Fish & Shellfish Immunology 31 (2011) 310–317

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Probiotics and herbal mixtures enhance the growth, blood constituents, and nonspecific immune response in *Paralichthys olivaceus* against *Streptococcus parauberis*

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ARTICLE INFO

Article history:
Received 2 March 2011
Received in revised form
19 May 2011
Accepted 21 May 2011
Available online 6 June 2011

Keywords:
Herbals
Nonspecific immune response
Paralichthys olivaceus
Probiotics
Streptococcus parauberis

ABSTRACT

The present study was reported the effect of probiotics and herbals mixture supplementation diet on growth, blood constituents, and nonspecific immune response in olive flounder *Paralichthys olivaceus* against *Streptococcus parauberis* on weeks 1, 2, 4, 6, 8, 10, and 12 after injected intraperitoneally (i.p.) with 50 μl of PBS (phosphate buffer saline) containing *S. parauberis* (2.1×10^7 CFU ml^{-1}). The initial weight did not significantly increased in supplementation diet group from 1 to 4 weeks, whereas it was significantly increased from weeks 6 to 12 as compared to fish fed without supplementation diet. The serum aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT) activities significantly increased from weeks 4 to 12 in infected fish fed with supplementation diet compared to fish fed without supplementation diet. However, the total protein (TP) and glucose (GLU) levels were significantly increased in infected fish fed with supplementation diet after 6 weeks. The phagocytic, respiratory burst, complement, and lysozyme activities significantly enhanced in infected fish fed with supplementation diet from weeks 4 to 12 as compared to fish fed without supplementation diet. These results suggested that different probiotics and herbals mixture supplementation diet enhanced the growth, blood biochemical constituents, and nonspecific immunity in olive flounder against *S. parauberis*.

1. Introduction

In South Korea, the mariculture industry was fast growing annually and its total mariculture production was 48,073 metric tonne in 2001; the production almost doubled within five years with 91,125 mt in 2006 [1]. Among the mariculture commodities, olive flounder *Paralichthys olivaceus* has become a commercially important marine fish species, with more than 43,000 mt cultured in Jeju Island, South Korea. Streptococcosis is one of the major problems in cultured marine fish culture industry in various countries, including Israel [2], Italy [3], Japan [4], Korea [5], and the USA [3]. The disease was first reported in cultured rainbow trout [6] and later in salmon, mullet, golden shiner, sea bass, and olive flounder [7]. The rapid expanding of olive flounder aquaculture industry in South Korea has become substantial economic losses due to bacterial pathogens, particularly *Streptococcus iniae*, *Streptococcus parauberis*, and *Lactococcus garvieae*, which are the major bacterial pathogens of streptococcosis [5,8–10].

In Korea, the farmers were used a large quantity of antibiotics to treat cultured fish from bacterial diseases. However, over use of antibiotics for bacterial disease in fish can lead to the emergence of drug-resistant strains and can create serious public health problems [11]. Several vaccine formulations have been successful in preventing different diseases under laboratory conditions but did not prove to be commercially viable because of their prohibitive cost of production, insufficient protection or lack of safety [12]. A number of biological and synthetic immunostimulants substances, such as levamisole, peptidoglycan, β-glucan, chitin, chitosan, yeast, and vitamin combinations have been used to heighten the nonspecific defence system capacity [13,14]. Recently the application of probiotics and herbals is increasingly used in disease control against bacterial fish pathogens especially in Asia and South America [13–16].

Panax ginseng, *Glycyrrhiza uralensis*, *Acanthopanax koreanum* are valuable agricultural commodity used in many traditional medicinal therapies of South Korea, including Japan, Vietnam, Pakistan, India and other Asian nations. *P. ginseng* contains protopanaxadiol,

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doi:10.1016/j.fsi.2011.05.020

protopanaxatriol, ocotillol, and oleanolic acid types [17,18]. *G. uralensis* investigated many chemical constituents [19,20] and *A. koreanum* contains phenylpropanoid compound [21]. Ginsenosides are considered to be the main active pharmacological constituent of ginseng that involved immunomodulatory roles in animals [21,22]. *G. uralensis* has been reported to have antibacterial, cytotoxic, and antioxidant effects [23,24] and *A. koreanum* suppresses interleukin-1 and TNF-alpha production in human monocytes/macrophages, and to suppress granuloma formation and fibrosis [25,26]. Our preliminary study indicated that significant antibacterial activity against fish pathogens including *S. parauberis* (unpublished data). However, there were no reports of these herbals on immune system in fish. Therefore, the present study was conducted for the first time the effects of different probiotics and herbals mixture supplementation diet on growth performances, blood constituents, and nonspecific immune responses in *P. olivaceus* against *S. parauberis*.

