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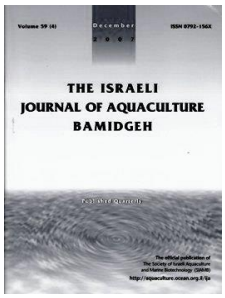

Evaluation of the Dietary Effect of Hallabong Peel Oil on Growth, Hematological, and Immune Gene Expression in Rock Bream, *Oplegnathus fasciatus* Challenged with *Edwardsiella tarda*

Subramanian Dharaneedharan[1]*, Yeoung-Hwan Jang[2], Dong-Hwi Kim[1], Chellam Balasundaram[3], Moon-Soo Heo[1]*

[1]Marine Pathogenic Microbes and Aquatic Disease Control Lab, School of Marine Life Sciences, Jeju National University, Jeju 690-756, South Korea.

[2]Jeju Special Self-Governing Province Ocean and Fisheries Research Institute, Pyoseon-myeon, Segwipo-si, Jeju 697-914, South Korea.

[3]Department of Animal Science, Bharathidasan University, Tamil Nadu, India.

Keywords*:* Hallabong; limonene; rock bream; immune gene; probiotic

Abstract

In the present study we evaluated the dietary effect of Hallabong peel oil (HPO) on growth, disease resistance, and immune gene expression of rock bream, *Oplegnathus fasciatus* challenged with *Edwardsiella tarda* after a 4 week feeding trial with 5 treatments: control-C, probiotic–P, HPO (0.1%), HPO (0.5%), and P+HPO, diets. All fish groups were assessed for growth performance, innate immune parameters, serum biochemical profile, and immune gene expression in head kidney on 2nd, and 4th week, and 1st, 3rd and 7th day post infection with *Edwardsiella tarda*. Fish fed the HPO enriched diets showed increased growth performance with significantly decreased (P>0.05) mortality compared with the control and probiotic diet groups. The positive effects of HPO enriched diet were also found in all assessed innate immune and biochemical parameters which included increased respiratory burst and lysozyme activity, with significantly increased erythrocyte and leukocytes counts, increased serum protein, decreased glucose, triglycerides, cholesterol level in serum compared with control diet fed fish. Moreover, the probiotic bacterial count in the intestine of fish was enhanced with the HPO diet and the P+HPO diet compared to fish fed the probiotic diet. The head kidney of HPO enriched diet fed fish showed up-regulated expression of inflammatory cytokines genes such as TNFα, IL-1β, and FST, after 4th week of feeding trial which was increased ~2 to 3 times on 1dpi and 3 dpi. These results indicate that limonene rich (91.26%), HPO enriched diets enhance growth and immunity and enhance disease resistance of *Oplegnathus fasciatus* challenged against *E. tarda.*

*Corresponding author: Email – msheo@jejunu.ac.kr; Tel: +8264543473; Fax: +82647563

Introduction

Due to several bacterial and viral diseases, aquaculture industries worldwide have suffered significant production losses (Wiens, 2009) either through mass mortality, decreasing growth and reproduction efficiency, or deteriorating quality of the final food product (FAO, 2013). Hence, more adequate preventive methods are required to maintain healthy aquaculture and to limit the outbreak of infectious diseases which threaten the stability of this vital food industry.

Several attempts have been made to control microbial infections by administering prophylactic and chemotherapeutic antimicrobial treatments (Cantas et al., 2013). Most of these agents are toxic, non-biodegradable, increase environmental pollution, and at times are not effective due to pathogen resistance (Shak et al., 2011). Disease outbreaks coupled with restricted use of chemical antibiotics have increased efforts to find other antibiotic alternatives.

In recent years, interest in the use of bioactive herbal immunostimulants (Bhuvaneswari, 2006), pro- and pre-biotics to improve the health status and to prevent and/or control pathogenic infection of fish, have greatly increased (Ganguly et al., Dimitroglou et al., 2011) Some herbal immunostimulants may disrupt the bacterial cell wall of pathogens, block the cellular metabolism, inhibit secretion of enzymes and interfere with bacterial signaling mechanism via *quorum* sensing (Citarasu, 2010). Several studies have reported the application of herbal extracts as phytobiotics, eco-friendly alternatives to chemical antibiotics for aquaculture species. These include, oregano essential oil, *Origanum heracleoticum* in channel catfish, *Ictalurus panctatus* (Zheng et al., 2009); lupin, *Lupinus perennis*, and mango, *Mangifera indica* in rainbow trout, *Oncorhynchus mykiss* (Awad et al., 2010); *Rubus coreanus* in Pacific Whiteleg shrimp, *Penaeus vannamei* (Subramanian et al., 2013), and white mulberry, *Morus alba* foliage methanolic extract in African Catfish, *Clarias gariepinus* (Sheikhlar et al., 2014).

Jeju hallabong (*Citrus kiyomi* × *ponkan*), is a hybrid seedless citrus fruit generally consumed throughout Korea and commonly known as 'Hallabong' named after Hallasan, the mountain where it was first grown since 1998 (Song et al., 2005; Kim et al., 2006). Hallabong is known for its sweet taste (sugar level above 12°Bx and acid content below 1.1%), large size and as a rich source of vitamin C (Lee et al., 2012). Studies on the effect of citrus peel essential oil in aquaculture are scarce. Hence, the present study was conducted to determine the dietary effect of Hallabong peel oil (HPO) on the growth, hematological and immune gene expression in rock bream, *Oplegnathus fasciatus* challenged with *Edwardsiella tarda.*

Materials and methods

Fish. Healthy cultured rock bream, *Oplegnathus fasciatus* (25.4 ± 0.13 g, *n* = 400) were obtained from a local fish farm in Jeju Island, Republic of Korea. The fish were transported and reared in a recirculating culture system in the Department of Aquatic Biomedical Science, Jeju National University. The recirculating culture system consisted of fifteen 1000 L circular tanks and one 1000 L filter tank with continuous aeration and temperature control. Culture water was partially replaced with sand filtered water once a week. The measured water quality parameters were: salinity 33 0.7 ppt, pH 7.5 0.6, dissolved oxygen 8.4 0.6 mg/L, ammonia 0.006 mg/L, and photoperiod of 14 h light:10 h dark cycle. Fish were fed a basal diet *ad libitum* twice a day at 09:00 and 15:00 h at a rate of 5% of their body weight (Table 1).

