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Immunomodulation and interferon gamma gene expression in sutchi cat fish, *Pangasianodon hypophthalmus*: effect of dietary fucoidan rich seaweed extract (FRSE) on pre and post challenge period

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Abstract

A 45-days feeding trial was conducted to study the immunomodulatory effect and interferon gamma gene expression of dietary fucoidan rich seaweed extract (FRSE) from *Sargassum wightii* on *Pangasianodon hypophthalmus* fingerlings. One hundred and eighty fingerlings were distributed into six experimental groups in triplicates. Each group was stocked with 10 fish and fed to satiation with iso-nitrogenous (34.96 ± 0.09–35.18 ± 0.03 CP%) and iso-caloric (368.65 ± 0.86–375.09 ± 0.26 Kcal/100 g) purified diets containing either 0% FRSE (control), 1% FRSE (TF_1), 2% FRSE (TF_2), 3% FRSE (TF_3), 3% seaweed powder (TS_3) or 6% seaweed powder (TS_6) in the feed. After feeding trial the experimental fish were challenged with *Aeromonas hydrophila.* Immunological parameters like respiratory burst activity, lysozyme activity, phagocytic activity and total leukocyte count (TLC) were increased with the increasing level of dietary FRSE, whereas serum Albumin/Globulin (A/G) ratio and blood glucose level exhibited decreasing trend ($P < 0.05$). Increased TLC, blood glucose level, respiratory burst activity, serum A/G ratio, lysozyme and phagocytic activities were recorded during the post-challenge period. Maximum expression of interferon gamma (IFN-γ) gene was recorded in FRSE fed groups than the control group both in pre and post challenge condition. After challenged with *A. hydrophila* the highest survival was observed in TF_2 and TF_3 groups whereas lowest survival was observed in the control group. Hence, dietary supplementation of FRSE at an optimum level of 2% reduced the stress and improved the immune status of *P. hypophthalmus* fingerlings.

Keywords: fucoidan rich seaweed extract, interferon gamma gene, pre and post challenge, immunomodulatory effect, *Sargassum wightii*, *Pangasianodon hypophthalmus*

Introduction

Over the past three decades, aquaculture has developed to become the fastest growing food producing sector in the world. Worldwide aquaculture production is expanding day by day, for which there is an increasing interest in fish health. Hence, immunomodulation has been proposed as a potential method to protect the cultured fish from infectious pathogens by increasing their innate immune system (Aoki 1992).

Pangasianodon hypophthalmus is the most popular fish among the fish farmers in South East Asian countries because of its fast growth rate and high stocking density. The intensive farming of pangasius led to a lot of disease problems because of highly endured stress and loss of immunity. This leads the fish more susceptible to bacterial diseases like haemorrhagic septicaemia or red disease which is caused by the bacteria, *Aeromonas hydrophila*. To safe guard the fish from the diseases and to increase the aquaculture production feeding of immunostimulants are ideal protective measures,

which improve their non-specific defense mechanism.

Many natural compounds especially nutraceuticals or phytochemicals have already used in animal feeds as immune-modulators, but their use in aquaculture is scanty. In this context, fucoidan, a polysaccharide which contains substantial percentages of L-fucose and sulphate easter groups, mainly derived from brown seaweeds, has shown promising result as an immune-stimulant. Fucoidan, being an antioxidant has the potential to scavenge the free radicals formed in the body of animal at the time of endured stress (Zhang, Yu, Zhou, Li & Xu 2003). Fucoidan has the property to stimulate the innate immune mechanism by the activation of macrophages and natural killer cells through different receptors and ultimately connect the adaptive immune defense mechanism by the cytokines which in turn stimulate the leucocytes to its enrichment and arrived at the site of infection. Fucoidan isolated from *Laminaria japonica* and its fractions have antioxidant activities, which are related to their immune-modulatory activities (Ren-na, Jian-ming, Chun-nian, Wei-wei & Qi 2008).

The immune-enhancing properties of fucoidan involve its ability to stimulate natural killer cells, which play an important part in the immune response by destroying cells infected with viruses (Maruyama, Tamauchi, Iizuka & Nakano 2006). Fucoidan can induce the production of interleukin-1 (IL-1) and interferon-γ (IFN-γ) *in-vitro*, enhance the functions of T- lymphocyte, B-cell, macrophages and natural killer cells (NK cell) and promote the primary antibody response to sheep red blood cell (SRBC) *in-vivo* (Yang, Sun & Xu 1995). Fucoidan from *Fucus vesiculosus* has immune-stimulating and maturing effects on dendritic cells (DCs), which are powerful antigen-presenting cells, via a pathway involving at least nuclear factor-κB (NF-κB) (Kima & Joo 2008).

The aqueous extracts of some of the brown seaweeds induced an increase in the respiratory burst activity of turbot phagocytes, suggesting that most of the immune-stimulatory capacity associated with polysaccharides (Castro, Zarra & Lamas 2004). The oral administration of *Sargassum fusiforme* polysaccharide extracts at an optimal level of 0.5% and 1.0% for 14 days effectively improved vibriosis resistance and enhanced immune activity in *Fenneropenaeus chinensis* (Huang, Zhou & Zhang 2006). The oral administration of *Sargassum polycystum* crude fucoidan (CF) inhibited the growth of *Vibrio harveyi, Staphylococcus aureus* and *Escherichia coli* at minimal inhibition concentrations (Chotigeat, Tongsupa, Supamataya & Phongdara 2004). The juveniles of *Marsupenaeus japonicas* fed with diets supplemented with *Undaria pinnatifida* fucoidan had potential serum antibacterial effect against *Vibrio harveyi* than the control group (Traifalgar, Kira, Tung, Michael, Laining, Yokoyama, Ishikawa & Koshio 2010).

In shrimp, the antiviral activity of fucoidan against WSSV was reported from different brown seaweeds by numerous workers (Takahashi, Uehara, Watanabe, Okumura, Yamashita, Omura, Yomo, Kawano, Kanemitsu, Narasaka, Suzuki & Itami 1998; Chotigeat *et al.* 2004; Cruz-Suárez, Hernández, Porchas-Cornejo, Coronado-Molina, Linné-Unzueta-Bustamante, Nieto-López, Tapia-Salazar & Ricque-Marie 2007; Immanuel, Sivagnanavelmurugan, Balasubramanian & Palavesam 2010). Although promising effect of dietary fucoidan has been reported in shrimp, but reports on the effect of fucoidan in fish is very limited.

Fucoidan is widely available from various cheap resource brown seaweeds. Hence, fucoidan has been one of focussed area of research to develop the functional foods or nutraceuticals for both in human and animal use. In this context, present work was designed to study the effects of dietary supplementation of fucoidan rich seaweed extract (FRSE), prepared from *S. wightii* on immunological responses of *P. hypophthalmus* challenged with *A. hydrophila*.

