

The Open Access Israeli Journal of Aquaculture – Bamidgeh

As from **January 2010** The Israeli Journal of Aquaculture - Bamidgeh (IJA) will be published exclusively as an **on-line Open Access (OA)** quarterly accessible by all AquacultureHub (<http://www.aquaculturehub.org>) members and registered individuals and institutions. Please visit our website (<http://siamb.org.il>) for free registration form, further information and instructions.

This transformation from a subscription printed version to an on-line OA journal, aims at supporting the concept that scientific peer-reviewed publications should be made available to all, including those with limited resources. The OA IJA does not enforce author or subscription fees and will endeavor to obtain alternative sources of income to support this policy for as long as possible.

Editor-in-Chief

Dan Mires

Editorial Board

Rina Chakrabarti Aqua Research Lab, Dept. of Zoology, University of Delhi, India

Angelo Colorni National Center for Mariculture, IOLR, Eilat, Israel

Daniel Golani The Hebrew University of Jerusalem, Israel

Hillel Gordin Kibbutz Yotveta, Arava, Israel

Sheenan Harpaz Agricultural Research Organization, Beit Dagan, Israel

Gideon Hulata Agricultural Research Organization Beit Dagan, Israel

George Wm. Kissil National Center for Mariculture, IOLR, Eilat, Israel

Ingrid Lupatsch Swansea University, Singleton Park, Swansea, UK

Spencer Malecha Dept. of Human Nutrition, Food & Animal Sciences, CTAHR, University of Hawaii

Constantinos Mylonas Hellenic Center for Marine Research, Crete, Greece

Amos Tandler National Center for Mariculture, IOLR, Eilat, Israel

Emilio Tibaldi Udine University, Udine, Italy

Jaap van Rijn Faculty of Agriculture, The Hebrew University of Jerusalem, Israel

Zvi Yaron Dept. of Zoology, Tel Aviv University, Israel

Copy Editor Miriam Klein Sofer

Published under auspices of
**The Society of Israeli Aquaculture and
Marine Biotechnology (SIAMB)**
&
University of Hawai'i at Mānoa
&
AquacultureHub
<http://www.aquaculturehub.org>



UNIVERSITY
of HAWAII
MĀNOA
LIBRARY



AquacultureHub
educate • learn • share • engage

ISSN 0792 - 156X

© Israeli Journal of Aquaculture - BAMIGDEH.

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL

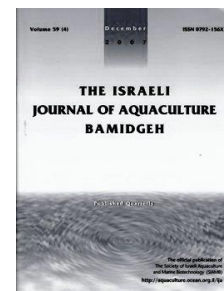
Phone: + 972 52 3965809

<http://siamb.org.il>



The IJA appears exclusively as a peer-reviewed on-line open-access journal at <http://www.siamb.org.il/>. To read papers free of charge, please register online at [registration form](#).

Sale of IJA papers is strictly forbidden.



Dietary Effect of *Lonicera japonica* on Immune Expression in Olive Flounder *Paralichthys olivaceus*, Challenged with *Vibrio anguillarum*

Subramanian Dharaneedharan¹, Dong-Hwi Kim¹, So-Hyun Park¹, Yeoung-Hwan Jang¹, Chellam Balasundaram², Moon-Soo Heo^{1*}

¹ Marine Applied Microbes and Aquatic Organism Disease Control Lab, Department of Marine Life Sciences, Jeju National University, Jeju 690-756, South Korea

² Department of Animal Science, Bharathidasan University, Tiruchirapalli-620 024, Tamilnadu, India

Keywords: immune response; *Lonicera japonica* leaf powder; olive flounder; *Vibrio anguillarum*

Abstract

Respiratory burst, lysozyme, phagocytic activities, and immune gene expression levels were assessed in olive flounder (*Paralichthys olivaceus*, 10.52±2.5 g) fed with 0%, 0.025%, 0.05%, 0.1%, 0.2%, and 0.4% *Lonicera japonica* leaf powder (LjLP) for four weeks. All flounder fish fed LjLP showed significantly increased respiratory burst, lysozyme and phagocytic activity. Total immunoglobulin levels in LjLP diet fed fish groups were higher than in fish fed the basal diet (BD). In flounder fed LjLP, immune gene expression and antioxidant activity were significantly enhanced after 4 weeks of culture. One week following a challenge with *Vibrio anguillarum*, fish fed with different concentrations of LjLP showed decreased cumulative mortality and enhanced immunity all compared to the BD diet group. Our findings have shown that *Lonicera japonica* leaf powder enhanced immune response and resistance to *V. anguillarum* infection in olive flounder.

* Corresponding author. e-mail: msheo@jejunu.ac.kr

Introduction

Japanese olive flounder *Paralichthys olivaceus*, is one of the most commercially important finfish species. It is widely cultured in East-Asia including Korea, Japan and China due to its rapid growth and high level of resistance to disease (Cho et al. 2013). Among the marine finfish species, production in Korea was highest, increasing from 3 mt in 1981 to 44,318 mt in 2003 (MAFO 2003). Due to intense culture throughout the country, production has suffered serious economic damage due to viral, bacterial and parasitic diseases (Oh and Choi 1998). There has been an increasing interest in the use of natural immunostimulants, pro- and pre-biotics as an alternative to the traditional use of chemical agents or vaccines to prevent or control pathogens (Ganguly et al. 2010); (Kim et al., 2015; Dimitroglou et al. 2011). Addition of natural immunostimulants as dietary supplements could minimize the risk of using chemicals. Natural immunostimulants constitute one of the most promising ways of controlling diseases in aquaculture (Nayak, 2010) and increasing resistance against pathogens by enhancing nonspecific defense mechanisms, increasing growth rate and nutrient metabolism (Ringø et al. 2010; Harikrishnan et al. 2011).