Plant materials and peel oil extraction. Fresh Hallabong fruit were purchased from Jeju Island traditional market and the peel oil sample was prepared following the cold press method described by Choi and Sawamura (2001). Briefly, the mesocarp and albedo layers were peeled from ~ 5 kg of fresh Hallabong and the peel oil (PO) was extracted by hand-pressing the flavedo, collected in brine solution on ice. Then the oil extract was centrifuged at 4000g for 15 mins at 4°C and the supernatant was dehydrated with anhydrous sodium sulphate at 4°C for 24 hours and stored at -25°C until further use.

Table 1. Formulation and proximate composition of rock beam *O. fasciatus* diet.

Ingredients (g/ 100 g diet)	*Composition (%)*
Fish meal	57.0
Wheat flour	20.0
Soy bean oil	5.7
Fish oil[a]	5.3
Mineral premix[c]	2.5
Vitamin premix[d]	3.0
Cellulose[b]	2.5
Binder	2.0
Antifungi	0.3
Antioxidant	0.35
Proximate composition (%)	
Crude protein	39.48
Crude Lipid	19.7
Ash	9.7
Fiber	2.7
Moisture	8.2
NFE	17.9
Gross energy (MJ/kg)	22.06

[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.
[e] Nitrogen-free extracts (NFE) = dry matter - (crude protein + crude lipid + ash + fibre).
[f] Gross energy (MJ/kg) calculated according to 23.6 kJ/g for protein, 39.5 kJ/g for lipid and17.0 kJ/g for NFE.

Gas chromatography-mass spectrometry analysis (GC-MS) Peel oil. One µl of extracted oil was injected and analyzed by GC-MS. The chemical composition of the hallbong peel oil was determined by the Technological Development Center (PADETEC) of the Federal University of Ceara using GC-MS with a Hewlett-Packard 5971 GC/MS apparatus (Avondale, PA, USA) under the following conditions: a 0.25 mm × 30 m polydimethylsiloxane DB-1 fused silica capillary column with a film thickness of 0.10 µm; helium as the carrier gas (1 ml/min); injector temperature of 250ºC; detector temperature of 200ºC. The column temperature ranged from 35-180ºC/min at 4ºC V/min, then 180-280ºC at 20ºC V/min. Mass spectra were obtained by electronic impact 70 eV. The compounds were identified by comparing their retention indices (RRI) with those reported in related literature and by comparison of their mass spectra with the Wiley library (Lin et al., 1999) or published mass spectra (Massada, 1976).

Experimental diet. Five different experimental diets: control (C), probiotic (P), 0.1% HPO, 0.5% HPO, and P+HPO, were prepared with commercial rock bream feed (Suhyup, S.Korea) according to Dairiki et al. (2013) with grain alcohol (known as ethyl alcohol) as a diluent for the hallabong peel oil. Proximate composition of the commercial feed was 35.5 g/kg crude protein, 120 g/kg moisture, 47 g/kg crude lipid, and 30 g/kg crude fiber. For every kg of feed, a portion of HPO in 100 ml of grain alcohol (100 µL for 0.1% HPO and 500 µL for 0.5% HPO) was sprinkled and mixed thoroughly. One kg of probiotic diet received 95 ml of grain alcohol, was left to dry for 2 hrs after which 5 ml of 1.4×10^6 CFU/mL of *Lactobacillus planctarum* (KCTC3104) was held in sterile saline. The control diet was mixed with 100 ml/Kg of grain alcohol. Similarly the P+HPO diet was mixed with 95 ml of grain alcohol, 250 µL (0.25%) of HPO, and 4.75 mL of 1.4×10^3 mL of probiotic bacterial cells prepared in sterile saline. The feeds were allowed to dry at 18°C under sterile conditions for 24 h after which they were packed in air tight plastic bags and stored at -18°C.

Feeding regime. A total of 400 fish were randomly divided into four diet groups (50 fish per group). Three replicate feeding experiments were conducted over seven

weeks (28 days of feeding trial and 21 days post challenge (dpi) with *E. tarda*). All fish groups were assessed for growth and serum biochemical parameters along with immune gene expression in head kidney using five randomly collected fish from each diet group (in triplicate) at the end of the 2nd and 4th week of feeding experiment and 1st, 3rd, and 7th day of post infection (dpi).

Growth performance. All fish were deprived of food 24 h before sampling. Then the following parameters were measured at the end of the feeding trial (4 weeks): Weight gain = W_2 (g) - W_1 (g); Specific growth rate (SGR) = 100 (ln W_2 - ln W_1) T^1; Feed conversion ratio (FCR) = feed intake/weight gain (g); Survival rate (%) = (final amount of fish/initial amount of fish) ×100;

Where W_1 is the initial weight, W_2 is the final weight and T is the number of days in the feeding period.