Material and methods

Seaweed collection and fucoidan rich seaweed extract (FRSE) preparation

Indian brown seaweed, *S. wightii* samples were collected during morning hours in the month of September from Mandapam, Ramanathapuram district of Tamil Nadu, India. Freshly collected matured, disease free and healthy *S. wightii* were thoroughly washed in freshwater to remove dirt and any other extraneous materials adhering to them and shade dried for about 3 days followed by pulverized to fine powder.

The dried seaweed powder of 100 g was taken into an appropriate sized vessel to which 1 L of 85% ethanol was added, followed by stirring with magnetic stirrer for 12 h. The alcohol was decanted

and the wet slurry was washed with acetone, centrifuged at 29 030 *g* for 10 min and the supernatant was decanted. The residual seaweed was dried at room temperature to remove the acetone completely. Furthermore, the dried residue was added with 700 mL distilled water and stirred for 1 h at 65°C. The hot mixture was filtered and the supernatant was collected. The pre-extracted residue was re-extracted with 350 mL of distilled water as done before and filtered again. Both extracts were mixed together and centrifuged at 29 030 *g* for 10 min. The supernatant was collected and 1% $CaCl_2$ was added by vigorous mixing, which was stored at 4°C for overnight to precipitate the alginic acid as a calcium salt of alginate. Next day morning, precipitated mixture was centrifuged and the brown colour pellet was removed by leaving the same colour aliquot. This supernatant was fucoidan rich seaweed extract (FRSE).

Quantification of FRSE

The fucoidan rich seaweed sample was dialysed and used for quantification. L-fucose content was analysed (Dubois, Gilles, Hamilton, Rebers & Smith 1956). Two millilitre of FRSE was pipetted into calorimetric tube and 50 μL of 80% phenol was added to it. Then 5 mL of concentrated sulphuric acid was added rapidly, the stream of acid was directed against the liquid surface rather than against the side of the test tube to obtain good mixing. The tube was allowed to stand for 10 min. Then, they were shaken and placed for 20 min in water bath at 37°C. The absorbance of the characteristic yellow-orange solution was measured at 480 nm. From the L-fucose content the fucoidan yield was calculated empirically that 1 μg of fucoidan is equivalent to 1.75 × fucose (μg) (Doner & Whistler 1973; Choosawad, Leggat, Dechsukhum, Phongdara & Chotigeat 2005).

Experimental diets

Purified ingredients such as casein (fat free), gelatin, dextrin, starch, cellulose, carboxy methyl cellulose (Himedia Chemical, Mumbai, Maharastra, India), cod liver oil, sunflower oil, BHT, vitamin and mineral mixture (PREEMIX PLUS, Mumbai, India) and choline chloride were used for feed formulation (Table 1). Six experimental diets with the same proximate composition with the crude protein and lipid levels at 35% and 8%, respectively, were prepared which contained graded levels of fucoidan rich seaweed extract (FRSE)/seaweed powder. The diets having graded levels of FRSE/seaweed powder were: 0% FRSE (control), 1% FRSE (TF_1), 2% FRSE (TF_2), 3% FRSE (TF_3), 3% seaweed powder (TS_3) and 6% seaweed powder (TS_6). All the ingredients were thoroughly mixed with required amount of water to make the dough except the oil, BHT and vitamin-mineral mix. Then, the dough was steam cooked for 10 min in an autoclave. After cooling BHT, oil and vitamin-mineral mix were mixed thoroughly to get even distribution of vitamin and mineral in the dough. Then, the dough was pressed through a hand pelletizer of 2 mm diameter to prepare pellet, which were dried at 40°C in hot air oven and finally packed and stored in refrigerator at 4°C until use.

Experimental animals

Fingerlings of iridescent shark or sutchi catfish, *Pangasianodon hypophthalmus* with a weight of 10.0–15.0 g were procured from Kakdwip Research Centre of Central Institute of Brackish water Aquaculture, West Bengal, India. The fish were air lifted with sufficient aeration to the wet laboratory, Central Institute of Fisheries Education. They were carefully transferred to a circular tank (1000 L) with black background supplied with vigorous aeration. The fish left undisturbed the whole night. To ameliorate the handling stress, the fish were given a mild salt treatment (2.0%) for 2 min in the next day morning and mild dose of $KMnO_4$ dip treatment. Vitamin C (2 mg L^{-1}) was also given to the fish as bath treatment. The stock was acclimatized under aerated conditions with a purified diet containing 35% crude protein for 1 month.

Fish rearing

The experiment was conducted for a period of 45 days in 18 black background plastic rectangular tubs (80 × 57 × 42 cm, 150 L capacity) covered with perforated lids. The tubs were initially washed and filled with $KMnO_4$ solution (4 mg L^{-1}) and were left overnight and washed with clean water in the next day morning before stocking with experimental fish. Totally one hundred and eighty fingerlings with initial weight ranging from 17.0 to 20.0 g were randomly distributed in six experimental groups with each of three replicates which following a completely randomized design. The total volume of the water in each tub was maintained

Table 1 Composition of experimental diets (g %)

Ingredients	Control	TF_1	TF_2	TF_3	TS_3	TS_6
Casein	33.00	33.00	33.00	33.00	33.00	33.00
Gelatin	7.25	7.25	7.25	7.25	7.25	7.25
Dextrin	16.75	16.75	16.75	16.75	16.75	16.75
Starch soluble	19.50	19.50	19.50	19.50	19.50	19.50
Cellulose	11.00	10.00	9.00	8.00	8.00	5.00
Cod liver oil	4.00	4.00	4.00	4.00	4.00	4.00
Sunflower oil	4.00	4.00	4.00	4.00	4.00	4.00
Vitamin mineral	2.00	2.00	2.00	2.00	2.00	2.00
CMC	2.00	2.00	2.00	2.00	2.00	2.00
Choline chloride	0.40	0.40	0.40	0.40	0.40	0.40
BHT	0.10	0.10	0.10	0.10	0.10	0.10
FRSE/ Seaweed powder	0.00	1.00	2.00	3.00	3.00	6.00
Total	100	100	100	100	100	100

Composition of vitamin mineral mix (PREEMIX PLUS) (quantity/2.5 kg). Vitamin A, 5 500 000 IU; Vitamin D3, 1 100 000 IU; Vitamin B2, 2000 mg; Vitamin E, 750 mg; Vitamin K, 1000 mg; Vitamin B6, 1000 mg; Vitamin B12, 6 mcg; Calcium Pantothenate, 2500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27 000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450 L-lysine, 10 g; DL-Methionine, 10 g; Selenium, 50 ppm.

equally and continuous aeration was provided in all the tubs throughout the experimental period. The fish were fed to satiation for 45 days in two frequencies daily one at 10.00 h in the morning and other at 18.00 h in the evening under normal light regime. The leftover feed and faecal matter were siphoned out in everyday morning with about 30–50% of water exchange. Water quality parameters viz. temperature, pH, dissolved oxygen, free carbon dioxide, total hardness, ammonia, nitrite and nitrate levels were recorded during the experimental period.