Plant-derived products with antimicrobial and/or immunostimulant properties have been used as therapeutic and/or prophylactic agents against fish pathogens. When used against bacteria, they disrupt the bacterial cell wall, block the synthesis of proteins and DNA, inhibit secretion of enzymes and interfere with bacterial signaling mechanism via quorum sensing (Citarasu, 2010). Natural immunostimulants are biocompatible, biodegradable, cost effective, eco-friendly (Ortuno et al. 2002) and their use in aquaculture of finfish and shellfish is growing. Dietary additives with such natural substances have been reported to improve survival, growth, immune resistance, and muscle quality of olive flounder (Kim et al. 2000; Jhon et al. 2009; Kim et al. 2002; Cho et al. 2006; Cho et al. 2007). Studies have shown that the use of Chinese herbs in shrimp and fish improves non-specific immune reaction such as bacteriolytic activity and leukocyte function (Luo, 1997; Chansue, 2000). Hot-water extract of many species of red algae have been used to increase resistance in common carp (*Cyprinus cyprinus*) to *Edwardsiella tarda*, and in yellowtail (*Seriola quinqueradiata*), to *Streptococcus* infection (Fujiki et al. 1992). Dietary guava leaf supplementation has been shown to enhance growth and non-specific immune response of *Penaeus monodon* (Yin et al. 2014).

Lonicera japonica, a species of honeysuckle native to eastern Asia including China, Korea and Japan is traditional medicine used to treat humans for fever, headache (Kumar et al. 2005), as an anti-inflammatory (Lee et al. 2001), and an immunomodulatory agent (Wu et al. 2004). Phytochemical studies have reported that *Lonicera* flower extract is rich in bioactive compounds such as Chlorogenic acid, which could activate macrophages through the calcineurin pathway in mammalian cells (Wu et al. 2004). A previous report has shown that *L. japonica* significantly increased blood neutrophil activity and promoted phagocytosis with neutrophils at suitable concentrations in bovine animals (Hu et al. 1992). Studies have shown that *Astragalus* and *Lonicera* extract supplemented with boron in fish feed could enhance immune response and disease resistance of cultured fish (Ardó et al. 2008). In the present study, an enriched fish diet was prepared using *Lonicera japonica* leaves powder (LjLP), and assessed for its effect on immune response and resistance to *V. anguillarum* in olive flounder *Paralichthys olivaceus*.

Material and methods

Preparation of *L. japonica* leaf powder. *Lonicera japonica* leaves were purchased from a herbal medicine shop, Jeju Dongmun traditional market (Jeju-do, South Korea), in February 2015. The leaves were sun-dried, and stored at room temperature. The dried *L. japonica* leaves were ground and sieved through a nylon sieve (300 mesh size).

Preparation of pelleted feed. Six diets containing different concentrations of *L. japonica* leaf were prepared (Table 1). *L. japonica* leaf powder was supplemented into a basal diet at six levels: 0% (basal diet), 0.025% (Lj1), 0.05% (Lj2), 0.1%, (Lj3), 0.2% (Lj4), and 0.4% (Lj5). The diet ingredients were also ground using a mixer grinder and sieved through a nylon sieve (300 mesh size). Experimental diets were prepared by mixing the dry ingredients with fish oil, vitamin and mineral mixture, guar gum, Carboxymethyl cellulose (Na CMC), and LjLP (replaced by cellulose in basal diet) after which 50% water was mixed in until a stiff dough was obtained. The dough was pelletized into 6.5 mm pellets, using a Wenger X-185 (Kansas, USA). The pellets were dried in a hot air oven at 90°C for 2 h and air dried at 25°C with an air conditioner and

an electric fan. All diets were stored at -20°C until use. The proximate analysis of the basal diet quantified according to the AOAC method comprised 49.9% crude protein, 10.7% crude lipid, 11.2% crude ash, and 18.2% crude carbohydrate (Table 1). All fish were hand-fed to apparent satiation twice a day (06:00 and 16:00 h), for 8 weeks.

Table 1. Formulation and proximate composition of the experimental diets for olive flounder.

Ingredients(g/100 g diet)	Diets					
	BD	Lj1	Lj2	Lj3	Lj4	Lj5
Fish meal	50.0	50.0	50.0	50.0	50.0	50.0
Soybean meal	8.0	8.0	8.0	8.0	8.0	8.0
Defatted rice bran	10.0	10.0	10.0	10.0	10.0	10.0
Wheat flour	13.0	13.0	13.0	13.0	13.0	13.0
Fish oil ^a	3.0	3.0	3.0	3.0	3.0	3.0
Soy lecithin	1.0	1.0	1.0	1.0	1.0	1.0
α-Potato	4.0	4.0	4.0	4.0	4.0	4.0
α-cellulose ^b	1.0	0.975	0.95	0.9	0.8	0.6
LjLP	0.0	0.025	0.05	0.1	0.2	0.4
Blood meal	2.0	2.0	2.0	2.0	2.0	2.0
Dextrin	2.0	2.0	2.0	2.0	2.0	2.0
Casein ^b	2.0	2.0	2.0	2.0	2.0	2.0
EPA + DHA ^a	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin and minerals premix	2.0	2.0	2.0	2.0	2.0	2.0
<i>Proximate analyses (% dry matter basis)</i>						
Moisture	10.0	9.87	10.2	10.2	10.3	10.0
Crude protein	49.9	48.9	50.8	51.2	50.3	51.1
Crude lipid	10.7	10.8	10.9	10.9	11.1	11.1
Crude ash	11.2	10.9	10.5	10.0	9.6	10.1
Crude carbohydrate	18.2	19.5	17.6	17.7	18.7	17.7

^a E-Wha oil, Pusan, Korea

^b United States Biochemical (Cleveland, OH) 44122

^c Premix (g/100 g) contains DL-calcium pantothenate, 0.5; choline bitartrate, 10; inositol, 0.5; menadione, 0.02; niacin, 0.5; pyridoxine-HCl, 0.1; riboflavin, 0.1; thiamine mononitrate, 0.1; DL-α-tocopheryl acetate, 0.2; retinyl acetate, 0.02; biotin, 0.01; folic acid, 0.02; B12, 0.0002; Cholecalciferol, 0.008; α-cellulose, 85.0.