Blood sampling and analysis. At the end of four weeks of the feeding trial, and 1st, 3rd, and 7th day after pathogenic challenge (dpi), blood samples (approximately 0.5 ml) of six fish/group were collected randomly from caudal vein using a vacutainer fitted 1ml 27-G needle. Individual fish were anesthetized with MS-222 ($NaHCO_3$ and tricaine methanesulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for 2 min. Individual fish were sampled only once to avoid the influence on the assays due to multiple bleeding and handling stress on the fish. To evaluate the blood physiological parameters and immunological assay, feeding was ceased for 24 h prior to sampling. Half of each blood sample was immediately used for hematological examination, while the other half was mixed with heparin anticoagulant and kept frozen at 4°C. The serum tubes were placed at room temperature and allowed to clot for 2 h. Sera were separated by centrifugation at 1500 g for 20 min and sera from the same groups were pooled before being stored at -70°C for biochemical and immunological analyses.

Erythrocyte and leukocyte count. Total erythrocyte and leukocyte counts were measured with a Neubauer hemocytometer in blood samples diluted in Hayem's and Turke's solution, respectively (Harikrishnan et al., 2003).

Respiratory burst activity. Respiratory burst activity was measured following Pieters et al. (2008). Fifty microlitres of blood was pipetted into the wells of 'U' bottom microtitre plates (NalgeNunc) and incubated for 1 h at room temperature to facilitate adhesion of the cells. The supernatant was gently removed and the adhered cells were washed three times with PBS. After washing, 50 µl of 0.2% (w/v) nitroblue tetrazolium (NBT, Sigma–Aldrich) in PBS was added to the wells and incubated for 1 h at room temperature. The supernatant was removed, and the cells were fixed with 100% (v/v) methanol for 3 min, and then washed three times with 30% (v/v) methanol. The plates were air-dried before 60 µl of 2 M potassium hydroxide (KOH, Sigma–Aldrich) and 70 µl dimethyl sulphoxide (DMSO, Sigma–Aldrich) were added to each well to dissolve the formazan blue crystals. The optimal density (OD) of the resulting solution was read in a microplate reader at 550 nm against a KOH/DMSO blank.

Serum biochemistry. Serum biochemical parameters, such as serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities, low density cholesterol (LDL), triglycerides (TG), hemoglobin (Hb), concentration of total protein (TP) and glucose (GLU), were determined in ch100 plus a blood chemistry autoanalyzer (DaeKwang Meditech, Korea) following manufacturer's instructions.

Lysozyme activity. The serum lysozyme activity was measured spectrophotometrically according to method of Ellis (1990). A suspension of *Micrococcus lysodeikticus* 0.02% (w/v) made up in 0.05 M PBS at pH 6.2 was used as substrate. Lyophilised hen egg white lysozyme was used as a standard. A 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 µg/ml equivalent of hen egg white lysozyme activity.

Intestinal microbiological analysis. Microfloral analyses were performed following Nikoskelainen et al. (2003) the difference being that the fish were starved for 30 h before sampling. The probiotic strain counts in the posterior intestines of fish were determined

by plate counting on MA. Six fish for each treatment and time were used for these studies.

Real-Time immune gene expression analysis using qPCR. For qPCR analysis of immune gene expression, total RNA was extracted from head kidney samples dissected from each experimental diet group at the end 2nd and 4th week of feeding trial (10th week) and 1st, 3rd and 7th day post infection (dpi) with *E. tarda* using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. To amplify rock bream immune genes such as Tumour necrosis factor-alpha (TNFα), FST, Cox-2, INF1 and IL-1β along with the internal control gene β-actin, primers were designed based on previous reports (Table. 2) and all primers were produced by Bioneer Corporation Public CO., Ltd. (Daejeon, South Korea).

Table 2. Primers used for the real-time PCR assay of five immune genes and one internal control β-actin gene of rock bream, *Oplegnathus fasciatus*.

	Gene	*Primer sequences (5' to 3')*	*Product size (bp)*	*Reference*
1	TNFα– Fw TNFα– Rev	GATCGCCTCTCCTGATGTTC ACCACCAAGCTGATGGTTTC	152	Hong et al., 2016
2	IL-1b– Fw IL-1b– Rev	ATCTGGAGACGGTGGACAAC GCTGATGTACCAGTCGCTGA	142	Hong et al., 2016
3	FST– Fw FST– Rev	GCTATGCTGCTGACCGCAATGA GCGTAGGACTGCAACTCCACAAC	103	Herath et al., 2015
4	CoX-2– Fw CoX-2– Rev	ACCTTGTGGAGTCGTTCACC CATGGAGAATCGCTTCCTGT	154	Hong et al., 2016
5	INF1– Fw INF1– Rev	GTGAAAACTCTTTGGATCTACTGGACACG GTGAAACCAAGTTTATCCTCAGCTGCTG	146	Hong et al., 2016
6	β-actin– Fw β-actin–Rev	TCATCACCATCGGCAATGAGAGGT TGATGCTGTTGTAGGTGGTCTCGT	98	Bae et al., 2013.

qPCR was carried out in the Mx3000P Real-time PCR System (Stratagene, USA) using SYBR Green. 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), 1.5 µL of template (0.1 µg cDNA), and 5 µL of DEPC-water. The thermal profile for 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 *O. fasciatus* 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 β-actin. The results are presented as means with standard deviations.

Statistics. The data (mean ± SE, standard error) were analyzed by one way analysis of variance (ANOVA) followed by Tukey's test to compare the means between individual treatments with SPSS (Ver. 22.0) at $P < 0.05$ levels.

Results

GC analysis of HPO components. Extracted phytochemicals and their relative percentages in the HPO are provided in figure 1. More than thirteen major compounds were detected by GC the most abundant constituent being limonene (91.26%) followed by sabinene (1.63%), myrcene (1.17%), and terpinene (0.86%).