Growth parameters and survival rate

Sampling for growth was done at every 15 days to assess the body weight of the fish. Fish were starved overnight before taking the weight. The weight was taken in an electronic balance. The growth performance was assessed using the following formulae:

$$\text{Weight gain}(\%) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{Specific growth rate (SGR)}\% = \frac{\text{Log}_e\text{final weight} - \text{Log}_e\text{initial weight}}{\text{Number of days}} \times 100$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed consumption (dry weight)}}{\text{Body weight gain (wet weight)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Net weight gain (wet weight)}}{\text{Protein fed}}$$

At the end of the experiment, all the experimental tubs were dewatered and the number of the experimental animals in each tub was counted and the survival rate (%) was calculated by the following formula:

$$\text{Survival rate (\%)} = \frac{\text{Total number of fish harvested}}{\text{Total number of fish stocked}} \times 100$$

Sampling

At the end of 45 days of feeding trial, the sampling was carried out for the analysis of the respiratory burst activity, phagocytic activity, blood glucose level and other blood parameters such as haemoglobin content, total erythrocytes, leukocyte count and haematocrit value. There were two fish from each replicate anaesthetized using clove oil (50 μL L^{-1}) and blood was drawn from the caudal vein (*Vena caudalis*) using a tuberculin syringe rinsed with 2.7% EDTA solution (an anti-coagulant) to prevent blood clotting. The blood samples from

the fish of the same replicate were pooled in an eppendorf tube containing a pinch of EDTA powder, shaken mildly to prevent haemolysis of blood and then kept at 4°C until use. For serum collection another six fish from each treatment was anaesthetized and blood was collected without anticoagulant treatment, allowed to clot for 3 h followed by serum was collected with micropipette and stored at −20°C until use.

Blood glucose

Blood glucose was estimated by the modified method of Nelson and Somogyi (Oser 1944). One hundred micro litre of blood sample was de-proteinized by mixing with 4.75 mL of 0.174 M zinc sulphate and 4.75 mL of 0.142 M barium hydroxide. The solution was mixed vigorously and filtered through a filter paper and the filtrate was collected in a dry test tube. Then 1 mL of alkaline copper sulphate solution was added in to 0.5 mL of filtrate. The test tubes were placed in a boiling water bath for 20 min. Then cooled to room temperature and 1 mL arseno molybdate reagent was added. The absorbance was recorded at 540 nm against blank.

Haematological parameters

The determination of haemoglobin was done by cyanmethaemoglobin method using Drabkin's fluid (Qualigens Fine Chemicals, Mumbai, India). Five millilitres of Drabkin's working solution was taken in a test tube followed by 20 μL of blood was added to it and the absorbance was measured using a spectrophotometer (MERCK-Thermo Electron, Madison, WI, USA) at a wavelength of 540 nm. The total erythrocyte and leucocyte were counted in a haemocytometer by taking 3980 mL of erythrocyte and leucocyte diluting fluids (Qualigens), respectively, in a separate clean glass vial and 20 μL of blood was added to it. Then the solution was mixed thoroughly to suspend the cells uniformly in the solution and the cells were counted in haemocytometer to calculate the total number of erythrocytes and leucocytes per millilitre of the blood sample by the following formula:

$$\text{Number of cells mL}^{-1} = \frac{\text{Number of cells counted} \times \text{dilution}}{\text{Area counted} \times \text{depth of fluid}}$$

The packed cell volume (PCV) was analysed by drawing non-dotted blood by capillary action into microhaematocrit tubes. The microhaematocrit tubes were sealed at one end with synthetic sealant. The sealed tubes were centrifuged in a microhaematocrit centrifuge for 5 min at 12 300 *g* measured using microhaematocrit reader. Then PCV was expressed as PCV percentage.

Respiratory burst activity

Nitroblue tetrazolium (NBT) assay following the method of Secombes (1990) subsequently modified by Stasiack and Bauman (1996) was used for the estimation of respiratory burst activity of the phagocytes. The wells of 'flat bottom' microtitre plates were loaded with 50 μL of blood samples and incubated at 37°C for 1 h to allow adhesion of cells. The supernatant was decanted and the wells were washed thrice with PBS. After washing, 50 μL of 0.2% NBT was loaded in the wells and incubated for 1 h. Then the cells were fixed with 100% methanol for 2–3 min and again washed thrice by using 70% methanol. The plates were air-dried for 5 min. After air drying, 60 μL of 2N potassium hydroxide and 70 μL of dimethyl sulphoxide were subsequently added in to all the wells to dissolve the formazon blue precipitate formed. Finally the OD of turquoise blue coloured solution was recorded in an ELISA reader at 540 nm.

Serum total protein, albumin and globulin

Serum total protein was estimated by the Biuret method (Reinhold 1953) using a Innoline Total protein kit. Albumin was estimated by the bromocresol green binding method (Doumas & Biggs 1972) using Innoline albumin kit. The absorbance of the standard and test was measured against a blank in a spectrophotometer at 550 nm and 578 nm, respectively, for total serum protein and albumin. Globulin values were obtained by subtracting the albumin values from the total serum protein values. The albumin/globulin ratio (A/G ratio) was calculated by dividing albumin values by globulin values.

Serum lysozyme activity

Serum lysozyme activity was carried out by using Anderson-Siwicki method (Anderson & Siwicki

1995). In the 96 well U bottom microtitre plate 150 μL of *Micrococcus luteus* suspension in phosphate buffer (A_{450} = 0.5–0.7) was taken and 50 μL of serum samples were added into it. The contents of microtitre plate were mixed thoroughly and immediately OD value was taken at 450 nm in ELISA reader. Then the plate was incubated at 25°C for 1 h and final OD was taken. This absorbance was compared with standard lysozyme of known activity by the same procedure. A unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 per minute and the lysozyme activity was expressed as U min^{-1} mg^{-1} protein of serum.

Phagocytic activity and phagocytic index

The phagocytic cells were detected with *Staphylococcus aureus* (Bangalore Genei, Hyderabad, India) by the method of Anderson – Siwicki (Anderson & Siwicki 1995). One hundred micro litres of blood sample was placed in the wells of microtitre plate followed by 100 μL of *S. aureus* 1×10^7 cells suspended in phosphate buffered saline (pH 7.2) was added and mixed thoroughly. This mixture was incubated for 20 min at room temperature. Then, 5 μL of this mixture was taken on to a clean glass slide and a smear was prepared. The smear was air dried for 5 min. After air drying, fixed with 95% ethanol for 5 min and again air dried. Then the smear was stained with Giemsa stain for 10 min. The sum of 100 neutrophils and monocytes from each smear were observed under the light microscope and the number of phagocytising cells and bacteria engulfed by the phagocyte were counted. The phagocytic activity defined as the number of phagocytising cells divided by the total number of phagocytes counted. The phagocytic index is expressed as the total number of bacteria engulfed by the phagocytes, divided by the total number of phagocytes containing engulfed bacteria.