^d Premix (g/100 g) contains Al, 0.12; Ca, 500; Cl, 10; Cu, 0.5; Co, 0.9; Na, 0.13; Mg, 50; P, 5000; K, 425; Zn, 0.3; Fe, 4; I, 0.5, Se, 0.02; Mn, 0.9.

Fish and experimental design. Healthy flounder *Paralichthys olivaceus*, (average weight 18±0.7 g) were obtained from a commercial fish farm in Jeju Island and acclimatized in 500 L aerated recirculation sea water system for one week and fed the basal diet. Their health status was examined immediately upon arrival. The fish were divided into six triplicate groups of 50 fish each and fed twice a day with 0%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4% LjLP respectively, diets at the rate of 5% of their body weight. The respective diets were fed for five weeks (four weeks experiment period and one week post-challenge). About five fish/tank/time (fifteen fish/experimental diet group) were randomly sampled weekly and assessed for various growth and immune parameters. After 4 weeks of feeding with the experimental diets, the remaining 30 fish in each group (n=3) was divided into two; (a) control and (b) challenged group with 10 and 20 fish respectively. Challenged groups were injected intraperitoneally with 100 µl PBS containing *V. anguillarum* at 3.3 × 10⁷ cfu/mL while control fish received 100 µl sterile PBS. All fish continued feeding on LjLP supplemented diets and the cumulative mortality was recorded for one week.

Growth performance. Groups of 30 fish in each experiment (n=3) were used to determine the growth performance such as percentage weight gain (WG), and feeding efficiency (FE). After 4 weeks of feeding these were calculated as follows:

WG% = final wt - initial wt / initial wt × 100 and

FE = final body weight - initial body weight/feed intake.

Blood sampling and separation of Leukocytes. Fish were anesthetized with 0.1 ppm of MS222 and blood was collected with a hypodermic syringe from the caudal vein at 0, 1,

3, 5 and 7 days post challenge with *V. anguillarum* (3.3×10^7 CFU). Heparin was used as an anticoagulant and leukocytes were separated from each blood sample by density gradient centrifugation. One mL of Histopaque®-1119 (Sigma) containing 100 ml of Bacto hemagglutination buffer, pH7.3 (Difco, USA) was dispensed into siliconized tubes, and then 1 mL of blood was layered carefully on top of the gradient. Sample preparations were centrifuged at $700 \times g$ for 15 min at 4°C. After centrifugation, plasma was collected and stored at -20°C for future analysis. Separated Leukocytes were gently removed and dispensed into siliconized tubes containing phenol red free Hank's Balanced Salt Solution (HBSS). Cells were then washed in HBSS and adjusted to 10^6 viable cells/mL.

Immune parameters of LjLP fed flounder fish challenged with V. anguillarum.

Respiratory burst activity of the isolated Leukocytes was quantified by the nitrobluetetrazolium (NBT) assay (Secombes, 1990). This measures the quantity of intra cellular oxidative free radicals as follows, 100 ml of Leukocyte suspension and an equal volume of cytochrome C (2 mg/L in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Sigma) at 1 µg/mL were placed in duplicate in microtitre plates. In order to test specificity, another 100 ml of leukocyte suspension and solutions of cytochrome C containing PMA and superoxide dismutase (SOD, Sigma) at 300 U/mL were prepared in duplicate in microtitre plates. Samples were then mixed and incubated at room temperature for 15 min. Extinctions were measured at 550nm against a cytochrome C blank in a multiscan spectrophotometer (Sepctr-Rainbow, TECAN Instrument, AG, Switzerland).

Phagocytosis activity of blood leukocytes was determined spectrophotometrically by the method described by Seeley et al. (1990). This assay involves the measurement of Congo red-stained yeast cells that have been phagocytosed. One mL of leukocyte suspension was mixed with 2 mL of the Congo red stained yeast cell suspension (providing a yeast cell : leukocyte ratio of 20:1). The mixtures were incubated at room temperature for 60 min and after which 1ml of ice-cold HBSS was added and 1 mL of Histopaque®-1077 solution was injected into the bottom of each sample tube. Samples were centrifuged at 850 g for 5 min to separate leukocytes from the free yeast cells. Leukocytes were harvested and washed twice in HBSS before being resuspension in 1 ml trypsin-EDTA solution (5.0 g/L trypsin and 2.0 g/L EDTA, Sigma) and incubated at 37°C overnight. The absorbance of the samples was measured at 510 nm using trypsin-EDTA as a blank.

Plasma lysozyme activity was measured spectrophotometrically by the method of Sankaran and Gurnani (1972). The lysozyme substrate comprised 0.02% (w/v) suspension of *Micrococcus lysodeikticus* (3×10^9 CFU/mL) made up in phosphate buffer (0.05M, pH 6.2) with lyophilized hen egg white lysozyme as a standard. A new standard curve was prepared for each assay. Standard solutions as well as samples were added to the substrate at 25 °C. The results are expressed as mg/mL equivalent of hen egg white enzyme activity.