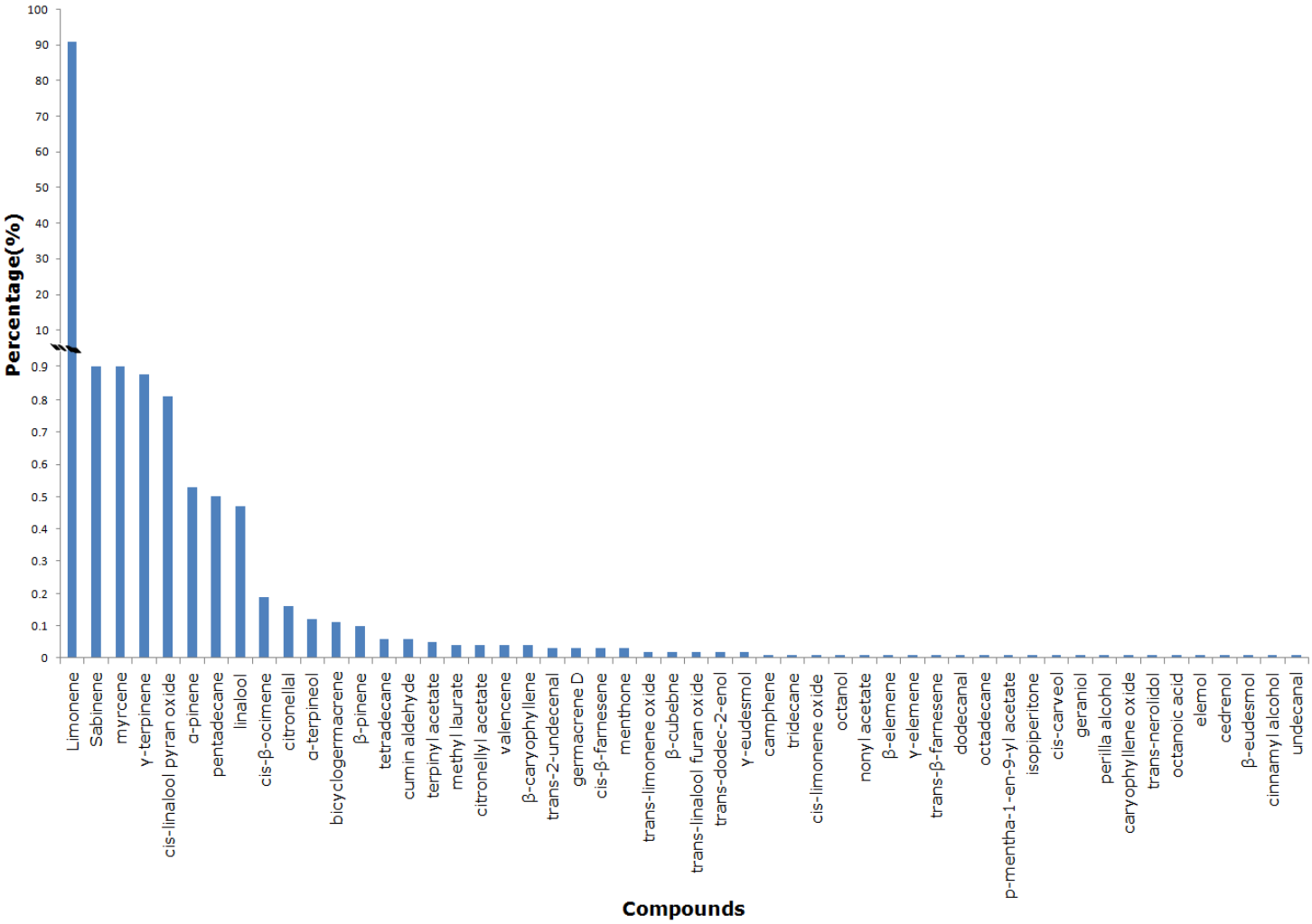

Fig.1. Composition of Hallabong peel oil in percentages (%).

Growth performance and disease resistance. Fish from all experimental diet groups showed a significant increase ($P<0.05$) in weight gain percentages compared to the control diet fed fish. However, among the experimental diet fish groups, the 0.5% HPO and P+HPO diet fed fish showed greater increased WG than the P and 0.1% HPO diet fish groups. Disease resistance against *E. tarda* was significantly ($P<0.05$) increased, and mortality decreased in the HPO diet fed groups compared to the probiotic and control diet fed fish (Table 3).

Dietary effect of HPO on fish erythrocyte and leukocytes count. Erythrocyte count (EC) increased with increasing concentration of HPO enriched diet compared to the control diet fed fish in both uninfected and challenged groups (Table 4). A similar pattern of increasing leukocyte count (LC) was found in HPO diet enriched fish groups when compared with those of the control and probiotic diet fed fish. However, P+HPO diet fed fish showed significant ($P<0.05$) increases in both EC and LC compared to the control and probiotic diet fish groups.

Table 3. Growth performance of *Oplegnathus fasciatus* fed with diets enriched with Hallabong peel oil for 4 weeks.