Pathogenic bacteria

The pathogenic bacteria, *Aeromonas hydrophila* was procured from the Aquatic Animal Health and Management Division, Central Institute of fisheries Education (CIFE), Mumbai. *A. hydrophila* was grown on nutrient broth (Himedia, Mumbai, India) for 24 h at 28°C. The culture broth was centrifuged at 3000 *g* for 10 min. The supernatant was decanted and the pellet was re-suspended in sterile phosphate buffer saline (pH 7.4). The OD of the solution was adjusted to 0.5 at 456 nm by serial dilution to obtain the final bacterial cell concentration of 1.8×10^7 CFU mL L^{-1} which is used for challenge study (Misra, Sahu, Pal, Xavier, Kumar & Mukherjee 2006; Christybapita, Divyagnaneswari & Michael 2007; Mohamad & Abasali 2010).

Challenge study

Five days after the initial sampling, five fish from each replicate of the experimental groups were injected intraperitoneally with 200 μL of bacterial suspension with bacterial cell concentration of 1.8×10^7 CFU mL^{-1}. Mortality was observed for 10 days in all the experimental groups. Post challenge sampling of the surviving fish were carried out on the 10th day for post challenge analysis. *A. hydrophila* was confirmed from the dead fish by isolating it from the dead fish. Survival was calculated using the following formula:

$$\text{Relative \% of survival} = \frac{\text{No of fish survived after challenge}}{\text{No of fish injected}} \times 100$$

Partial sequencing of interferon gamma 2 gene from *P. hypophthalmus*

The cloning for partial sequencing of interferon gamma 2 gene was done from spleen and head kidney samples collected from *P. hypophthalmus.* Total RNA was extracted using Trizol reagent (Invitrogen Bioservices India, Bangalore, Karnataka, India) following standard protocol. The cDNA was synthesized using the first strand cDNA synthesis kit (Fermentas, Hanover, MA, USA) following manufacturer's instructions. The PCR reaction was performed with the following degenerate interferon-γ2 forward (CCTCCCGAAGAACATCAAGG) and reverse (TTAGCTTGACGTCGTCTCC) primers and β-actin forward (GACTTCGAGCAGGAGATGG) and reverse (CAAGAAGGATGGCTGGAACA) primers. PCR amplification was carried out by normal cyclic conditions with 50°C annealing temperature in the Peltier thermal Cycler (Biorad MJ Research, Gurgaon, Haryana, India). The resulting PCR product of the expected size was gel excised and isolated using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), cloned in a bacterial vector using the InsTA

PCR cloning kit (Fermentas) and transformed in *Escherichia coli* competent cells following the manufacturer's instructions. White colonies were selected from X-Gal/IPTG ampicillin LB agar plates and grown in LB/ampicillin liquid media and plasmids were further purified using the QIAprep spin miniprep kit (Qiagen). The cloned products were sequenced using universal primers M13 by ABI prism 377automatic sequencer (Bangalore Genei). The sequence was analysed using BLAST (Basic Local Alignment Search Tool) software in the NCBI (National Centre for Biotechnology Information) GenBank nucleotide database for finding homology with other sequences and submitted to NCBI GenBank.

Expression using semi-quantitative RT-PCR

The semi-quantitative analysis of *P. hypophthalmus* interferon-γ 2a was done after 45 days of FRSE diet feeding and again after artificial infection with *A. hydrophila* was studied by RT-PCR analysis. The primer sequences used in RT-PCR analysis are PHIFN_F (GGCTCAGGATGAAAAGGTGA) and PHIFN_R (CTCTGGGTCAGTGGGTCATT). The both forward (PHIFN_F) and the reverse (PHIFN_R) primers are having same base pair length i.e. 20 numbers. The relative changes in intensity of bands were measured using Gel-Pro Software Version 4.5.

Statistical analysis

The effect of FRSE on the immunomodulation of *P. hypophthalmus* was done with one way ANOVA. The mean values for pre- and post-challenge parameters were compared by Student's *t*-test. Duncan's multiple range test was used to determine the significant differences between the treatment means. All statistical analysis was done using the software package SPSS (version 16).

Results

Quantification of fucoidan rich seaweed extract (FRSE)

The dialysed fucoidan rich seaweed extract was quantified by measuring the L-fucose content and the L-fucose content was multiplied by the factor 1.75. The L-fucose content was determined by colorimetric method and the quantity of L-fucose was 31.61 mg g^{-1} dried seaweed powder. Hence, the fucoidan content was 55 mg g^{-1} or 5.5 g/100 g dried seaweed powder.

Physico-chemical parameters of water

The physico-chemical parameters of water such as temperature (°C), pH, dissolved oxygen (mg L^{-1}), free carbon dioxide (mg L^{-1}), total hardness (mg L^{-1}), ammonia (mg L^{-1}), Nitrite – N (mg L^{-1}), Nitrate – N (mg L^{-1}) were recorded during the experiment and the range of physico-chemical parameter values of all the treatments are presented in Table 2.

Growth parameters and survival rate

The growth parameters such as weight gain percentage, specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) of the experimental groups were listed in the Table 3. The weight gain percentage was found to be significantly different ($P < 0.001$) among the various treatment groups. The highest SGR was found in TF_3 which was significantly higher ($P < 0.001$) than the all other groups. The FCR of different experimental groups varied significantly ($P < 0.001$). The best FCR was found in TF_3 group. And the same way the highest PER was found in TF_3 treatment group. The survival rates of the fish in the different experimental groups are given in the Table 3. There was no mortality observed in the TF_1, TF_2, TF_3 and TS_6 groups.

Blood glucose

Control group showed higher glucose level than the FRSE diet fed treatment groups during pre-challenge condition. However, there was no significant variation among the treatment groups ($P > 0.05$). Similarly control group in post challenge condition showed highest value and lowest value was recorded in TF_3, TF_2 and TS_6 groups (Fig. 1). There was no significant difference ($P > 0.05$) between pre challenge and post challenge blood glucose level of different treatments except control group ($P < 0.05$).