The plasma total immunoglobulin assay was performed according to the method by Buss et al. (1997). The microplates were pre-coated with 100 mL of serum samples diluted in 50 mM carbonate buffer, pH 9.6 at 4°C overnight, and the unbound immunoglobulin was removed by washing twice with 0.25% Tween-20 in PBS pH 7.2. After blocking with 1% BSA in PBS, pH 7.2, 100 ml of horseradish peroxidase-labelled goat anti-trout Ig (Kir-kegaard and Perry Laboratories, KPL) diluted in PBS was added and incubated for 1h at room temperature. After washing twice with 0.25% Tween-20 in PBS, the level of bound antibody was measured by adding 100 ml of 0.1 mg/ml ABTS substrate (Diammonium 2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonate), Sigma) in 0.1 M citrate buffer with 0.01% hydrogen peroxide at pH 5 and incubated for 30min at room temperature. The OD was read with a microplate reader (Sepctr-Rainbow, TECAN Instrument, Switzerland) at 405 nm.

Immune gene expression of LjLP fed flounder fish challenged with V. anguillarum

For qPCR analysis of immune gene expression, total RNA was extracted from the isolated leukocytes from control and LjLP fed flounder using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. To amplify flounder immune genes such as tumor necrosis factor-alpha (TNF α), and IL-1b along with β -actin as internal control, primers were designed based on our previous reports (Jang et al. 2014). The primer sequences are: TNF α -F 5' AGATCCATGACCTGCTGA3', TNF α -R 5' GGACCTCTCATAGGCACA 3', IL-1bf 5' TGCACCCTTCACCCACCA 3', IL-1bR 5' CGACACGCTCCAGATGCA 3', β -actinF 5' AGGCGCAGAGCCTTGATG 3' and β -actinR 5'

GTC AAGCGCCAAAATAACTG 3'. All primers were produced by Bioneer Corporation Public CO., Ltd. (Daejeon, South Korea).

The qPCR was carried out in the Mx3000P_ Real-time PCR System (Stratagene, USA) using SYBR Green. The amplifications were performed in a 96-well plate in a 25 μ L reaction volume containing 12.5 μ L of 2 \times Brilliant III Ultr-Fast SYBR[®] Green Master Mix (Agilent Technologies), 2.5 μ L (each) of the forward and reverse primers (10 μ M), 1 μ L of template (1 μ g cDNA), and 9 μ L of DEPC-water. The thermal profile for the qPCR was 95 $^{\circ}$ C for 10 min, followed by 25 cycles of 95 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 60 s and 72 $^{\circ}$ C for 60 s. After PCR amplification, melt-curve analysis was conducted with a thermal profile cycle of 95 $^{\circ}$ C for 60 s, 55 $^{\circ}$ C for 30 s and 95 $^{\circ}$ C for 30 s to confirm that there was only one amplified product and its size was verified on ethidium bromide-stained 2% agarose gels in Tris acetate-EDTA buffer. Data analysis of the qPCR was performed with the MxPro – Mx3000P Multiplex Quantitative PCR system Software. The qPCR standard curve of each gene was prepared using the plasmid vector containing *P. olivaceus* specific cDNA fragment as a template. It calculated the relative expression ratio (R) of mRNA according to the formula $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct(\text{test}) - \Delta Ct(\beta\text{-actin}))}$ (Livak and Schmittgen, 2001). Real-time PCR efficiencies were acquired by the amplification of dilution series of cDNA according to the equation $10^{(-1/\text{slope})}$ and were consistent between target genes and b-actin. The results are presented as means with standard deviations.

Statistical analysis. All statistical analyses were performed using SPSS program version 17.0 (SPSS, Chicago, IL, USA), an independent samples T test was conducted to compare the significant differences of immune parameters and gene expression between treatments. A probability (P) value of less than 0.05 was considered significant.

Results

Growth. Percent weight gain (WG) of all *LjLP* fed fish groups showed a greater increase after four weeks of feeding than the control fish fed with basal diet (Fig. 1). Feeding efficiency also increased significantly with *LjLP* enriched diet fed fish groups when compared with control fish group (Fig. 2).

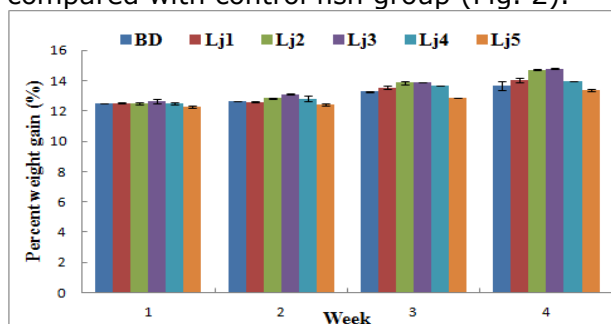


Fig. 1. Growth performance (WG%) of *Paralichthys olivaceus* (n=50) fed with basal diet (BD) and *L. japonica* (Lj) leaf powder enriched diet for 4 weeks. Data represent the mean \pm SE from triplicate samples. Statistical differences ($P < 0.05$) from the control group are indicated by asterisk.

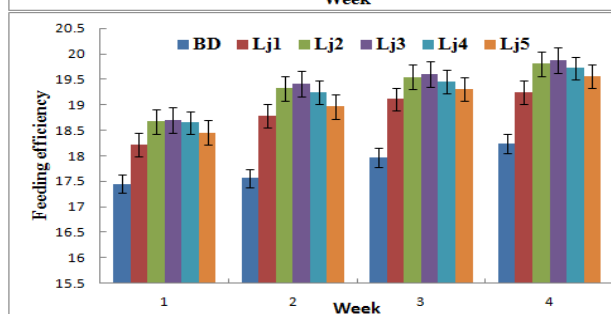


Fig. 2. Feeding efficiency (FE) of *P. olivaceus* (n=50) fed basal diet (BD) and *L. japonica* (Lj) leaf powder enriched diet for 4 weeks. Data represent the mean \pm SE from triplicate samples.