Index	*C diet*	*P diet*	*0.1%HPO diet*	*0.5%HPO diet*	*P+HPO*
WG (%)	23.16 ± 1.14^{c}	32.76 ± 1.32^{b}	35.56 ± 0.14^{ab}	36.87 ± 0.11^{a}	35.51 ± 1.04^{ab}
SGR	2.22 ± 0.09^{c}	2.56 ± 0.14^{b}	2.59 ± 0.1^{ab}	2.62 ± 0.32^{a}	2.60 ± 0.17^{ab}
FCR	2.18 ± 0.22^{c}	1.86 ± 0.20^{ab}	1.85 ± 0.07^{ab}	1.89 ± 0.21^{a}	1.83 ± 0.11^{b}
SR (%)	53 ± 0.11^{c}	78 ± 0.32^{ab}	84 ± 0.14^{a}	85 ± 0.31^{a}	81 ± 0.12^{ab}

*Data are presented as mean ± S.D (n = 5). Values in each row with different superscripts shows significant difference (P < 0.05). WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate.

Table 4. Hematological and biochemical indices of Oplegnathus fasciatus fed with diets enriched with Hallabong peel oil for 4 weeks of feeding trial and 7 days of post challenged with E. tarda.

Parameters	*C diet*	*P diet*	*0.1%HPO diet*	*0.5%HPO diet*	*P+HPO*
Erythrocytes (10^6/mm^3)	0.83 ± 0.12^{c}	0.92 ± 0.18^{b}	0.97 ± 0.21^{ab}	1.06 ± 0.11^{a}	0.96 ± 0.13^{ab}
Leukocytes (10^3/mm^3)	11.7 ± 0.5^{c}	12.2 ± 0.7^{b}	15.41 ± 0.11^{ab}	19.17 ± 0.13^{a}	14.02 ± 0.22^{ab}
Aspartate aminotransferase	48.24 ± 0.15^{c}	29.27 ± 0.14^{b}	27.25 ± 0.19^{ab}	24.02 ± 0.07^{a}	26.42 ± 0.12^{ab}
Alanine aminotransferase (IU/L)	3.27 ± 0.21^{c}	2.3 ± 0.11^{b}	2.1 ± 0.13^{ab}	1.94 ± 0.18^{a}	2.17 ± 0.22^{ab}
LDL cholesterol (g/dL)	124.21 ± 0.15^{c}	82.12 ± 0.17^{b}	74.24 ± 0.21^{ab}	52.11 ± 0.2^{a}	57.43 ± 0.14^{ab}
Triglycerides (mg/dL)	38.41 ± 0.14^{c}	33.12 ± 0.15^{b}	29.24 ± 0.05^{ab}	23.94 ± 0.12^{a}	30.41 ± 0.3^{ab}
Total protein (g/dL)	2.92 ± 0.14^{c}	3.78 ± 0.24^{b}	4.12 ± 0.07^{ab}	4.61 ± 0.27^{a}	4.22 ± 0.31^{ab}
Glucose (g/dL)	84.25 ± 0.5^{c}	51.04 ± 0.03^{b}	44.21 ± 0.7^{ab}	43.11 ± 0.02^{a}	46.2 ± 0.06^{ab}

*Data are presented as mean ± S.D (n = 5). Data in the same row with different superscript are significantly different (p < 0.05).

Respiratory burst activity. Figure 2 shows the respiratory burst activity of uninfected and challenged fish fed with control, probiotic, and HPO enriched diets. The blood respiratory burst level was significantly enhanced (*P<0.05*) in both 0.1% and 0.5% HPO diet fed fish followed by P+HPO diet fed fish for one week compared to the control and probiotic diet fish.

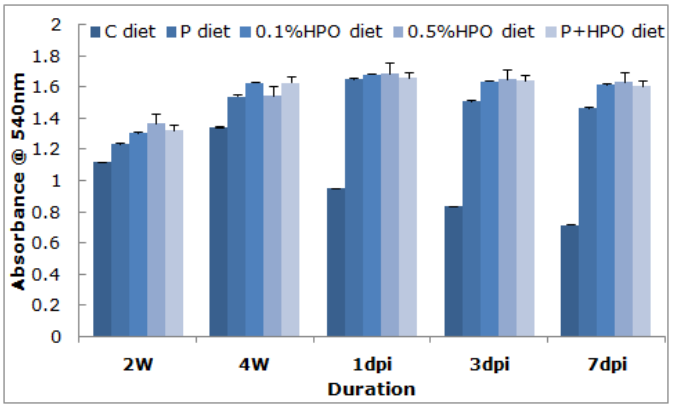

Fig.2. Respiratory burst activity of pre- and post-challenged rock bream fed with control – C diet, probiotic – P diet, HPO enriched diet - 0.1%, HPO enriched diet - 0.5%, and P+HPO diets for 36 days. n=5 in triplicate.

Serum biochemistry and lysozyme activity. Biochemical responses of uninfected and challenged rock bream fed with control, probiotic, and HPO enriched, diets are shown in Table 5. Total serum protein level of fish fed with HPO and P+HPO diets, increased significantly (*P<0.05*) while other parameters such as total glucose, AST, ALT, triglycerides, low density cholesterol levels decreased significantly (*P<0.05*) compared to the probiotic and control diet fed fish groups. However, serum lysozyme activity was significantly enhanced (*P<0.05*) in all the experimental diet fed fish when compared with the control diet fed fish (Fig. 3). However in the HPO enriched diet fed fish it differed significantly (*P<0.05*) from the probiotic diet group.

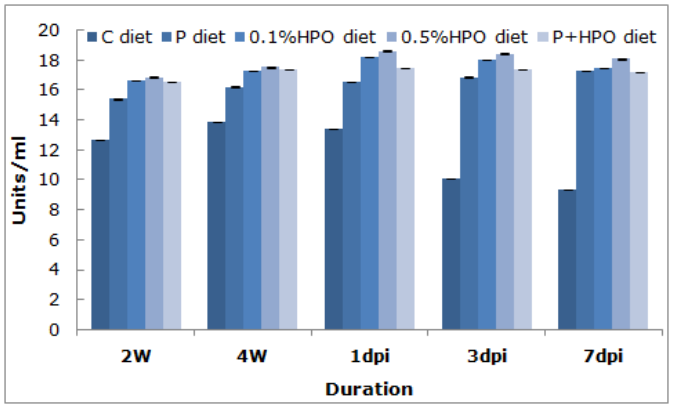

Fig.3. Lysozyme activity of pre- and post-challenged rock bream fed with control –C diet, probiotic –P diet, HPO enriched diet (0.1%), HPO enriched diet (0.5%), and P+HPO diet for 36 days. n=5 in triplicate.