2. Materials and methods

2.1. Fish

Healthy olive flounder (mean ± SD, body weight 163 ± 2.8 g) were obtained from local farm in Jeju Island, South Korea (33°24′N, 126°32′E) in March 2009. Fish were transported alive in plastic bags containing water enriched with oxygen. They were examined health status immediately upon arrival [27]. They were acclimated for 2 week in 1000 l tanks filled with sea water and provided with continuous aeration using electric air-pumping compressors; 50% of the water was exchanged twice a week to remove waste feed and faecal materials. The water temperature 18 ± 3.2 °C, pH 7.97 ± 1.24, salinity 33.6 ± 1.8 g l^{-1}, dissolved oxygen concentration 6.78 ± 1.56 mg l^{-1}, and photoperiod 14 h light:10 h dark cycle were maintained during the experimental period. Prior to the experiment, fish were fed a standard diet without probiotics or herbals (Table 1) *ad libitum* twice a day at 09:00 and 15:00 h at a rate of 5% of their body weight.

2.2. Probiotic bacterial procurement, growth and harvest

The probiotic bacterial strains of *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus acidophilus*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* were obtained from the Korean culture center of micro-organisms (KCCM), South Korea. All bacteria were culture 30 °C and their culture mediums were mentioned in Table 2. After one day of culture, the bacteria were harvested by centrifuging at 15000 *g* at 4 °C for 10 min. The bacterial pellets were washed three times with sterile peptone water (NaCl, 0.85% and Polypeptone, 0.1%) for further use. These bacteria were identified as probiotics with related biochemical and molecular study and effectively inhibit the growth of fish pathogens based our preliminary study.

2.3. Herbal extract

The stems of *A. koreanum*, *G. uralensis* Fischer, and *P. ginseng* were collected from Jeju Island, South Korea in March 2009. The stems were washed in sterile distilled water, shade-dried, powdered, and stored at −20 °C until further use. The extraction was done following the methods of Harikrishnan et al. [13] The herbal extract were prepared with 1.2 kg of *A. koreanum*, 80 g of *G. uralensis* Fischer, and 40 g of *P. ginseng* in addition to 30 g of Molasses (EMNARA Co. Ltd) and 4 g of Chitooligosaccharides (KITTOLIFE Co. Ltd) were evenly mixed (w/w) and placed into sterilized 5 l glass tanks with 2 l of sterile distilled water and mixed well. The tank was tightly covered with aluminium foil, kept for 7 d at room temperature and agitated daily to ensure complete digestion. The extract was then filtered through sterile muslin cloth. The filtrate was collected and the water was evaporated using a rotary vacuum evaporator (Buchi SMP). The residue obtained after evaporation in a sterilized screw-cap glass container for further use.

Table 1
Formulation and chemical composition (g 100 g^{-1}) of diets for olive flounder.

Ingredients	Concentration (%)
Fish meal	50
Soy bean meal	8
Wheat flour	10
Defated rice bran	9
α-potato starch	4
α-cellulose[a]	0.5
Squid liver oil[b]	4
Blood meal	2
Dextrin	2
Casein[a]	3
EPA + DHA[b]	0.5
Vitamin premix[c]	4
Minerals premix[d]	3
Additions to standard diet	
Herbal extract	0.5
Lactobacillus plantarum	0.1
Lactobacillus acidophilus	0.1
Lactobacillus brevis	0.1
Bacillus subtilis	0.1
Saccharomyces cerevisiae	0.1

[a] United States Biochemical (Cleveland, OH) 44122.
[b] E-Wha oil, Pusan, Korea.
[c] Contains (as g 100 g^{-1} 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.
[d] Contains (as mg 100 g^{-1} 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.