The immune parameters of *LjLP* fed fish. Figure 3 shows the immune parameters such as respiratory burst activity, phagocytic activity and lysozyme activity of control and *LjLP* fed fish. The respiratory burst activity and phagocytic activity of challenged *LjLP* fed fish groups were significantly higher ($P < 0.05$) than that of basal diet fed challenged control fish group. No significant difference was observed in the lysozyme activity in *LjLP* and basal diet fed fish groups. However, the lysozyme activity showed a significant increase ($P < 0.05$) after 2 weeks in *LjLP* fed fish groups particularly in Lj2 and Lj3 feed groups.

Similar enhancement was also found in total immunoglobulin level in *Lj*LP supplemented diet group (Figure 4).

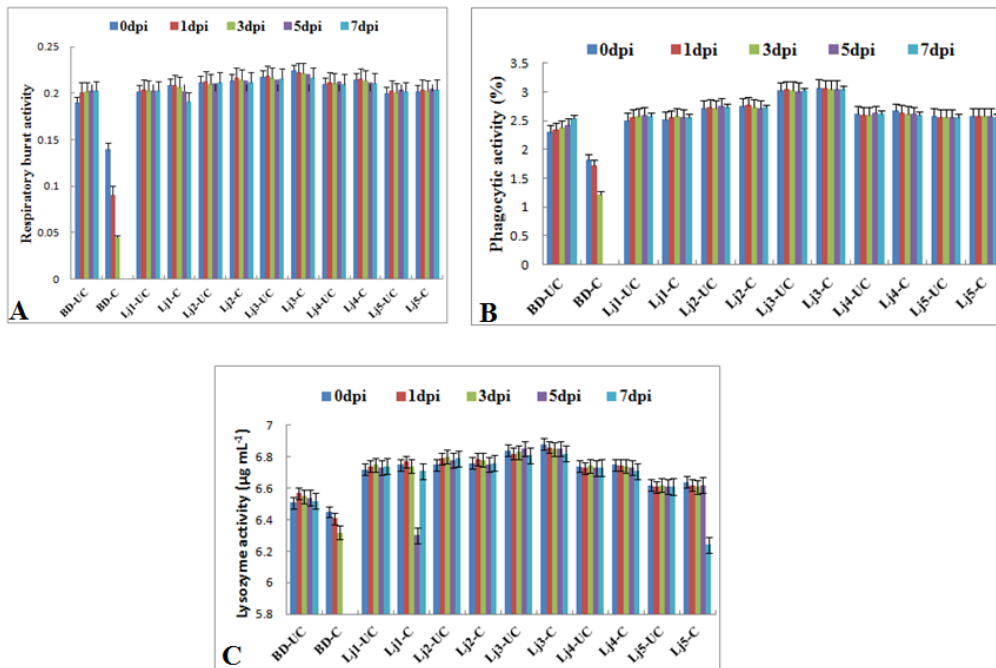


Fig. 3. Immune activity of *P. olivaceus* (n=5) fed with basal diet (BD) and *L. japonica* (Lj) leaf powder enriched diet and challenged with *V. anguillarum*. A) Respiratory burst activity; B) Phagocytic activity; C) Lysozyme activity. Data represent the mean \pm SE from triplicate samples.

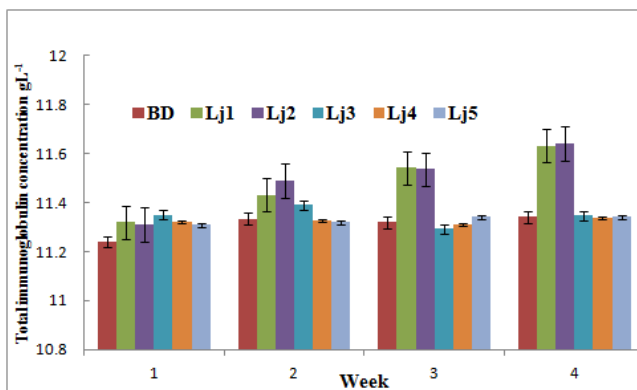


Fig. 4. Total immunoglobulin concentration of *P. olivaceus* (n=5) fed with basal diet (BD) and *L. japonica* (Lj) leaf powder enriched diet for 4 weeks. Data represent the mean \pm SE from a triplicate samples.

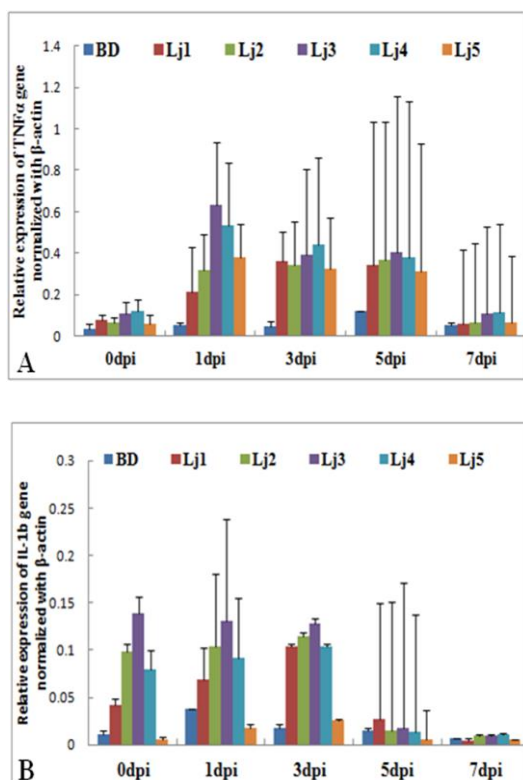


Fig. 5. Effect of *Lonicera japonica* Leaf powder enriched diet on immune gene expression of olive flounder, *P. olivaceus*. A) Relative expression of TNF α ; B) relative expression of IL-1b mRNA level. (n=5)