Intestinal microflora. No bacterial species were detected in the intestine of control, 0.1% and 0.5% HPO diet fed fish after four weeks of the feeding trial. The probiotic and P+HPO diet fed fish groups showed 2.4×10^3 and 3.2×10^3 cells which increased to 4.4×10^5 and 8.6×10^6 cells after challenge with *E. tarda.* (Table 5).

Table 5. *Lactobacillus planctarum* cell counts of rock bream, after 4 weeks of feeding trial and 3 weeks post challenge with *E. tarta*.

Weeks	C diet	P diet	0.1%HPO diet	0.5%HPO diet	P+HPO
1st	ND	2.4×10^3	ND	ND	3.2×10^3
2nd	ND	3.2×10^2	ND	ND	3.5×10^4
3rd	ND	3.6×10^3	ND	ND	4.1×10^4
4th	ND	3.7×10^3	ND	ND	5.6×10^5
5th	ND	4.2×10^4	ND	ND	6.4×10^5
6th	ND	4.4×10^5	ND	ND	3.6×10^6
7th	ND	3.6×10^4	ND	ND	8.6×10^6

Immune gene response for HPO enriched diet. Dietary inclusion of HPO significantly enhanced the mRNA expression of immune related genes namely TNF-α, FST, and IL-1β (Fig. 4), on 2nd and 4th weeks when compared with probiotic and control diet groups. HPO and P+HPO diets increased the synthesis of TNF-α, FST, and IL-1β transcript which were enhanced ~2 to 3 times on 1dpi and 3dpi and gradually decreased on 7dpi. However, in probiotic diet fed fish, immune gene enhancement commenced on 3dpi and reduced on 7dpi particularly in TNF-α, FST, and IL-1β gene. The expression level of Cox2 and INF1 gene showed no significant variation ($P<0.05$) among the experimental diet fed fish groups; however their expression level was found to be significantly enhanced ($P<0.05$) when compared with control diet fed fish group (Fig. 5).