Blood haemoglobin, Total erythrocyte count (TEC), Total leucocyte count (TLC) and Haematocrit value

Significant variation ($P < 0.001$) in both pre and post challenge haematological parameters were

Table 2 Physico-chemical parameters of water during the experimental period of 45 days for different experimental groups

Treatments	Temperature (°C)	pH	Dissolved oxygen (ppm)	Free CO_2 (ppm)	Hardness (ppm)	Ammonia (ppm)	Nitrite (ppm)	Nitrate (ppm)
Control	25.2–27.4	7.6–8.2	6.0–6.5	ND	230–241	0.06–0.08	0.085–0.088	1.0–1.1
TF_1	25.6–28.4	7.7–8.0	6.1–6.8	ND	232–242	0.06–0.01	0.086–0.087	0.98–0.99
TF_2	25.2–27.8	7.8–8.1	6.2–6.6	ND	236–241	0.05–0.07	0.084–0.085	1.05–1.2
TF_3	25.4–27.5	7.5–8.4	6.1–6.5	ND	224–248	0.07–0.09	0.088–0.089	0.89–1.0
TS_3	25.8–27.9	7.7–8.2	5.8–6.4	ND	222–252	0.03–0.07	0.078–0.087	0.76–0.94
TS_6	25.6–28.2	7.8–8.2	5.7–6.6	ND	220–260	0.05–0.09	0.081–0.083	0.79–0.82

ND, not detected.

Table 3 Growth parameters and survival rate of different experimental groups fed different experimental diets at the end of the experiment

Treatments	Wt. gain (%)	SGR	FCR	PER	Survival rate (%)
Control	$40.72^{a} \pm 1.36$	$0.76c^{a} \pm 0.021$	$3.57^{c} \pm 0.116$	$0.81^{a} \pm 0.026$	96.67 ± 3.33
TF_1	$52.36^{b} \pm 1.27$	$0.94^{b} \pm 0.018$	$2.86^{b} \pm 0.063$	$1.03^{b} \pm 0.023$	100.00 ± 0
TF_2	$54.85^{b} \pm 0.88$	$0.97^{b} \pm 0.012$	$2.75^{b} \pm 0.035$	$1.06^{b} \pm 0.013$	100.00 ± 0
TF_3	$61.84^{c} \pm 1.37$	1.07 ± 0.018	$2.47^{a} \pm 0.060$	$1.19^{c} \pm 0.029$	100.00 ± 0
TS_3	$50.65^{b} \pm 1.16$	$0.91^{b} \pm 0.017$	$2.93^{b} \pm 0.070$	$1.00^{b} \pm 0.023$	96.67 ± 3.33
TS_6	$51.93^{b} \pm 2.36$	$0.93^{b} \pm 0.034$	$2.89^{b} \pm 0.119$	$1.01^{b} \pm 0.043$	100.00 ± 0
P value	0.001	0.001	0.001	0.001	0.571

Mean values in the same column with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE $n = 3$
SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio.

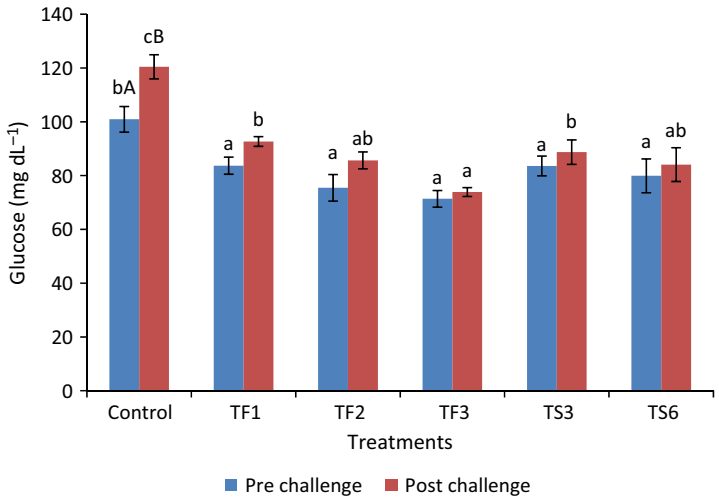

Figure 1 Pre- and post-challenge blood glucose (mg dL^{-1}) of different experimental groups. Mean values in the experimental group with different superscript (a,b,c) differ significantly ($P < 0.05$). Mean values in the experimental groups with different superscript (A,B) between pre and post challenge group under each treatment vary significantly ($P < 0.05$). Data expressed as Mean ± SE $n = 3$. Control – 0% FRSE, TF_1 – 1% FRSE, TF_2 – 2% FRSE, TF_3 – 3% FRSE, TS_3 – 3% seaweed powder, TS_6 – 6% seaweed powder.

found among the various treatment groups (Table 4). Feeding of FRSE diet significantly ($P < 0.001$) affected the haemoglobin, TEC and TLC with the highest value in 3% FRSE diet fed group and the lowest in the control group. Same trend was also noticed in the post-challenged groups. But in case of post challenge condition, TEC values of all the treatments groups were less than their pre-challenged counterpart. The highest haematocrit values in both pre and post challenge conditions were found in TF_3 groups which was significantly different from all the other treatment

groups ($P < 0.001$). There was significant difference in pre and post challenge HCT value in TF_1, TF_3 and TS_6. The lowest HCT value was found in the control groups both in pre and post challenge condition and no significant ($P > 0.05$) difference between pre and post challenge condition.

Respiratory burst activity

There was significant difference ($P < 0.001$) in the respiratory burst activity among the various treatment groups separately in both pre- and post-challenge conditions (Fig 2). In both, pre and post-challenged groups highest NBT activity was observed in 3% FRSE diet fed group and least activity was recorded in the control group. There was no significant difference between the pre-challenge group of TF_1, TS_3 and TS_6 treatments. As well as no significant difference was found among the post challenge group of TF_1, TF_2, TS_3 and TS_6 groups However, there were no significant ($P > 0.05$) changes in NBT values between pre and post challenge condition of the respective treatment groups i.e. between pre challenge TF1 and post challenge TF1.

Serum protein, albumin, globulin and albumin to globulin ratio

There was significant difference ($P < 0.001$) in serum protein, albumin, globulin and A/G ratio level among the various treatment groups in both pre and post challenge conditions, separately (Table 5). In both pre and post-challenge condition the serum protein level increased with the increasing FRSE content in the diets. But during the post challenge condition serum protein level was lower than the serum protein content of pre challenge condition. And the values for TF_2 and TF_3 were similar ($P > 0.05$) in pre and post challenge condition. There was significant difference ($P < 0.05$) between pre and post challenge serum protein in control, TF_2 and TF_3 experimental groups. Serum globulin content also exhibited the same trend during pre and post challenge condition as like serum protein. Highest serum globulin content was found in TF_3 group. The significant difference ($P < 0.05$) between pre and post challenge globulin content was found in control, TF_2, TF_3 and TS_6 groups.

The pre and post challenge serum albumin content of different experimental groups significantly differs among the different treatments ($P < 0.05$).