2.4. Standard diet with probiotics and herbals for supplementation diet

The formulated fish feed was prepared in the laboratory using soy bean and fish meal as the protein sources. The prepared standard feed (g kg^{-1}) was composed of 50% fish meal, 8% Soy bean meal, 10% wheat flour, 9% defated rice bran, 0.5% α-potato starch, 0.5% α-cellulose, 4% squid live oil, 2% blood meal, 2% dextrin, 3% casein, 0.5% EPA + DHA, and 7% vitamin and mineral premix (w/v) with a crude approximate composition of 51.6% crude protein, 12.5% crude fat, 18.4% crude starch, and 12.3% crude ash (Table 1). To prepare the probiotics-enriched diet, the required amount of bacterial suspension and herbal extracts (Tables 1 and 2) were sprayed into the feed slowly, mixing part by part in a drum mixer, after which it was air-dried under sterile conditions for 12 h. The pellets were dried in an oven at 30 °C for 18 h, packed, and stored in

Table 2
List of probiotics strains used for the experimental diet.

Bacterial strain	Strain no.	Culture medium
Lactobacillus plantarum	KCCM 11322	Man Rogosa Sharpe broth (Difco Co.)
Lactobacillus brevis	KCCM 11904	Man Rogosa Sharpe broth (Difco Co.)
Lactobacillus acidophilus	KCCM 40265	Man Rogosa Sharpe broth (Difco Co.)
Bacillus subtilis	KCCM 11316	Nutrient broth (Difco Co.)
Saccharomyces cerevisiae	KCCM 11201	YM broth (Difco Co.)

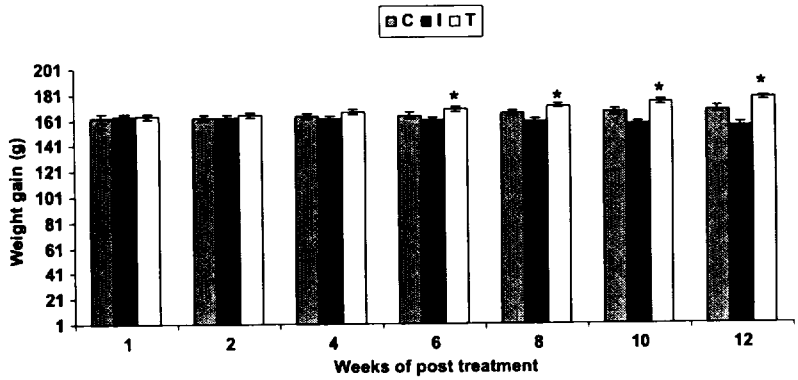

Fig. 1. Growth performance of olive flounder (mean ± SD) uninfected control (C) and infected fish fed with (T) or without (I) probiotics and herbals mixture supplementation diet for chosen weeks. Statistical differences ($P < 0.05$) from the control group of the same week as indicated by asterisks.

a freezer at −20 °C until used. The bacterial count in each at 7.8×10^8 CFU ml^{-1} and viability of the incorporated bacterial cells in the feed was assessed by spreading onto triplicate plates of same culture media in Table 2.

2.5. Pathogen and culture conditions

S. parauberis was isolated from diseased olive flounder and identified based on their phenotypic, biochemical, molecular characteristics, and were stored at −70 °C in tryptic soy broth (TSB) containing 10% glycerol until further use [9,10].

2.6. Experimental design

The effect of growth performances, blood biochemical constituents, and nonspecific immune responses of *P. olivaceus* ($n = 200$) were injected intraperitoneally (i.p.) with 50 μl of PBS (phosphate buffer saline) containing *S. parauberis* (2.1×10^7 CFU ml^{-1}). On Day 7 post-infection, the infected fish were divided into infected fish given with (T; $n = 100$) or without (I; $n = 100$) probiotics and herbals mixture supplementation diet at 5% of their body weight for 12 weeks. Another 100 fish were used for control (C) injected i.p. with 50 μl of PBS alone and fed without supplementation diet. All groups were maintained separately in three replicate groups.

2.7. Growth performance

Growth performance or weight gain (WG) was calculated on week 1, 2, 4, 6, 8, 10, and 12. Weight gain (%) = 100 × (average weight gain/average initial body weight), where average weight gain = [(final total weight + sampled fish weight) − initial total weight] ÷ average of initial and final number of fish.

