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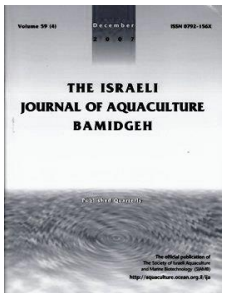

Dietary Effect of *Lonicera japonica* on Immune Expression in Olive Flounder *Paralichthys olivaceus,* Challenged with *Vibrio anguillarum*

Subramanian Dharaneedharan[1], Dong-Hwi Kim[1], So-Hyun Park[1], Yeoung-Hwan Jang[1], Chellam Balasundaram[2], 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 (*Lj*LP) for four weeks. All flounder fish fed *Lj*LP showed significantly increased respiratory burst, lysozyme and phagocytic activity. Total immunoglobulin levels in *Lj*LP diet fed fish groups were higher than in fish fed the basal diet (BD). In flounder fed *Lj*LP, 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 *Lj*LP 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 (*Lj*LP), 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 *Lj*LP (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
*Lj*LP	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
Proximate analyses (% dry matter basis)						
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% *Lj*LP 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 x 10^7 cfu/mL while control fish received 100 µl sterile PBS. All fish continued feeding on *Lj*LP 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 – intial 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×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 (Sepectr-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-EDTAsolution (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 (Sepectr-Rainbow, TEcan Instrument, Switzerland) at 405 nm.

*Immune gene expression of Lj*LP *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 *Lj*LP 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 (TNFα), and IL-1b along with ß-actin as internal control, primers were designed based on our previous reports (Jang et al. 2014). The primer sequences are: TNFα-F 5′ AGATCCATGACCTGCTGA3′, TNFα –R 5′ GGACCTCTCATAGGCACA 3′, IL-1bf 5′ TGCACCCTTCACCCACCA 3′, IL-1bR 5′ CGACACGCTCCAGATGCA 3′, ß-actinF 5′ AGGCGCAGAGCCTTGATG 3′ and ß-actinR 5′

GTCAAGCGCCAAAAATAACTG 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 µL reaction volume containing 12.5 µL of 2× Brilliant III Ultr-Fast SYBR® Green Master Mix (Agilent Technologies), 2.5 µL (each) of the forward and reverse primers (10 µM), 1 mL of template (1 µg cDNA), and 9 µL of DEPC-water. The thermal profile for the qPCR was 95°C for 10 min, followed by 25 cycles of 95°C for 30 s, 60°C for 60 s and 72°C for 60 s. After PCR amplification, melt-curve analysis was conducted with a thermal profile cycle of 95°C for 60 s, 55°C for 30 s and 95°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 $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct\ (test)-\Delta Ct\ (\beta\text{-actin}))}$ (Livak and Schmittgen, 2001). Real-time PCR efficiencies were acquired by the amplification of dilution series of cDNA according to the equation $10^{(-1/slope)}$ 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 *Lj*LP 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 *Lj*LP enriched diet fed fish groups when compared with control fish group (Fig. 2).

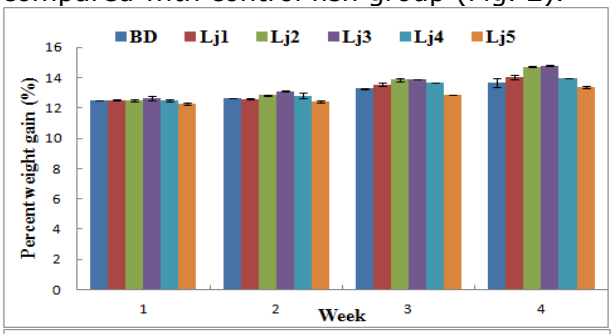

Fig. 1. Growth performance (WG%) of *Paralichthys olivaceus* (n=50) fed with basal diet (BD) and *L. japonica* (Lj) leaf powder enriched diet for 4 weeks. Data represent the mean±SE from triplicate samples. Statistical differences (P<0.05) from the control group are indicated by asterisk.

