags and acclimated for two weeks in 1000-l tanks filled with sea water during which half the water was exchanged twice a week to remove waste feed and fecal materials, and fish were fed a formulated diet *ad libitum* twice a day (09:00 and 15:00) at 5% of their body weight (Table 1).

*Experimental design*. At the start of the trial, olive flounder (*n* = 150) and parrot fish (*n* = 150) were divided into two groups and challenged with 50 µl at 2.6 × 10^6 CFU/ml *Edwardsiella tarda*. Groups were maintained separately in triplicate tanks. One group of each species was administered a probiotic or herbal supplemented diet (T) and the other an unsupplemented control diet (C). On weeks 1, 2, 4, 6, 8, 10, and 12 post-challenge, fish were individually anesthetized with MS-222 (NaHCO_3 and tricaine methane sulphonate; Sigma Chemicals) at 1:4000 in dechlorinated water for 2 min and blood samples were taken for biochemical and immunological assay. Mortality was recorded daily for 30 days and expressed as cumulative mortality. Weight gain (%) was calculated on week 12 as 100(avg wt gain/avg initial body wt), where average weight gain = [(final total wt + sampled fish wt) - initial total wt]/avg of initial and final number of fish. Feed efficiency was calculated at week 12 as 100(wt gain/ feed intake). Throughout the experiment, water temperature was 18±3.2°C, pH 7.97±1.24, salinity 33.6±1.8 mg/l, dissolved oxygen concentration 6.78±1.56 mg/l, and photoperiod 14 h light:10 h dark.

*Diets*. Formulated fish feed was prepared in the laboratory using soybean and fishmeal as the protein sources (Table 1). The proximate composition of the feed was 51.6% crude protein, 12.5% crude fat, 18.4% crude starch, and 12.3% crude ash. This feed served as the unsupplemented control (C). To prepare the supplemented feed (T), the required amount of herbal extract was added to the feed and the required amount of bacteria was gradually sprayed onto the feed. The feed was mixed part by part in a drum mixer, dried in an oven at 30°C for 18 h, packed, and stored in a freezer at -20°C until use.
Effects of probiotic and herbal supplemented diets on fish immune system

Table 1. Diet formulation (g/100 g).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal diet</td>
<td></td>
</tr>
<tr>
<td>Fishmeal</td>
<td>53</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>7</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>8</td>
</tr>
<tr>
<td>Defatted rice bran</td>
<td>9</td>
</tr>
<tr>
<td>α-potato starch</td>
<td>4</td>
</tr>
<tr>
<td>Squid liver oil (^2)</td>
<td>4</td>
</tr>
<tr>
<td>Blood meal</td>
<td>2</td>
</tr>
<tr>
<td>Dextrin</td>
<td>2</td>
</tr>
<tr>
<td>Casein (^1)</td>
<td>3</td>
</tr>
<tr>
<td>α-cellulose (^1)</td>
<td>0.5</td>
</tr>
<tr>
<td>EPA+DHA (^2)</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin premix (^3)</td>
<td>4</td>
</tr>
<tr>
<td>Minerals premix (^4)</td>
<td>3</td>
</tr>
<tr>
<td>Supplement</td>
<td></td>
</tr>
<tr>
<td>Herbal extract</td>
<td>0.5</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>0.1</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>0.1</td>
</tr>
<tr>
<td>Lactobacillus bretilis</td>
<td>0.1</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0.1</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>0.1</td>
</tr>
</tbody>
</table>

\(^1\) United States Biochemical, Cleveland, OH 44122, USA
\(^2\) E-Wha oil, Pusan, Korea
\(^3\) Contains (g/100 g premix): DL-calcium pantothenate 0.5, choline bitartrate 10, inositol 0.5, menadione 0.02, niacin 0.5, pyridoxine-HCl 0.05, riboflavin 0.1, thiamine mononitrate 0.05, DL-α-tocopheryl acetate 0.2, retinyl acetate 0.02, biotin 0.005, folic acid 0.018, B\(_12\) 0.0002,cholecalciferol 0.008, α-cellulose 85.0.
\(^4\) Contains (mg/100 g premix): Al 0.12, Ca 500, Cl 10, Cu 0.5, Co 0.9, Na 0.125, Mg 50, P 5000, K 425, Zn 0.3, Fe 4, I 0.45, Se 0.02, Mn 0.9.

Probiotic bacteria. The bacteria *Lactobacillus plantarum* (KCCM 11322), *L. brevis* (KCCM 11904), *L. acidophilus* (KCCM 40265), *Bacillus subtilis* (KCCM 11316), and *Saccharomyces cerevisiae* (KCCM 11201) were obtained from the Korean Culture Center of Micro-organisms (KCCM) in South Korea and cultured in Man Rogosa Sharpe broth (Difco Co.) at 30°C. After one day of culture, the bacteria were harvested by centrifuging at 15,000 g at 4°C for 10 min. The bacteria pellets were washed three times with sterile peptone water (NaCl 0.85% and polypeptone 0.1%).