2.8. Sample collection

On 1, 2, 4, 6, 8, 10, and 12 weeks of feeding, six fish were randomly collected in each groups and individual fish were anaesthetised with MS-222 ($NaHCO_3$ and tricaine methanesulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for 2 min than collect blood samples. Fish were bled immediately after capture to eliminate possible effects of stress on analyzed parameters [28]. Blood was collected from the caudal vasculature using a 1-ml syringe and 25-gauge needle. Part of the blood was heparinized and the rest was allowed to clot at room temperature. Serum was preserved at −70 °C or used immediately for analysis. The heparinized blood samples from each treatment were immediately used for the phagocytic assay and NBT assay. Head kidney leucocytes cells were obtained following the methodology described by Secombes [29].

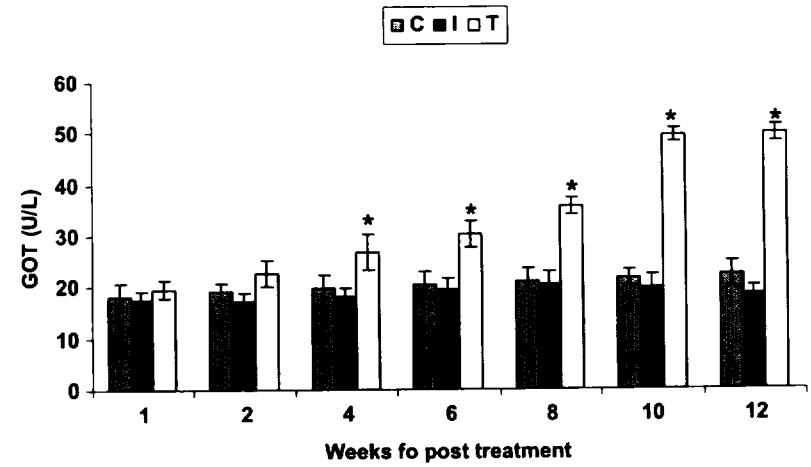

Fig. 2. Serum glutamic oxaloacetic transaminase (GOT) of olive flounder (mean ± SD, $n = 6$) uninfected control (C) and infected fish fed with (T) or without (I) probiotics and herbals mixture supplementation diet for chosen weeks. Statistical differences ($P < 0.05$) from the control group of the same week as indicated by asterisks.

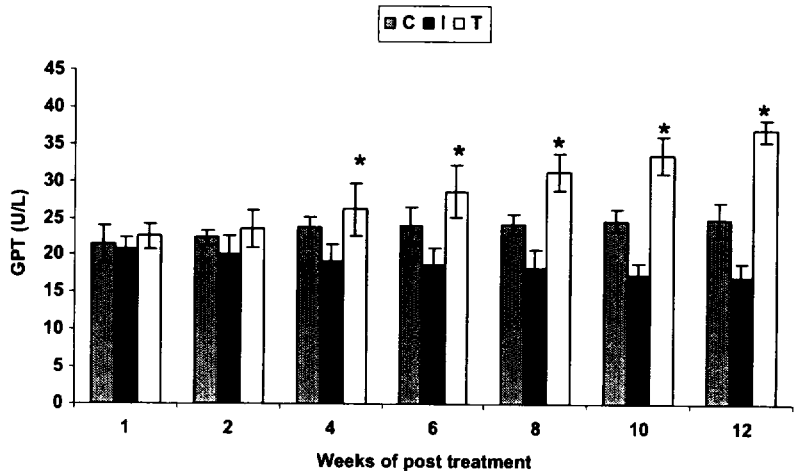

Fig. 3. Serum glutamic pyruvic transaminase (GPT) of olive flounder (mean ± SD, $n = 6$) uninfected control (C) and infected fish fed with (T) or without (I) probiotics and herbals mixture supplementation diet for chosen weeks. Statistical differences ($P < 0.05$) from the control group of the same week as indicated by asterisks.

2.9. Blood biochemistry

We determined the serum biochemical analysis such as serum aspartate aminotransferase (SGOT: U l^{-1}), alanine aminotransferase (SGPT: U l^{-1}) activities and concentration of total protein (TP: g dl^{-1}) and glucose (GLU: mg dl^{-1}) were determined in ch100plus blood chemistry autoanalyzer (SEAC, Italy) using analysis kits (STANBIO, Texas, USA).