Real-time PCR expression of LjLP fed fish immune genes. The cDNA obtained from the control and LjLP diet fed fish groups at different sampling periods after challenge with *V. anguillarum* were subjected to real-time PCR expression of two immune related genes, TNF- α and IL-1b, all normalized with the internal control gene β -actin (Fig. 5). The expression profile of both genes was significantly higher ($P>0.05$) from 0th day of post infection (dpi) to 3rd dpi than the basal diet-fed control fish. The Lj3 diet group showed significantly increased expression of TNF α from 1st to 5th day compared to the basal diet fed fish group. However, the expression profile of IL-1b gene shows increased expression level from the challenge day till the third day in all the LjLP diet fed fish groups.

Challenge with *V. anguillarum* and survival. All of the unchallenged basal diet and LjLP enriched diet fed flounder survived. In contrast, the challenged flounder group recorded mortality 1 day after challenge. The highest mortality was observed in basal diet fed fish (78.9%) while LjLP diet fed fish groups showed increased survival rate (Table 2). Among the LjLP diet group Lj3 with 99.1 \pm 2.4% showed highest survival rate followed by Lj2 with 98.6 \pm 2.1%. Altogether there was a significant increase ($P>0.05$) in survival of fish challenged with *V. anguillarum* after being fed the LjLP diet

Table 2. Effect of *Lonicera japonica* leaf powder on the survival of olive flounder challenged with 3.3×10^7 cfu/mL *vibrio anguillarum* (-C) or saline (-UC). Values are mean \pm SE (n = 10 fish in each group in triplicate).

Diet group	Survival (%)				
	0dpi	1dpi	3dpi	5dpi	7dpi
BD-UC	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
BD-C	100 \pm 0	71.4 \pm 1.2 ^{c*}	21.1 \pm 3.3 ^{c**}	NA	NA
Lj1-UC	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
Lj1-C	100 \pm 0	98.4 \pm 2.4	83.1 \pm 3.7	78.2 \pm 2.5	73.4 \pm 3.1
Lj2-UC	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
Lj2-C	100 \pm 0	98.6 \pm 2.1 ^{b*}	95.3 \pm 2.2 ^{b*}	95.1 \pm 2.4 ^{b*}	92.1 \pm 3.1 ^{b*}
Lj3-UC	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
Lj3-C	100 \pm 0	99.1 \pm 2.4 ^{a**}	98.7 \pm 1.1 ^{a**}	98.4 \pm 0.5 ^{a**}	98.2 \pm 2.3 ^{a**}
Lj4-UC	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
Lj4-C	100 \pm 0	96.2 \pm 2.3	94.7 \pm 2.7	93.2 \pm 2.1	92.6 \pm 2.1
Lj5-UC	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
Lj5-C	100 \pm 0	93.4 \pm 2.7	91.5 \pm 3.2	88.6 \pm 3.3	85.2 \pm 2.8

*-diet group shows significant difference; **-diet group shows highly significant difference ($P<0.05$).

Discussion

The application of herbal extracts and their products in aquaculture is an efficient approach and an alternative to conventional chemical additives (Jian and Wu, 2003; Sivaram et al. 2004). Supplementation of plant extracts from several species of marine algae and Chinese and Indian herbs have been reported to enhance fish and shellfish immune system efficiency ((Bhuvanewari and Balasundaram, 2006). It has also been used to modulate the innate immune system of tilapia with elevated phagocytosis throughout the experimental period (Yin et al. 2008). Phagocytosis and killing activity of phagocytic cells is the primary defense mechanism against bacterial pathogens (Neumann et al. 2001; Rao et al. 2006). Fish phagocytes are able to produce the superoxide anion (O_2^-) during a cellular mechanism called respiratory burst (Lee et al. 1998; Sahu et al. 2007; Ardó et al. 2008) where the free anion (O_2^-) kills invading bacterial pathogens (Abbas et al, 2010).

Lysozyme activity is a measurable humoral component of a non-specific defense mechanism that is enhanced after the activation of the immune system. In the present study, flounder fed a LjLP enriched diet showed elevated phagocytosis, respiratory burst, and lysozyme activity.

Total protein and total immunoglobulin levels of LjLP diet groups were enhanced compared to the basal-diet group. Fish fed a Lj5 (0.4%) diet showed an increased level of total immunoglobulin. Previous reports on sturgeons also showed varying levels of total immunoglobulin when exposed to different immunostimulants (Sakai et al. 1992).

Tumor necrosis factor alpha (TNF- α) and interleukin (IL) are two well-studied cytokines in fish, which enhance various cellular responses such as phagocytosis, chemotaxis, macrophage proliferation, and lysozyme synthesis (Dinarello, 1997; Goetz et al. 2004). The real-time PCR expression of these two cytokine genes in leucocytes of fish fed the LjLP diet showed a significant increase from 0 dpi to 5 dpi and 0 dpi to 3 dpi in the TNF- α and IL-6 genes respectively when compared with the basal diet group.

Following 4 weeks of being fed a LjLP enriched diet, fish challenged with *V. anguillarum* showed improved survival rates compared to the control group. Highest survival (99.1%) was observed in Lj3 group followed by Lj2 (96%). This indicates that the LjLP supplemented fish diet acted as an immunostimulant and enhanced the immune status of fish.