Table 4 Pre- and post challenge haematological parameters of different experimental groups

Treatments	Hb (g %)		TEC (10^6/mm^3)		TLC (10^3/mm^3)		HCT (%)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Control	$9.43^{aB} \pm 0.17$	$8.46^{aA} \pm 0.08$	$1.69^{aB} \pm 0.03$	$1.51^{aA} \pm 0.05$	$167.13^{aA} \pm 0.90$	$179.06^{aB} \pm 0.86$	$13.33^{aA} \pm 0.95$	$11.79^{aA} \pm 0.09$
TF_1	$11.33^{cB} \pm 0.24$	$10.27^{bA} \pm 0.14$	$1.94^{bcB} \pm 0.02$	$1.68^{bA} \pm 0.05$	$187.65^{bA} \pm 4.05$	$199.14^{bA} \pm 3.21$	$16.28^{bcA} \pm 0.16$	$17.59^{cB} \pm 0.19$
TF_2	$12.77^{dB} \pm 0.23$	$11.58^{cA} \pm 0.31$	$2.13^{cB} \pm 0.01$	$1.61^{abA} \pm 0.05$	$214.46^{cA} \pm 3.48$	$227.72^{cB} \pm 4.15$	$20.00^{dA} \pm 0.63$	$15.63^{bA} \pm 0.62$
TF_3	$14.46^{eB} \pm 0.34$	$12.94^{dA} \pm 0.42$	$2.65^{dB} \pm 0.09$	$2.44^{cA} \pm 0.05$	$242.96^{dA} \pm 3.68$	$260.65^{dB} \pm 1.49$	$26.48^{eB} \pm 0.34$	$24.67^{eA} \pm 0.14$
TS_3	$10.60^{bB} \pm 0.08$	$9.96^{bA} \pm 0.07$	$1.81^{abB} \pm 0.04$	$1.63^{abA} \pm 0.04$	$174.46^{aA} \pm 4.07$	$173.81^{aA} \pm 5.31$	$14.04^{abA} \pm 0.24$	$14.22^{bA} \pm 0.15$
TS_6	$11.61^{cB} \pm 0.11$	$10.39^{bA} \pm 0.35$	$2.10^{cB} \pm 0.09$	$1.76^{bA} \pm 0.04$	$194.33^{bA} \pm 2.01$	$207.55^{bB} \pm 1.91$	$18.03^{cdA} \pm 0.20$	$19.30^{dB} \pm 0.25$
P-Value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Mean values in the same column with different superscript (a,b,c,d) differ significantly ($P < 0.05$).
Mean values in the same row with different superscript (A,B) between pre and post challenge group under each treatment vary significantly ($P < 0.05$).
Data expressed as Mean ± SE $n = 3$.
Hgb, blood haemoglobin; TEC, total erythrocyte count; TLC, total leucocyte count; HCT, haematocrit value; Pre-pre challenge; Post-post challenge.

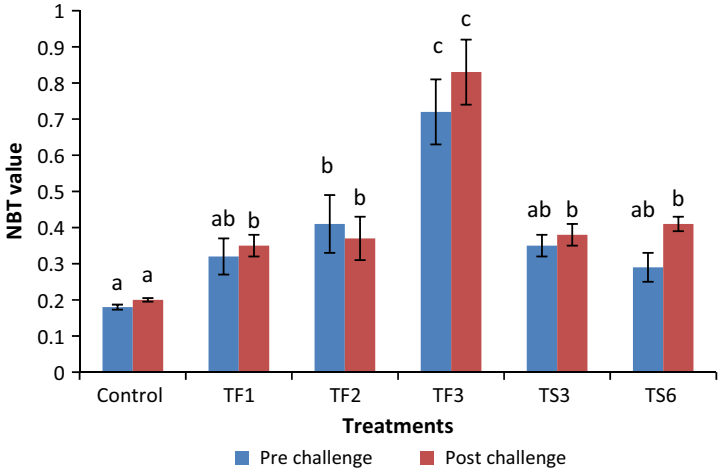

Figure 2 Pre- and post-challenge NBT value of different experimental groups. Mean values in the experimental group with different superscript (a,b,c) differ significantly ($P < 0.05$). Data were expressed as Mean ± SE $n = 3$. Control – 0% FRSE, TF_1 – 1% FRSE, TF_2 – 2% FRSE, TF_3 – 3% FRSE, TS_3 – 3% seaweed powder, TS_6 – 6% seaweed powder.

There was significant difference exists between pre and post challenge serum albumin level only in TF_2 and TF_3 ($P < 0.05$). Highest A/G ratio was found in the control groups of both pre and post challenge conditions, whereas least values were recorded in the FRSE fed groups. There was no significant ($P > 0.05$) changes exists between pre and post challenge A/G ratio of different experimental groups except the control group ($P < 0.05$).

Serum lysozyme and phagocytic activity

In pre-challenge condition, the highest serum lysozyme activity was found in TF_2 group ($P < 0.001$), whereas in post-challenge condition the maximum activity recorded in TF_3 group ($P < 0.001$). The lowest activity was found in control group in both cases ($P < 0.001$). The post challenged group exhibited significantly higher value than their pre-challenged counterpart. There was clear significant difference ($P < 0.05$) observed between pre and post challenge serum lysozyme activity of all the experimental groups. The serum phagocytic activity and phagocytic index exhibited increasing trend as the increase in FRSE and seaweed powder incorporated diet both in pre and post challenge experimental groups. The higher value of phagocytic activity and phagocytic index was observed in the TF3 experimental treatment in both pre and post challenge condition. There was significant difference ($P < 0.001$) in serum phagocytic activity and phagocytic index among, both the pre and post challenge experimental groups. A clear significant difference ($P < 0.05$) between the pre- and post- challenge serum phagocytic activity, and phagocytic index was noticed due to feeding of fucoidan rich seaweed extract incorporated diet (Table 6).

Cumulative mortality percentage and relative percentage of survival

There was no mortality recorded in both in TF_2 and TF_3 group and highest mortality was recorded in the control group when challenged with *A. hydrophila* (Fig. 3). The survival trend in TF_1, TS_3 and TS_6 were almost similar. There was no much significant difference in the survival rate of different experimental groups except the control group ($P < 0.05$).

Sequencing and identification of IFN-γ2 gene

Fortunately, we got two different sequences and which were analysed and confirmed as pIFN-γ 2a and pIFN-γ 2b. Sutchi catfish interferon gamma 2a and 2b gene sequences were submitted to the NCBI GenBank and possessing the accession numbers JN 185453 and JN 185454 respectively. pIFN-γ2a and pIFN-γ 2b genes showed maximum homology of (89%) and 88% with *Ictalurus punctatus* interferon

Table 5 Pre- and post challenge serum total protein, albumin, globulin and A:G ratio of different experimental groups