2.10. Immunological assays

The phagocytic activity was followed by Pang and Zou [30] and the respiratory burst activity measured the production of intracellular O_2^- was determined using the nitroblue tetrazolium method (NBT) following Cook et al. [31]. The alternative complement activity (ACH_{50}) was determined following Ortuno et al. [32] and the lysozyme activity was measured according to the turbidimetric method described by Hultmark [33].

2.11. Statistics

Experimental data are presented as mean ± SE and were analyzed with 1-way ANOVA followed by Tukey's test to compare the means between individual treatments in SPSS at a significance level of $P < 0.05$.

3. Results

3.1. Growth performance

There was no significant difference in infected fish fed with probiotics and herbals mixture supplementation diet on week 1–4. However the final weight significantly increased infected fish after fed with supplementation diet after 6–12 weeks compared to infected fish fed without supplementation diet (Fig. 1).

3.2. Serum biochemical constituents

There was no significantly enhance the serum aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT) activities of infected fish fed with different probiotics and herbals mixture supplementation diet on 1–2 weeks compared to infected fish fed without supplementation diet (Fig. 2 and 3). On the other hand, the total protein (TP) and glucose (GLU) levels did not significantly increased in infected fish fed with supplementation diet between 1

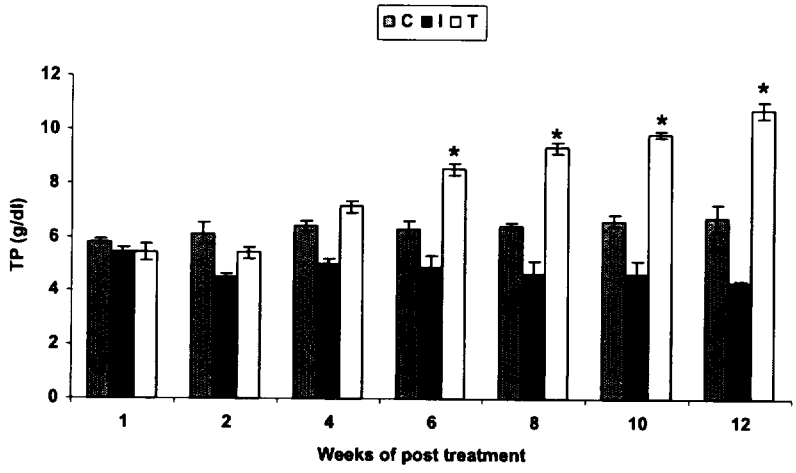

Fig. 4. Serum total protein (TP) of olive flounder (mean ± SD, $n = 6$) uninfected control (C) and infected fish fed with (T) without (I) probiotics and herbals mixture supplementation diet for chosen weeks. Statistical differences ($P < 0.05$) from the control group of the same week as indicated by asterisks.

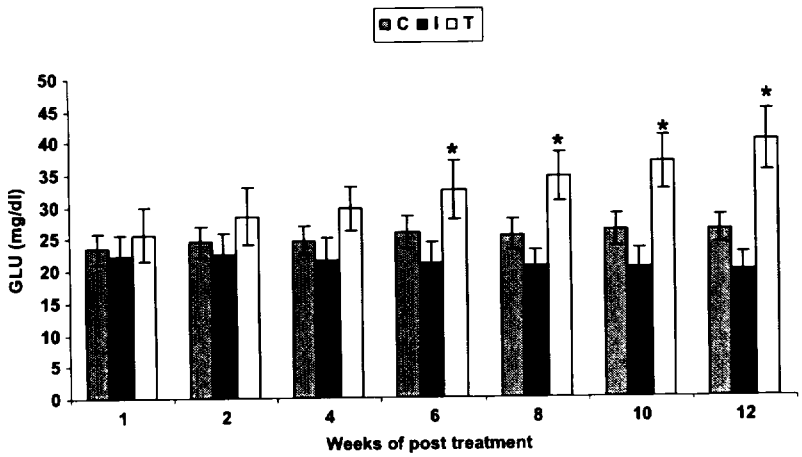

Fig. 5. Serum glucose (GLU) of olive flounder (mean ± SD, $n = 6$) uninfected control (C) and infected fish fed with (T) or without (I) probiotics and herbals mixture supplementation diet for chosen weeks. Statistical differences ($P < 0.05$) from the control group of the same week as indicated by asterisks.

and 4 weeks while it significantly increased from weeks 6 to 12 (Fig. 4 and 5).