Herbs. The stems of *Acanthopannax korearum*, *Glycyrrhiza vralensis*, and *Panax ginseng* were collected from Jeju Island, South Korea, in March 2008, washed in sterile distilled water, shade-dried, powdered, and extracted following the methods of Hankirshnan et al. (2010b). Thirty grams of molasses (Emnara Co. Ltd., South Korea) and 4 g of chitooligosaccharides (Kittolife Co. Ltd., South Korea) were evenly mixed (w/w) and placed into sterilized 5-l glass tanks. The extracted herbs were added, and the tanks were tightly covered with aluminum foil, kept for seven days at room temperature, and agitated daily to ensure complete digestion. The mixture was filtered through a sterile muslin cloth, the water was evaporated with a rotary vacuum evaporator (Buchi SMP, India), and the resultant herbal extract was stored in a sterilized screw-cap glass container.

Pathogen. *Edwardsiella tarda* was isolated from diseased olive flounder and provided by Prof. Moon-Soo Heo of the Jeju National University. The bacteria were cultured on heart infusion agar (Difco, USA) containing 2% NaCl at 25°C and biochemically and molecularly cauterized following Kim et al. (2001).

Blood sampling and separation of leukocytes. Blood was drawn from the caudal vein of six fish in each group. Leukocytes were separated from the anterior kidney as described by Chung and Secombes (1988). The anterior kidney was aseptically dissected and homogenized in *Leibovitz*-15 (L-15) medium (Sigma, St. Louis, MO, USA) supplemented with 0.2% heparin, 1% penicillin-streptomycin solution (10,000 units/ml penicillin + 10 mg/ml streptomycin; Sigma), and 2% fetal calf serum (FCS; Gibco BRL, Grand Island, NY, USA), and filtered through a 100-μm nylon mesh. The resulting cell suspension was carefully poured onto a 34%/51% Percoll (Sigma) density gradient and centrifuged at 450 × g for 30 min at 4°C. The cells at the interface were collected and washed with L-15 containing 0.1% FCS by centrifugation at 450 × g for 10 min, and the concentration of viable cells was determined by trypan blue exclusion.

Biochemical assay. Activity of serum glutamic oxaloacetic transaminase (SGOT; U/l) and serum glutamic pyruvic transaminase (SGPT; U/l), and concentrations of total protein (TP; g/dl) and glucose (GLU; mg/dl) were determined in a CH-100 plus blood chemistry autoanalyzer (SEAC, Italy) using analysis kits (Stanbio, Texas, USA).

NBT. Production of intracellular O\(_2\)\(_{-}\) was evaluated using nitroblue tetrazolium (NBT) reduction analysis. The effect of glucans on the respiratory burst of fish phagocytes was evaluated by two types of experiments. Leukocytes (2 × 10\(^6\)/ml) were suspended in L-15 containing 0.1% FCS, then transferred to 96-well plates and incubated at 18°C for 2 h. Non-adherent cells were removed by washing and cell monolayers were maintained in L-
Intracellular production of superoxide anions was estimated, based on the formation of formazan crystals in the cells. A volume of 100 µl leukocyte solution (2 × 10⁶/ml) was mixed with 100 µl/ml NBT (0.2% in phosphate buffered saline) containing zymosan (Sigma). After incubation at room temperature for 60 min with regular mixing, the plates were centrifuged at 500 × g for 3 min, and the supernatants were discarded. The cells were washed twice with Hanks’ balanced salt solution and fixed in 70% methanol. The formazan crystals formed were dissolved by adding 0.12 ml potassium hydroxide (KOH) and 0.14 ml dimethyl sulfoxide (DMSO). Alternatively, to study the effects of the soluble components of the glucan suspensions, cells were incubated for 1 or 2 h with the supernatants plus NBT. After turquoise-blue solutions were obtained, absorbance was measured at 620 nm using a multiscan spectrophotometer with KOH/DMSO as the blank.

Lysozyme activity. Serum lysozyme activity was measured spectrophotometrically according to the method of Ellis (1990). A volume of 0.02% (w/v) suspension of Micrococcus lysodeikticus made up in 0.05 M phosphate buffer (pH 6.2) was used as the substrate with lyophilized hen egg white lysozyme as the standard. A new standard curve was prepared for each assay. Standard solutions and samples were added to the substrate at 25°C. Results were expressed as µg/ml equivalent of hen egg white lysozyme activity.

Statistics. Experimental data were analyzed with one-way ANOVA followed by Tukey’s test to compare treatment means in SPSS at a significance level of p<0.05. Results are presented as means±SE.

Results

There were no significant differences in initial weight in either species, or following challenge with E. tarda, in fish fed the control or supplemented diet. However, 12 weeks after challenge, final weight and feed efficiency were significantly higher in fish fed the supplemented diet than in fish fed the control in both species (Fig. 1).