Treatments	Total Protein (g dL^{-1})		Albumin (g dL^{-1})		Globulin (g dL^{-1})		A:G ratio	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Control	$3.00^{aB} \pm 0.09$	$2.68^{aA} \pm 0.08$	$0.98^{aA} \pm 0.03$	$1.00^{aA} \pm 0.03$	$2.02^{aB} \pm 0.08$	$1.68^{aA} \pm 0.07$	$0.40^{dA} \pm 0.02$	$0.60^{\ dB} \pm 0.03$
TF_1	$4.51^{bA} \pm 0.23$	$4.24^{bA} \pm 0.22$	$0.97^{aA} \pm 0.09$	$0.99^{aA} \pm 0.09$	$3.54^{bA} \pm 0.14$	$3.25^{bA} \pm 0.13$	$0.27^{abA} \pm 0.01$	$0.30^{abA} \pm 0.01$
TF_2	$7.00^{cB} \pm 0.04$	$6.67^{cA} \pm 0.10$	$1.20^{bA} \pm 0.02$	$1.22^{bB} \pm 0.01$	$5.80^{cB} \pm 0.08$	$5.45^{cA} \pm 0.05$	$0.21^{aA} \pm 0.01$	$0.23^{aA} \pm 0.01$
TF_3	$7.74^{cB} \pm 0.11$	$7.44^{cA} \pm 0.06$	$1.46^{cA} \pm 0.01$	$1.49^{cB} \pm 0.02$	$6.28^{cB} \pm 0.11$	$5.95^{cA} \pm 0.08$	$0.24^{aA} \pm 0.02$	$0.26^{aA} \pm 0.02$
TS_3	$3.82^{abA} \pm 0.23$	$3.50^{abA} \pm 0.22$	$0.93^{aA} \pm 0.05$	$0.95^{aA} \pm 0.05$	$2.89^{abA} \pm 0.21$	$2.55^{abA} \pm 0.2$	$0.33^{bA} \pm 0.03$	$0.38^{bA} \pm 0.04$
TS_6	$4.23^{bA} \pm 0.47$	$3.89^{bA} \pm 0.46$	$1.20^{bA} \pm 0.07$	$1.22^{bA} \pm 0.06$	$3.0^{bB} \pm 0.5$	$2.66^{bA} \pm 0.10$	$0.42^{cA} \pm 0.03$	$0.49^{cA} \pm 0.04$
P-value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Mean values in the same column with different superscript (a,b,c,d) differ significantly ($P < 0.05$).
Mean values in the same row with different superscript (A,B) between pre and post challenge group under each treatment vary significantly ($P < 0.05$).
Data were expressed as Mean ± SE $n = 3$.
A/G ratio, albumin/globulin ratio; Pre-pre challenge; Post-post challenge.

gamma 2a (DQ124250) and 2b (DQ124251) gene sequences respectively. The longer transcript designated IFN-γ2a is contains nine additional nucleotides (GTAAGAAAA), which encode the tripeptide val-arg-lys (VRK) found. The shorter transcript, IFN-γ2b is identical to IFN-γ2a except for the absence of the nine aforementioned nucleotides.

Semi-quantitative RT PCR analysis and expression level of IFN-γ2a

The cDNA samples derived from head kidney collected from differential experiment groups before and 24 h after infection with *A. hydrophila* were used to examine the expression levels of interferon gamma 2a, β-actin was used as reference control to normalize the errors in initial RNA concentration. In pre-challenge condition the expression level of IFN-γ2a level was increased with increasing level of FRSE and seaweed powder incorporated diet fed groups. The expression level was less in control group than all the other experimental groups. In post-challenge condition, though TF_3 group show maximum expression there was no marked difference in the expression level of IFN-γ2a gene in different experimental groups (Fig. 4) as revealed from Gel-Pro analysis (Fig. 5).

Discussion

There are plenty of herbal extracts used as immunostimulants in aquaculture to enhance the non-specific immune system of cultured fish species (Sakai 1999; Rao, Das, Jyotyrmayee & Chakrabarti 2006; Sahu, Das, Pradhan, Mohapatra, Mishra & Sarangi 2007; Ardo, Yin, Xu, Varadi, Szigeti, Jeney & Jeney 2008). The main components of the non-specific immune system are monocytes, granulocytes, neutrophils, macrophages and humoral elements, like lysozyme, agglutinin and metal-ion binding proteins (Secombes & Fletcher 1992; Sakai 1999; Rao *et al.* 2006; Sahu, Das, Pradhan *et al.* 2007; Ardo *et al.* 2008). Hence, evaluating fish blood parameters might be a useful tool to understand the impact of immunostimulants on fish health. In the present study, fucoidan rich seaweed extract from *S. wightii* was found to have significant stimulatory effect on non-specific immune parameters in *P. hypophthalmus*, following a challenge with *A. hydrophila.*

Several methods were used to extract fucoidan from different species, such as *Dictyota mertensis,*

Table 6 Pre- and post-challenge phagocytic activity, phagocytic index and serum lysozyme activity (U mg protein^{-1}) of different experimental groups

	Phagocytic activity (%)		Phagocytic index		Serum lysozyme activity	
Treatments	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Control	$10.14^{aB} \pm 0.14$	$8.58^{aA} \pm 0.27$	$1.36^{aB} \pm 0.02$	$1.22^{aA} \pm 0.04$	$31.48^{aA} \pm 0.45$	$42.67^{aB} \pm 0.91$
TF_1	$10.58^{aA} \pm 0.39$	$12.15^{bB} \pm 0.24$	$1.44a^{bA} \pm 0.01$	$1.56^{cB} \pm 0.01$	$54.44^{cA} \pm 1.16$	$66.14^{dB} \pm 1.10$
TF_2	$14.48^{bA} \pm 0.57$	$16.21^{cA} \pm 0.30$	$1.62^{cA} \pm 0.02$	$1.74^{dB} \pm 0.03$	$67.27^{eA} \pm 0.51$	$82.30^{eB} \pm 1.40$
TF_3	$20.70^{cA} \pm 0.36$	$23.96^{dB} \pm 0.85$	$1.84^{dA} \pm 0.03$	$1.97^{eB} \pm 0.03$	$60.08^{dA} \pm 1.93$	$87.53^{fB} \pm 1.58$
TS_3	$10.30^{aA} \pm 0.09$	$10.80^{abB} \pm 0.11$	$1.41^{bA} \pm 0.02$	$1.45^{bA} \pm 0.02$	$48.65^{bA} \pm 0.36$	$52.58^{bB} \pm 0.62$
TS_6	$12.40^{abA} \pm 0.74$	$13.76^{bcA} \pm 0.78$	$1.51^{bA} \pm 0.03$	$1.55^{cA} \pm 0.01$	$54.79^{cA} \pm 0.93$	$60.23^{cB} \pm 1.01$
P-value	0.001	0.001	0.001	0.001	0.001	0.001

Mean values in the same column with different superscript (a,b,c,d) differ significantly ($P < 0.05$).
Mean values in the same row with different superscript (A,B) between pre and post challenge group under each treatment vary significantly ($P < 0.05$).
Data were expressed as Mean ± SE $n = 3$.
Pre-pre challenge; Post-post challenge.