3.3. Phagocytic activity

The phagocytic ability of blood leucocytes in infected fish fed with probiotics and herbals mixture supplementation diet did not significantly enhanced between 1 and 2 weeks. However, the phagocytic activity significantly enhanced from weeks 4 to 12 compared to infected fish fed without supplementation diet (Fig. 6).

3.4. Respiratory burst activity

There was no significant respiratory burst activity found in infected fish fed with probiotics and herbals mixture supplementation diet on 1 and 2 weeks compared to infected fish fed without supplementation diet. However, it was significantly enhanced from weeks 4–12 compared to infected fish fed without supplementation diet (Fig. 7).

3.5. Alternative complement activity

Serum alternative complement activity did not significantly enhanced in infected fish fed with probiotics and herbals mixture supplementation diet on week 1 and 2. On the other hand, the serum alternative complement activity significantly enhanced in infected fish fed with supplementation from weeks 4–12 compared to infected fish fed without supplementation diet (Fig. 8).

3.6. Serum lysozyme activity

The serum lysozyme activity did not significantly increased in infected fish fed with probiotics and herbals mixture supplementation diet on 1 and 2 weeks compared to infected fish fed without supplementation diet. However, the serum lysozyme activity significantly enhanced fish fed with supplementation diet from weeks 4 to 12 compared to infected fish fed without supplementation diet (Fig. 9).

4. Discussion

In the present study, *S. parauberis* infected olive flounder after administration with different probiotics and herbals mixture supplementation diet showed significant weight gain compared to infected fish fed without supplementation diet. On the contrary, some of medicinal herbs and their mixture in diets induced higher growth performance than the fish fed the control diet in Japanese flounder [34], greasy grouper [35], shrimp [36], and abalone [37].

Serum SGOT and SGPT activities of infected olive flounder fed with different probiotics and herbals mixture supplementation diet

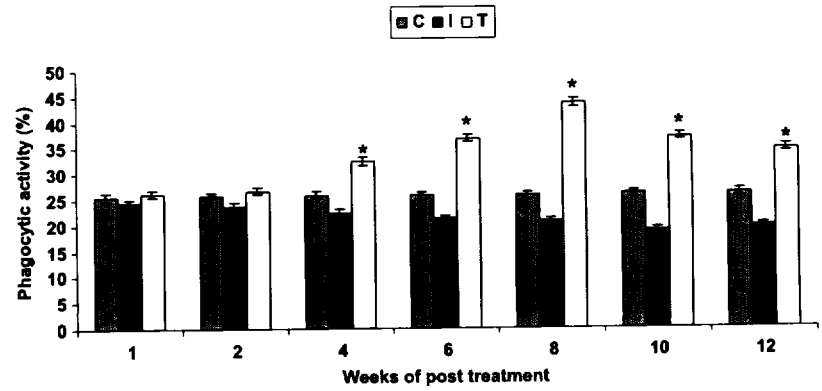

Fig. 6. Phagocytic activity of isolated phagocytic cells from olive flounder (mean ± SD, $n = 6$) uninfected control (C) and infected fish fed with (T) or without (I) probiotics and herbals mixture supplementation diet for chosen weeks. Statistical differences ($P < 0.05$) from the control group of the same week as indicated by asterisks.

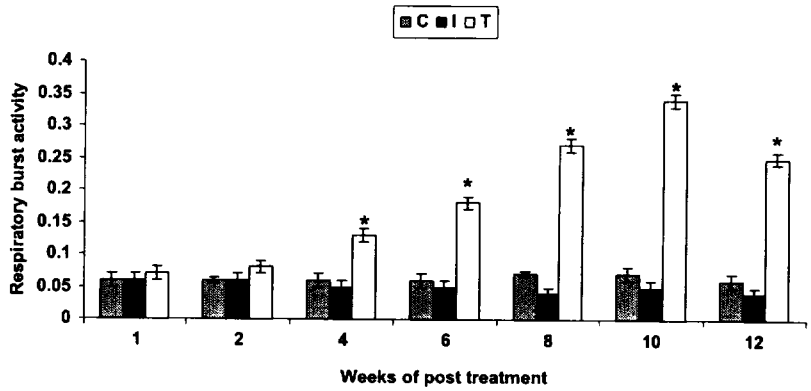

Fig. 7. Respiratory burst activity of olive flounder (mean ± SD, $n = 6$) uninfected control (C) and infected fish fed with (T) or without (I) probiotics and herbals mixture supplementation diet for chosen weeks. Statistical differences ($P < 0.05$) from the control group of the same week as indicated by asterisks.