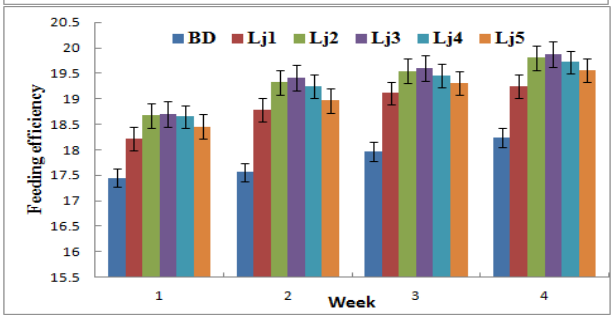

Fig. 2. Feeding efficiency (FE) of *P. olivaceus* (n=50) fed basal diet (BD) and *L. japonica* (Lj) leaf powder enriched diet for 4 weeks. Data represent the mean±SE from triplicate samples.

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 *Lj*LP fed fish. The respiratory burst activity and phagocytic activity of challenged *Lj*LP 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 *Lj*LP and basal diet fed fish groups. However, the lysozyme activity showed a significant increase (P<0.05) after 2 weeks in *Lj*LP 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).

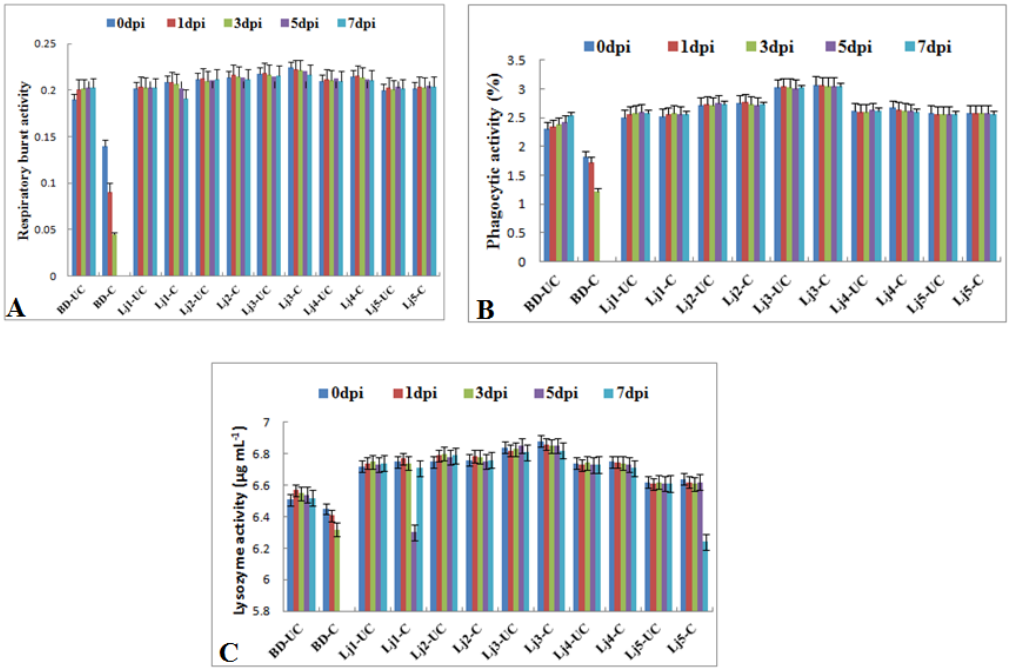

Fig. 3. Immune activity of *P. olivaceus* (n=5) fed with basal diet (BD) and *L. japonica* (Lj) leaf powder enriched diet and challenged with *V. anguillarum*. A) Respiratory burst activity; B) Phagocytic activity; C) Lysozyme activity. Data represent the mean±SE from triplicate samples.

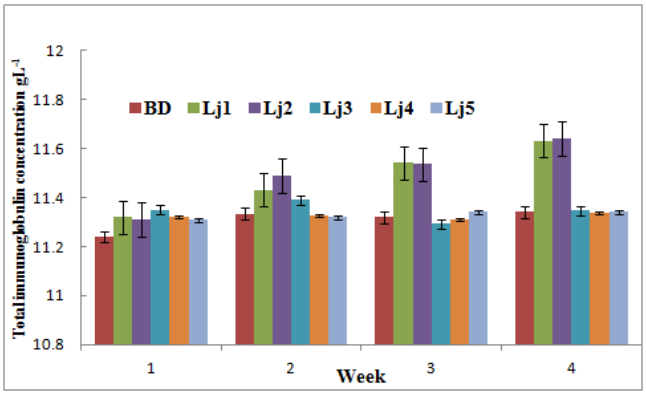

Fig. 4. Total immunoglobulin concentration of *P. olivaceus* (n=5) fed with basal diet (BD) and *L. japonica* (Lj) leaf powder enriched diet for 4 weeks. Data represent the mean±SE from a triplicate samples.