Our results show a positive immunostimulatory effect of *Lonicera japonica* leaf powder in flounder, particularly with 0.1% and 0.2% in the fish diet, improving immunity and increasing disease resistance of flounder against *V. anguillarum* infection. The present study also suggests that *Lonicera japonica* leaf powder could be used as an aquatic feed additive in order to enrich nutritional value in the diet.

Acknowledgment

The authors gratefully acknowledge the National Research Foundation of Korea (NRF) for the grant funded by the Korean government (2013HIB8A2032163 & 2013RIAIA403011090).

References

- Ardó L., Yin G., Xu P., Váradi L., Szigeti G., Jeney Z. and G. Jeney,** 2008. Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the nonspecific immune response of Nile tilapia (*Oreochromis niloticus*) and resistance against *Aeromonas hydrophila*. *Aquaculture*, 275:26–33.
- Abbas A.K., Lichtman A.H. and S. Pillai,** 2010. Cellular and molecular immunology (updated 6/E). Saunders Elsevier Philadelphia, PA, USA
- Bhuvanewari R. and Balasundaram C. ,** 2006. Traditional Indian Herbal Extracts used in Vitro Against Growth of the Pathogenic Bacteria –*Aeromonas hydrophila* *The Israeli Journal of Aquaculture – Bamidgeh* 58(2), 89-96.
- Buss H., Chan T.P., Sluis K.B., Domigan N.M. and C.C. Winterbourn,** 1997. Protein carbonyl measurement by a sensitive elisa method. *Free Radical Biology and Medicine*, 23 (3):361–366.
- Chansue N., Ponpornpisit A., Endo M., Sakai M. and Y. Satoshi,** 2000. Improved immunity of tilapia, *Oreochromis niloticus* by C-UPIII, a herb medicine. *Fish Pathol.* 35:89-90.

- Cho S.H., Jeon G.H., Kim H.S., Kim D.S. and C. Kim,** 2013. Effects of dietary *Scutellaria baicalensis* extract on growth, feed utilization and challenge test of olive flounder (*Paralichthys olivaceus*). *Asian-Australasian Journal of Animal Sciences*, 26(1), 90–96.
- Cho S.H., Lee S., Park B.H., Ji S., Lee J.H., Bae J. and S. Oh,** 2007. Effect of dietary inclusion of various sources of green tea on growth, body composition and blood chemistry of juvenile olive flounder *Paralichthys olivaceus*. *Fish Physiol. Biochem.* 33:49–57.
- Cho S.H., Lee S.M., Park B.H., Ji S.C., Kwon M., Kim Y., Lee J.H., Park S. and H.K. Han,** 2006. Effect of dietary inclusion of various sources of green tea on immune system and challenging test of juvenile olive flounder *Paralichthys olivaceus*. *J. Aquacult.* 19:84–89.
- Citarasu T,** 2010. Herbal biomedicines: a new opportunity for aquaculture industry. *Aquac. Int.* 18:403–414.
- Dimitroglou A., Merrifield D.L., Carnevali O., Picchiatti S., Avella M., Daniels C., Güroy D. and S.J. Davies,** 2011. Microbial manipulations to improve fish health and production – a Mediterranean perspective. *Fish Shellfish Immunol.*, 30:1–16.
- Dinarello C.A.** 1997. Interleukin-1. *Cytokine Growth Factor Rev.*, 8:253–65.
- Fujiki K., Matsuyama H. and T. Yano,** 1992. Effect of hot-water extracts from marine algae on resistance of carp and yellowtail against bacterial infections. *Sci. Bull. Facul. Agr. Kyushu Univ.*, 45:137–41.
- Ganguly S., Paul I. and S.K. Kumar Mukhopadhyay,** 2010. Application and Effectiveness of Immunostimulants, Probiotics, and Prebiotics in Aquaculture: A Review. *The Israeli Journal of Aquaculture - Bamidgeh* 62(3), 2010, 130–138.
- Goetz F.W., Planas J.V. and S. MacKenzie,** 2004. Tumor necrosis factors. *Dev. Comp. Immunol.* 28:487–97.
- Harikrishnan R., Kim M.C., Kim J.S., Balasundaram C. and M.S. Heo,** 2011. Protective effect of herbal and probiotics enriched diet on haematological and immunity status of *Oplegnathus fasciatus* (Temminck & Schlegel) against *Edwardsiella tarda*. *Fish Shellfish Immunol.*, 30:886–893.
- Hu S., Cai W., Ye J., Qian Z. and Z. Sun,** 1992. Influence of medicinal herbs on phagocytosis by bovine neutrophils. *Zentralbl Veterinarmed Reihe A.*, 39:593–599.
- Jang Y.H., Dharaneedharan S. and M.S. Heo,** 2014. Efficacy of formalin-killed *Pseudomonas anguilliseptica* vaccine on protection and immune gene expression in farmed olive flounder, *Paralichthys olivaceus*. *Vaccine*, 32:1808–1813.
- Jhon B., Kim M., Kim Y. and M. Heo,** 2009. Effects of the culture broth of lactic acid bacteria cultured in herb extract on growth promotion and nonspecific immune response of aquacultured fish. *J. Life Sci.*, 19:87–93.
- Jian J. and Z. Wu,** 2003. Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, *Pseudosciaena crocea* (Richardson). *Aquaculture*. 218:1–9.
- Kim S-S., Shin S-J., Han H-S., Kim J.-D., and K-J., Lee,** 2015. Effects of dietary *Spirulina pacifica* on innate immunity and disease resistance against *Edwardsiella tarda* in olive flounder *Paralichthys olivaceus*, 9 pages. *The Israeli Journal of Aquaculture - Bamidgeh*, [IJA 65.2015.1152](#)
- Kim J.H., Moon Y.B., Jeong C.H., D.S. Kim,** 2000. Utilization of dietary herb obosan III. Growth of juvenile olive flounder, *Paralichthys olivaceus*. *J. Aquacult.* 13:231–238.
- Kim K., Bai S., Koo J., Wang X. and S. Kim,** 2002. Effects of dietary *Chlorella ellipsoidea* supplementation on growth, blood characteristics, and whole-body composition in juvenile Japanese flounder *Paralichthys olivaceus*. *J. World Aquac. Soc.*, 33:425–431.
- Kolman H,** 2001. The humoral effects of epin in siberian sturgeon (*Acipenser baeri* Brandt). *Arch Polish Fish* 9:61–69.
- Kumar N., Singh B., Bhandari P., Gupta A.P., Uniyal S.K., V.K. Kaul,** 2005. Bi-flavonoids from *Lonicera japonica*. *Phytochemistry*, 66:2740–2744.
- Lee J.H., Ko W.S., Kim Y.H., Kang H.S., Kim H.D. and B.T. Choi,** 2001. Anti-inflammatory effect of the aqueous extract from *Lonicera japonica* flower is related to