were significantly increased in this study from weeks 4 to 12, but their serum TP and GLU contents levels were significantly enhanced from weeks 6 to 12 compared to infected fish fed without supplementation diet. It is well known that the liver is rich in SGOT and SGPT, and that damage to it may result in high serum SGOT and SGPT activities [38]. However, some herbals have been reported to reduce serum SGOT and SGPT activities in other animals [39,40]. The mixed different probiotics or herbal may synergistic effect for growth, biochemical, and immune system in the present against pathogen. This synergistic effect of herbs has also been reported in Japanese flounder [41] and rock bream [42].

The probiotics bacteria, such as *Bacillus*, *Vibrio*, and *Lactobacillus* are widely used in freshwater aquaculture for the improvements of survival, growth, immune system and, inhibition of pathogenic bacteria [43–45]. Further, the probiotic bacteria were enhancing the enzyme activity [43,44] and disease resistance [46]. Medicinal herbs such as *P. ginseng* enhanced immunity [21,22], *G. uralensis* an antibacterial and antioxidant effects [23,24], and *A. koreanum* suppresses interleukin-1 and TNF-alpha production in monocytes/macrophages, and to suppress granuloma formation and fibrosis in human and animals [25,26].

Phagocytic cells are the most important cellular components of the innate immune system and also its primitive defence mechanism in fish [29]. Phagocytes produce toxic oxygen forms during a process called respiratory burst [47]. Since superoxide anion is the first product to be released from the respiratory burst, the measurement of O_2^- has been accepted as a precise way of measuring respiratory burst [48]. In the present study, infected fish fed with supplementation diet did not enhance the phagocytic activity on week 1–4 whereas it was observed significant increased from weeks 6 to 12.

The respiratory burst activity significantly enhanced in infected fish fed with probiotics and herbals mixture supplementation diet from weeks 4–12 but not on weeks 1 and 2. Similarly, the lysozyme activity has been enhanced after 2, 4, and 6 weeks of feeding diets with *Carnobacterium divergens* B33 and *Lactobacillus rhamnosus* JCM 1136 in rainbow trout [49,50], *S. cerevisiae* in seabream [51], and *Lactobacillus plantarumin* in grouper [52], respectively. In vertebrates phagocytic cells, i.e. monocytes/macrophages and neutrophils are able to generate superoxide anion (O_2^-), which is a measure of respiratory burst activity, hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) and hydroxyl radical (OH^-), all of which are highly microbiocidal [53,54]. Possibly, such enhanced nonspecific factors of the immune system may have provided defence against infection by the pathogen. The increased oxidative killing mechanism, as observed with blood superoxide anion and head kidney macrophage peroxidase an activity in rainbow trout fed SM1, has been correlated with enhanced pathogen killing capacity of phagocytes and disease resistance in fish [55] and so is likely in the improvement of disease resistance.

The bactericidal activity of complement has been well recognized as one of the key killing mechanisms of clearing bacteria in fish and other animals [54]. The present study indicates that the complement activity did not enhance significantly in infected fish fed with supplementation diet on weeks 1 and 2 whereas after 4