Fig. 1. (a) Growth performance and (b) feed efficiency of olive flounder and parrot fish (mean±SD) fed a control diet or a diet supplemented with probiotic bacteria and an herbal extract for 12 weeks after challenge with Edwardsiella tarda. IW = initial wt, FW = final wt, WG = wt gain. Statistical differences between groups in each species (p<0.05) are indicated by asterisks.

Serum glutamic pyruvic transaminase (SGPT) activity and glucose (GLU) were significantly higher in both species in fish fed the supplemented diet than in the control (Fig. 2). In both species, serum glutamic oxaloacetic transaminase (SGOT) was significantly higher in weeks 6-12 in fish fed the supplemented diet than in the control. Values were significantly higher in fish fed the supplemented diet than in the control for total protein (TP) in weeks 10-12, respiratory burst activity as measured by superoxide anion production in anterior kidney leukocytes (NBT), and serum lysozyme activity in weeks 2-12. Cumulative mortality after 30 days was 10% and 5% for olive flounder and parrot fish fed the supplemented diet, respectively, and 50% and 45% for those fed the control diet (Fig. 3).
Discussion

The continued use of imported marine fish in the cultured flounder industry constitutes a first-order environmental threat due to the significant risk of spreading new diseases. Lactic acid bacteria as a dietary supplement has been proposed as an alternative mode of improving the growth and health of cultured Atlantic cod and rainbow trout (Gildberg et al., 1997; Robertson et al., 2000; Nikoskelainen et al., 2001) and enhancing immunity (Harikrishnan et al., 2010a).
In the present study, fish challenged with *E. tarda* showed significantly better weight gain and feed efficiency when fed the probiotic and herbal supplemented diet than the control. Likewise, dietary medicinal herbs and mixtures induced better growth performance and feed efficacy in Japanese flounder (Kim et al., 1998a), Nile tilapia (Kim et al., 1998b), greasy grouper (Sivaram et al., 2004), shrimp (Immanuel et al., 2004), and abalone (Lee et al., 2001). The significantly better serum glutamic pyruvic transaminase (SGPT) activity, glucose (GLU) contents, and glutamic oxaloacetic transaminase (SGOT), together with the total protein (TP) level that significantly differed from weeks 10 to 12, indirectly suggest that probiotics and herbs promote cellular lipid, and fatty acid utilization and metabolism as energy sources, resulting in good growth performance and protein accumulation. Liver is rich in GOT and GPT, and may result in high SGOT and SGPT activity (Vaglio and Landtiscina, 1999). Dietary inclusion of herb mixtures, as opposed to single herbs, often provides cooperative action to physiological functions. The synergistic effect of herbs has been reported in Japanese flounder (Kim et al., 2000), Nile tilapia (Kim et al., 1998b), and rock bream (Kim et al., 2003).

Respiratory burst (NBT) activity due to increased oxidation in phagocytes stimulated by foreign agents is an important indicator of the innate defense mechanism in fish (Miyazaki, 1998). Reactive species can destroy invading pathogens (Hassett and Cohen, 1989). In the present study, respiratory burst activity was significantly higher in fish fed the supplemented diet during weeks 2-12. Indeed, production of reactive oxygen species significantly increased after week 2, suggesting inhibition of pathogens (Olivier et al., 1985).

Lysozymes attack mainly gram-positive bacteria and some gram-negative bacteria in conjunction with serum complements (Lie et al., 1989). In this study, serum lysozyme activity was significantly higher in fish fed the supplemented feed than in the control, as in goldfish fed a probiotic and herbal supplemented diet (Harikrishnan et al., 2010a) and rainbow trout fed a *L. rhamnosus* supplemented feed for 30 d (Panigrahi et al., 2004). It is generally accepted that lysozyme is involved in the defense against micro-organisms.

In the present study, cumulative mortality in fish fed the supplemented diet and challenged with a pathogen was 5-10%. Similarly, immunity and disease resistance against pathogens improved when a probiotic and herbal supplemented diet was given to goldfish (Harikrishnan et al., 2010a), carp (Harikrishnan et al., 2010e), olive flounder (Harikrishnan et al., 1010b,c,d), rohu (Rao et al., 2006), and catla (Rao and Chakrabarti, 2005).

In conclusion, the dietary administration of a probiotic and herbal supplemented diet did not affect growth performance or feed utilization in olive flounder and parrot fish. The supplemented diet enhanced biochemical constituents and innate immune response, and lowered mortality. Substituting antibiotics with probiotics and herbals could be a valuable new, secure, and efficient rearing technique for farmed fish. Further studies are warranted to ascertain the cellular and molecular mechanisms of the effects of probiotics and herbals when administered for a shorter period and at a lower dose.

**Acknowledgements**

This work was supported by the National Foundation of Korea (NRF) grant funded by the Korean government (MEST), no. 2011-0006155.

**References**


Effects of probiotic and herbal supplemented diets on fish immune system