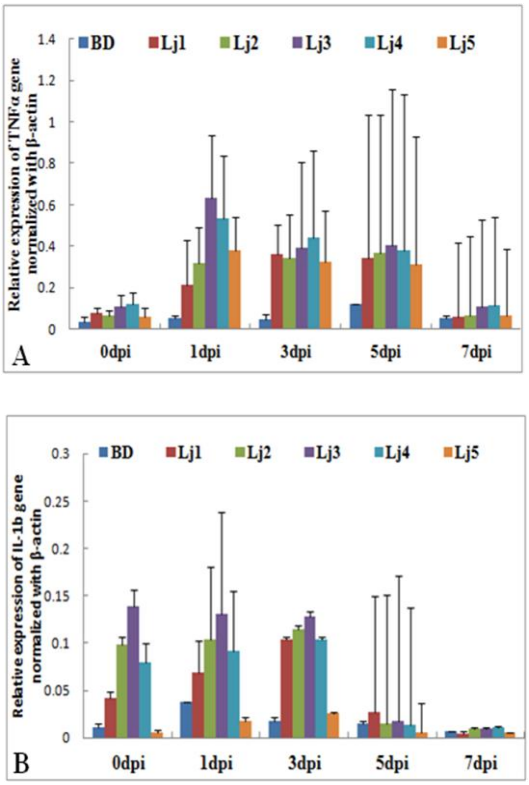

Fig. 5. Effect of *Lonicera japonica* Leaf powder enriched diet on immune gene expression of olive flounder, *P. olivaceus.* A) Relative expression of TNFα; B) relative expression of IL-1b mRNA level. (n=5)

Real-time PCR expression of LjLP fed fish immune genes. The cDNA obtained from the control and *Lj*LP 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 TNFα 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 *Lj*LP diet fed fish groups.

Challenge with V. anguillarum and survival. All of the unchallenged basal diet and *Lj*LP 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 *Lj*LP diet fed fish groups showed increased survival rate (Table 2). Among the *Lj*LP diet group Lj3 with 99.1±2.4% showed highest survival rate followed by Lj2 with 98.6±2.1%. Altogether there was a significant increase (P>0.05) in survival of fish challenged with *V. anguillarum* after being fed the *Lj*LP diet

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

	Survival (%)				
Diet group	*0dpi*	*1dpi*	*3dpi*	*5dpi*	*7dpi*
BD-UC	100±0	100±0	100±0	100±0	100±0
BD-C	100±0	71.4±1.2 ^{c}*	21.1±3.3 ^{c}**	NA	NA
Lj1-UC	100±0	100±0	100±0	100±0	100±0
Lj1-C	100±0	98.4±2.4	83.1±3.7	78.2±2.5	73.4±3.1
Lj2-UC	100±0	100±0	100±0	100±0	100±0
Lj2-C	100±0	98.6±2.1^{b}*	95.3±2.2 ^{b}*	95.1±2.4^{b}*	92.1±3.1^{b}*
Lj3-UC	100±0	100±0	100±0	100±0	100±0
Lj3-C	100±0	99.1±2.4^{a}**	98.7±1.1 ^{a}**	98.4±0.5^{a}**	98.2±2.3^{a}**
Lj4-UC	100±0	100±0	100±0	100±0	100±0
Lj4-C	100±0	96.2±2.3	94.7±2.7	93.2±2.1	92.6±2.1
Lj5-UC	100±0	100±0	100±0	100±0	100±0
Lj5-C	100±0	93.4±2.7	91.5±3.2	88.6±3.3	85.2±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 ((Bhuvaneswari 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 *Lj*LP enriched diet showed elevated phagocytosis, respiratory burst, and lysozyme activity.

Total protein and total immunoglobulin levels of *Lj*LP diet groups were enhanced compared to the basal-diet group. Fish fed a *Lj*5 (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-a) 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 *Lj*LP 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 *Lj*LP enriched diet, fish challenged with *V. anguillarum* showed improved survival rates compared to the control group. Highest survival (99.1%) was observed in *Lj*3 group followed by *Lj*2 (96%). This indicates that the *Lj*LP 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.

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