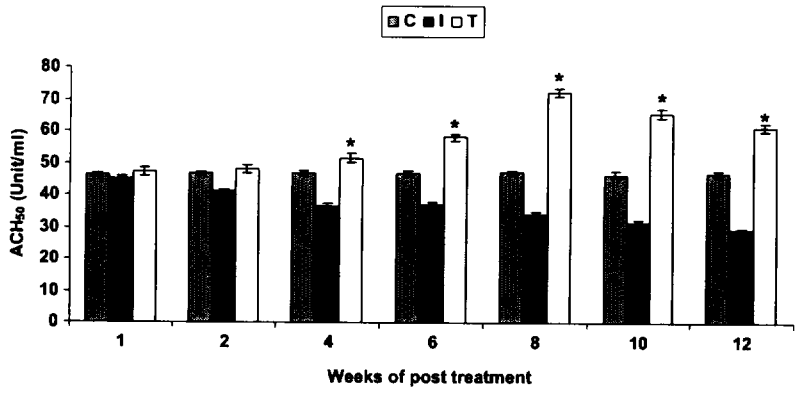

Fig. 8. Alternative complement activity of olive flounder (mean ± SD, $n = 6$) uninfected control (C) and infected fish fed with (T) or without (I) probiotics and herbals mixture supplementation diet for chosen weeks. Statistical differences ($P < 0.05$) from the control group of the same week as indicated by asterisks.

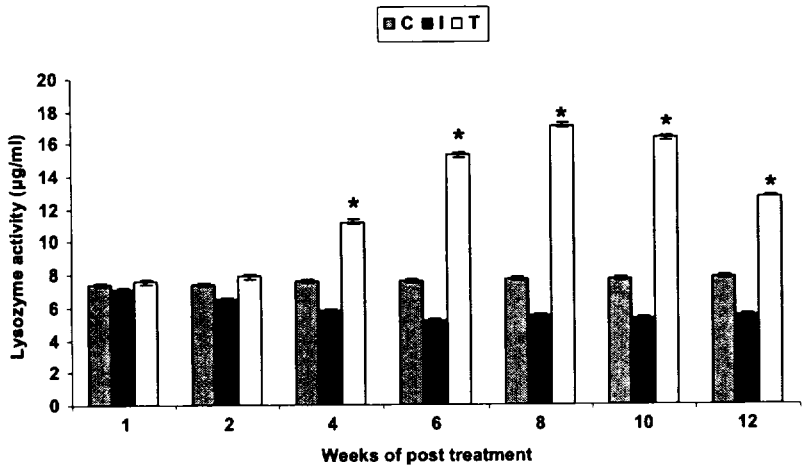

Fig. 9. Changes in serum lysozyme activity of olive flounder (mean ± SD, $n = 6$) uninfected control (C) and infected fish fed with (T) or without (I) probiotics and herbals mixture supplementation diet for chosen weeks. Statistical differences ($P < 0.05$) from the control group of the same week as indicated by asterisks.

weeks it was significantly enhanced. This result are agreement in findings of Cuesta et al. [56] where intraperitoneal administration of propolis on gilthead seabream did not result in a significant change in alternative complement activity while oral administration only provoked very low variations in this activity.

Lysozyme is known to attack mainly Gram-positive bacteria in conjunction with complements [57]. The serum lysozyme activity in the present study did not significantly enhanced in infected fish after fed with supplementation diet on weeks 1 and 2, but it was significantly increased after 4–12 weeks. This result is in agreement with increase in lysozyme activity due to administration with different probiotics and herbals supplementation diet in our recent study in the goldfish [13]. Lysozyme is a humoral nonspecific defence protein widely distributed in fish [57] and it hydrolyses N-acetylmuramic acid and N-acetylglucosamine, which are constituents of the peptidoglycan layer of bacterial cell walls.

In the present results are in agreement with the administration of probiotics and herbals mixture supplemented diet has been reported to be capable of better immunity and disease resistance in goldfish [13], tilapia [58], and carp [59] against pathogens. In conclusion, the dietary administration with different probiotics and herbals mixture does not affect growth performances whereas significantly enhanced the biochemical constituents and nonspecific immune responses. Therefore probiotics and herbals mixture supplementation diet substituting treatments for chemotherapy or vaccines treatment. Further studies are needed on the cellular and molecular mechanisms of the effect of probiotics and herbals mixture diet with reduced length of exposing time and doses.

Acknowledgements

RH is grateful financial assistance through KOSEF Postdoctoral Fellowship and BK 21 program of the Ministry of Education, South Korea and Council of Scientific and Industrial Research (CSIR), India for the award of Research Associateship. We are grateful to the UGC-SAP and DST-FIST, Government of India for the facilities made available to support this work. All authors thanks to Miss. Jayalakshmi Harikrishnan, Department of Tourism Management, Jeju National University, South Korea for her help with the statistical analysis.

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