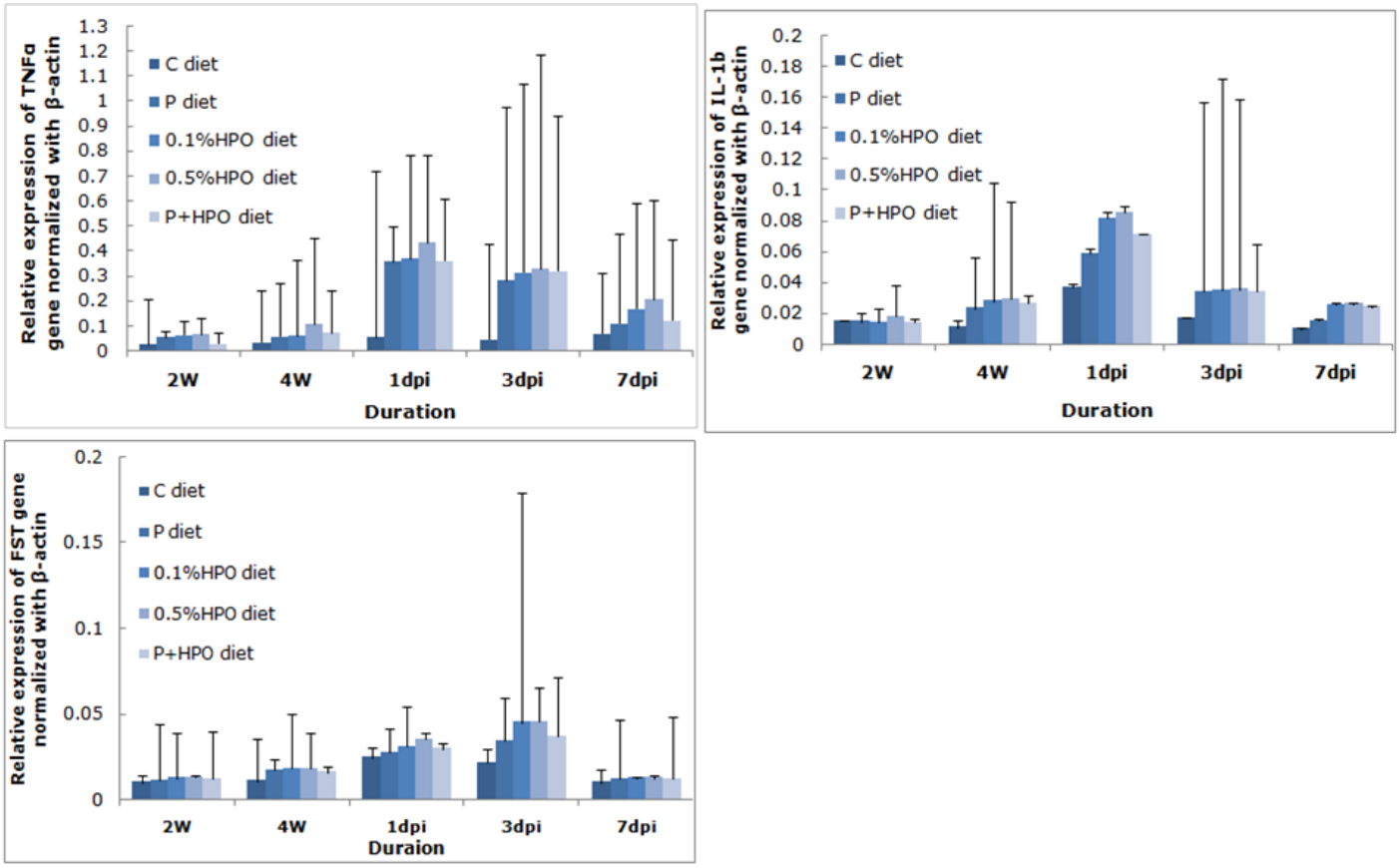

Fig. 4. Relative mRNA expression of TNFα, IL-1b, and FST gene transcripts in head kidney of rock bream, *O. fasciatus*, fed with control, probiotic, HPO (0.1% and 0.5%) and P+HPO diet, by SYBR green qPCR. All samples were normalized using β-actin expression as an internal control. Relative levels of target genes mRNA were analyzed by the $2^{-\Delta Ct}$ (the Ct value of the target gene minus the Ct value of the β-actin gene) method. Data are presented as mean ± S.D.

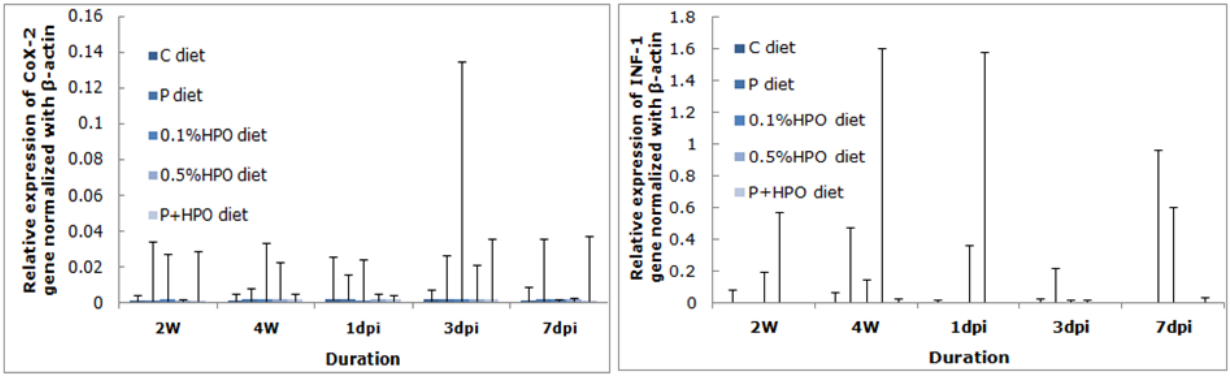

Fig. 5. Relative mRNA expression of CoX-2 and INF-1 gene transcripts in head kidney of rock bream, *O. fasciatus*, fed with control, probiotic, HPO (0.1% and 0.5%) and P+HPO diet, by SYBR green qPCR. All samples were normalized using β-actin expression as an internal control. Relative levels of target genes mRNA were analyzed by the $2^{-\Delta Ct}$ (the Ct value of the target gene minus the Ct value of the β-actin gene) method. Data are presented as mean ± S.D.

Discussion

Hallabong peel oil (HPO) was found to be rich in monoterpene hydrocarbons such as limonene-α (91.26%), sabinene, myrcene, γ-terpinene, with lower proportion of aliphatic and sesquiterpene hydrocarbons which was consistent with previous reports (Choi, 2003; Tajkarimi et al., 2010). These compounds are secondary metabolites known to possess antimicrobial, antiviral, and antifungal properties (Fitzgerald et al., 2003; Schnitzler et al., 2011; Tajkarimi et al., 2010). In citrus fruits, essential oil is accumulated largely in

the peel which is presently considered as waste in most countries, and information about the use of such hydrocarbon rich peel in fish disease management is scarce. The present study is the first to report on the dietary effect of Hallabong peel oil in growth, biochemical, and immune gene enhancement in rock bream, *Oplegnathus fasciatus* challenged with *E. tarda.*

In this study, growth performance such as weight gain, SGR, and FCR were found to increase with diets enriched with HPO. The highest level was achieved with the 0.5% HPO diet and exceeded the probiotic diet fed fish. These results are consistent with results obtained in *Oreochromis mossambicus* fed a diet enriched with *Citrus sinensis* peel oil (Acar et al., 2015) and in juvenile *Labeo victorianus* fed with *Citrus limon* peel oil (Ngugi et al., 2016). Dietary inclusion of HPO increased survival rate from 58.2% (control diet fed fish) to 93.5% (0.5% HPO diet fed fish), and survival rate was higher than that of probiotic diet fed fish (91.2%).

E. tarda, a Gram negative major fish pathogenic bacterium causes edwardsiellosis disease in economically important aquaculture fish species such as tilapia, koi carp, flounder, salmon, eel, etc with clinical symptoms such as lesions, pigment loss, necrotic abscesses, bulged kidneys and internal organs (Plumb. 1999). However, in this study, all the experimental diet (HPO and probiotic diet) groups except the control diet fed fish, showed clinical symptoms with improved survival rate after challenge with the *E. tarda* pathogen. A similar result of decreased mortality after infection with *A. hydrophila* was documented in *Ictalurus punctatus* when fed a diet enriched with oregano essential oil (Zheng et al., 2009).