inhibition of NF-kappaB activation through reducing I-kappaB α degradation in rat liver. *International Journal of Molecular Medicine*, 7:79–83.

Lee S.J., Son K.H., Chang H.W., Kang S.S. and H.P. Kim, 1998. Anti-inflammatory activity of *Lonicera japonica*. *Phytoth. Res.*, 12:445-447.

Livak K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using Real-Time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods*, 25:402-408.

Luo R., 1997. Induction of immunity substance in *Penaeus chinensis* by Chinese herbal medicine. *Oceanol. Limnol. Sin.*, 28:573-7.

Nayak S.K., 2010. Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol.*, 29:2–14.

Neumann N.F., Stafford J.L., Barreda D., Ainsworth A.J. and M. Belosevic, 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defence. *Dev. Comp. Immunol.*, 25:807–825.

Oh M.J. and T.J. Choi, 1998. A new rhabdovirus (HIRRV-like) isolated in Korea from cultured olive flounder, *J. Fish Pathol.*, 11:129–136.

Ortuno J., Cuesta A., Rodriguez A., Esteban M.A. and J. Meseguer, 2002. Oral administration of yeast, *Saccharomyces cerevisiae*, enhances the cellular innate immune response of gilthead seabream (*Sparus aurata* L.). *Vet. Immunol. Immunopathol.*, 85:41-50.

Rao Y.V., Das B.K., Jyotirmayee P. and R. Chakrabarti, 2006. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol.*, 20:263–273.

Ringø E., Olsen R.E., Gifstad T.Ø., Dalmo R.A., Amlund H., Hemre G.I. and A.M. Bakke, 2010. Prebiotics in aquaculture: a review. *Aquac. Nutr.*, 16:117–136.

Sahu S., Das B.K., Mishra B.K., Pradhan J. and N. Sarangi, 2007. Effect of *Allium sativum* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *J. Appl. Ichthyol.*, 23:80–86.

Sakai M., Kamiya H., Ishii S., Atsuta S. and M. Koybayashi, 1992. The immunostimulating effects of chitin in rainbow trout, *Oncorhynchus mykiss*, 413-417. In Shariff, M., Subasinghe, R.P. and Arthur, J.R. (eds.). *Diseases in Asian Aquaculture I. Fish Health Section*, Asian Fisheries Society, Manila, Philippines.

Sankaran K. and S. Gurnani, 1972. On the variation in the catalytic activity of lysozyme in fishes. *Indian J. Biochem. Biophys.*, 9:62-165.

Secombes C.J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Stolen JS, Anderson DP, Robertson BS, vanMuiswinkel WB, editors. *Techniques in fish immunology*. Fair Haven: SOS Publications. 137-54.

Seeley K.R., Gillespie P.D. and B.A. Weeks, 1990. A simple technique for the rapid spectrophotometric determination of phagocytosis by fish macrophages. *Mar. Environ. Res.*, 30:123-8.

Sivaram V., Babu M.M., Immanuel G., Murugadass S., Citarasu T. and M.P. Marian, 2004. Growth and immune response of juvenile greasy groupers (*Epinephelus tauvina*) fed with herbal antibacterial active principle supplemented diets against *Vibrio harveyi* infections. *Aquaculture*, 237, 9–20.

MAFO, 2003. The Maritime Affairs and Fisheries Office. Statistical year book of maritime affairs and fisheries, Ministry of Maritime Affairs and Fisheries, Korea.

Wu H., Luo J., Yin Y and Q. Wei, 2004. Effects of chlorogenic acid, an active compound activating calcineurin, purified from *Flos Lonicerae* on macrophage. *Acta Pharmacologica Sinica*, 12:1685–1689.

Yin G., Ardo L., Jeney Z., Xu P. and G. Jeney, 2008. Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) enhance non-specific immune response of tilapia, *Oreochromis niloticus*, and protection against *Aeromonas hydrophila*. 269-282. In Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M. and Subasinghe, R.P. (eds.). *Diseases in Asian Aquaculture VI*. Fish Health Section, Asian Fisheries Society, Manila, Philippines. 505pp.

Yin X.L., Li Z.J., Yang K., Lin H.Z. and Z.X. Guo, 2014. Effect of guava leaves on growth and the non-specific immune response of *Penaeus monodon*. *Fish shellfish immunol*, 40:190-196.