Disease resistance of fish is usually associated with humoral and cell mediated immunity (Erdal et al., 1991; Hadidi et al., 2008; Lin and Shiau, 2005). Several immune parameters were assessed in order to determine the protective effect of dietary Hallabong peel oil on disease resistance in rock bream. This study reveals increased EC and LC counts in blood, enhanced serum protein with decreased glucose levels in HPO enriched diet fed fish when compared with control diet fed rock bream. Comparable results of increased erythrocytes, leukocyte count, and serum protein levels in *Lobeo rohita* fingerlings (Sahu et al., 2007) and common carp (Gopalakannan and Arul, 2006) were obtained when fed with dietary herbal immunostimulants. An increase in serum protein levels is thought to be associated with a stronger innate immune response and a fundamental defense mechanism in fish (Wiegertjes et al., 1996). Such mechanisms are formed by a series of essential functions that keep host cells alive, healthy, and protected from pathogens.

In HPO enriched diet fed fish, other biochemical parameters like serum glucose, low density cholesterol and triglycerides showed a declining pattern when compared with control diet fed fish. Reduction in the serum glucose level is recognized as an important indicator of hormonal response to stressors (Morgan and Iwama, 1997). This result suggests that HPO contains compounds that reduce primary stress in fish. Previous studies have demonstrated that citrus fruit peels are more effective in lowering cholesterol and triglyceride levels in other animals as they are rich in limonene polymethoxylated flavones (Kurowska and Manthey, 2004). A similar decreasing trend was found with two liver enzymes namely AST and ALT in HPO diet fed rock bream indicating the non toxic nature of HPO as it protects the liver from damage (Giannini et al., 2005).

Lysozyme is a major defense molecule of the innate immune system which is directly linked with elevated oxygen consumption (Biller-Takahashi et al., 2013) and its reactive derivatives (i.e. hydrogen peroxide and hydroxyl radicals) which are capable of destroying invading pathogens (Neumann et al., 2001; Secombes and Olivier, 1997). Dietary intake of HPO increased the level of lysozyme and respiratory burst activity and in turn induced the release of cytokines and initiated inflammatory response in rock bream.

In fish inflammatory response, TNF-α and IL-1β, are pro-inflammatory cytokines (Koj, 1998; Scapigliati et al., 2001) which act synergistically to mediate resistance to infections by controlling intracellular pathogen replication (Haugland et al., 2007). In the

present study, gene expression of TNF-α and IL-1β were up-regulated by HPO in the head kidney of rock bream fed an HPO enriched diet. Similar constitutive expression of TNF-α in all gilthead sea bream tissues has been reported (Garcia-Castillo et al., 2002) and increases of TNF-α and IL-1β after pathogen exposure has been also reported for different fish species (Garcia-Castillo et al., 2002; Mulder et al., 2007; Pelegrin et al., 2001). In our study we found increases of cytokine expression in all dietary treatments in the period post-infection with *E. tarda*. Previous studies also suggest that in fish challenged with bacterial pathogens; pro-inflammatory cytokines can be over-expressed earlier in systemic immune tissues than in local tissues were the pathogens to proliferate, as suggested by other authors (Lindenstrom et al., 2004; Poisa-Beiro et al., 2008; Sigh et al., 2004). Excessive production of pro-inflammatory cytokines is latently harmful to the host (Koj (1998). TGF-β, an important fish anti-inflammatory cytokine, is produced to inhibit excessive activation of immune response (Raida and Buchmann, 2008). In the present study, gene expression of FST was up-regulated by dietary HPO in head kidney which indicated that excessive activation of the inflammatory response was also depressed by Hallabong essential oil. The follistatin (FST) gene encodes a monomeric glycoprotein that plays a role in binding and inhibiting the function of members of the transforming growth factor (TGF)-β superfamily. Thus, FST facilitates a wide variety of functions, ranging from muscle growth to inflammation and immunity (Tortoriello et al., 2001). A previous study reported the up-regulation (~3.4 times) of FST gene transcript, 6 hours after infection with *E. tarda* in rock bream (Funkenstein et al., 2009). Similarly, studies in rats have shown that dietary supplementation increased expression of TGF-β, mRNA, and TGF-β protein content (Narita et al., 1995).

The HPO enriched diet also showed increased *L. planctarum* cells in the intestine of P+HPO diet fed fish when compared with the probiotic diet fed fish, indicating that the presence of HPO could improve the colonization of beneficial bacteria and in turn help to improve the health status of the fish. Gills, skin, and gastrointestinal tract have been demonstrated to be portals of entry for many microbial pathogens (Evelyn, 1996). The probiotic bacteria might be able to protect the host from pathogens by blocking the integumental attachment sites (adhesion receptors). *L. planctarum* colonizing epidermal mucus may originate from the intestinal tract via feces. Good adhesion ability to mucosal surfaces is a prerequisite for bacterial colonization of *Lactobacillus rhamnosus* to fish mucus (Nikoskelainen et al., 2001) and human mucus (Kirjavainen et al., 1998; Nikoskelainen et al., 2003; Ouwehand et al., 1999). To our knowledge, this is the first study reporting the wide ranging enhancement of probiotic bacterial colonization of *L. planctarum* in fish gut (10^1-10^6 cfu/mL). Continuous feeding of probiotic diet is essential to maintain the probiotic bacterial count in fish gut (Kim et al., 2010). The present study demonstrates that Hallobong peel oil could have a greater effect on increasing the probiotic count than probiotic feed.

The results of the present study suggest that dietary inclusion of Hallabong peel oil can enhance growth, immunity, and disease resistance in rock bream, *O. fasciatus* challenged with *E. tarda.* Hence, HPO has the potential to be used in aquaculture for fish disease prevention and management particularly against edwardsiellosis.

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