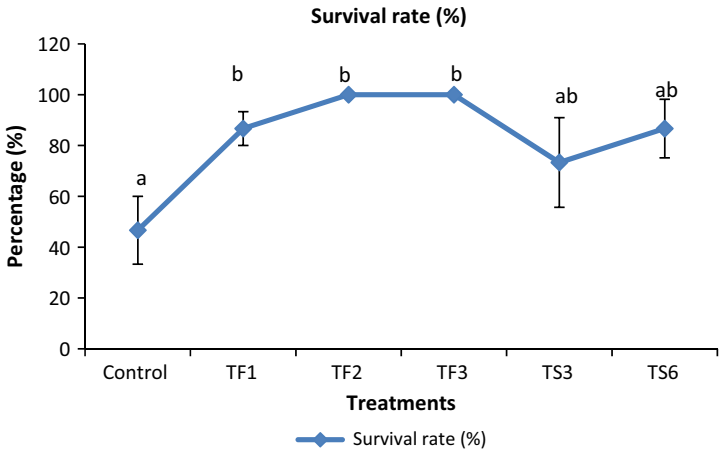

Figure 3 Cumulative relative percentage of survival of *P. hypophthalmus* fingerlings of different experimental group challenged with *A. hydrophila.* Mean values in the experimental group with different superscript (a,b) differ significantly ($P < 0.05$). Data were expressed as Mean ± SE $n = 15$. Control – 0% FRSE, TF_1 – 1% FRSE, TF_2 – 2% FRSE, TF_3 – 3% FRSE, TS_3 – 3% seaweed powder, TS_6 – 6% seaweed powder.

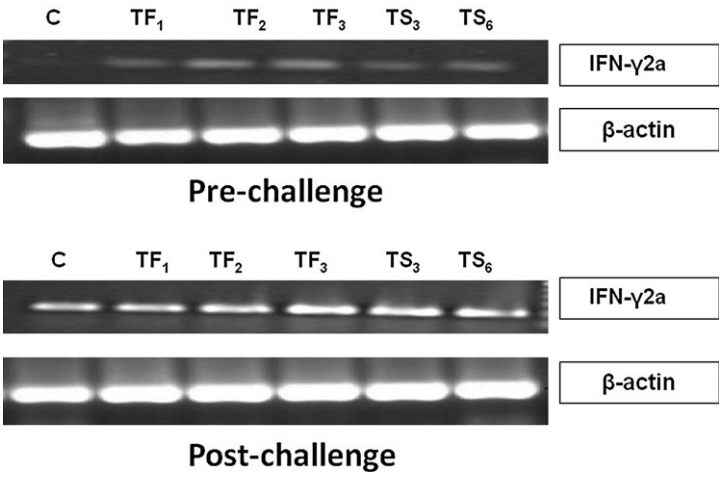

Figure 4 IFN-γ2a and β-actin expression levels in head kidney tissue before and after infection (pre- and post challenge) sequentially. C – 0% FRSE, TF_1 – 1% FRSE, TF_2 – 2% FRSE, TF_3 – 3% FRSE, TS_3 – 3% seaweed powder, TS_6 – 6% seaweed powder.

Padina gymnospora and *Sargassum vulgare* by Maxatase (Dietrich, Farias, De Abreu, Leite, De Silva & Nader 1995), *Facus vesiculosus, Laminaria brasiliensis* and *Ascophyllum nodusum* by papain (Pereira, Mulloy & Mourao 1999), *Sargassum horneri* by 10% TCA (Hoshino, Hayashi, Hayashi,

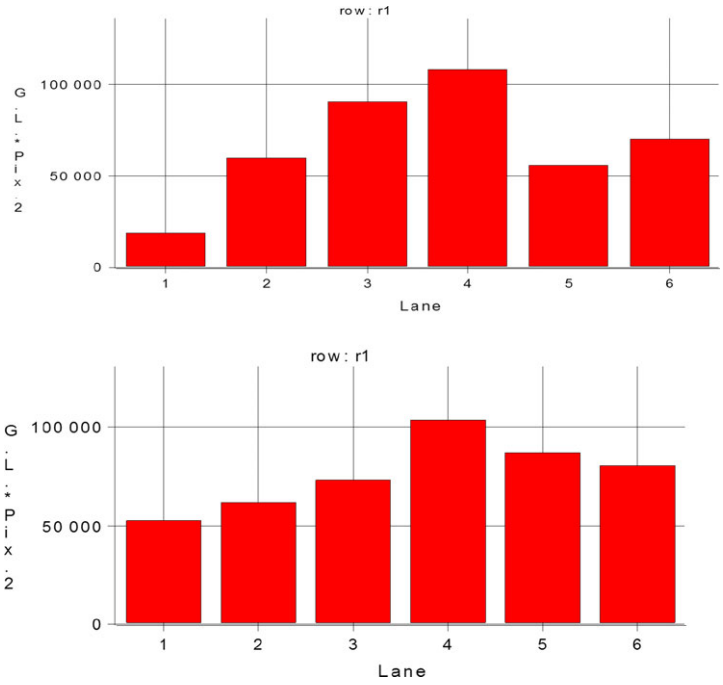

Figure 5 IFN-γ2a expression levels in head kidney tissue before (pre-challenge) and after (post challenge) infection with *A. hydrophila* sequentially (Gel-Pro analysis). In both the graph the numbers 1, 2, 3, 4, 5 & 6 represents. C – 0% FRSE, TF_1 – 1% FRSE, TF_2 – 2% FRSE, TF_3 – 3% FRSE, TS_3 – 3% seaweed powder and TS_6 – 6% seaweed powder respectively.

Hamada, Lee & Sankawa 1998) and *Sargassum horneri, Sargassum ilicifolium, Padina canaliculata* and *Sargassum polycystum* by HCl (Wang & Zhae 1985; Velayutham & Jayachandran 1991; Colliec, Vidal & Jozefonviez 1994; Chotigeat *et al.* 2004). The yield varied widely, depending on the method used and the seaweed species. In the present study, fucoidan content of *Sargassum wightii* was 5.5%. This is in agreement with (Wang & Zhae 1985; Velayutham & Jayachandran 1991; Eluvakkal, Sivakumar & Arunkumar 2010), who reported that the fucoidan content from *Sargassum wightii, Sargassum ilicifolium* and *Sargassum horneri* was 7.15%, 4.2% and 3.9% respectively.

In the present study, inclusion level of FRSE and seaweed powder showed significant effect on the weight gain percentage and specific growth rate of the fingerlings of *P. hypophthalmus* than the control group. The current result agrees with the previous findings, indicating the growth-promoting effects of fucoidan in shrimps (Immanuel *et al.* 2010; Traifalgar *et al.* 2010). In the present study, FCR was significantly different in FRSE diet fed groups and seaweed power fed groups than the control group. This is expected as there was increase in weight gain due to feeding of different concentration of FRSE. This is in agreement with the results of (Immanuel *et al.* 2010; Traifalgar *et al.* 2010) in shrimps. The PER was also significantly affected ($P < 0.005$) by graded level feeding of FRSE blended diets. Growth rate of *P. hypophthalmus* fingerling were influenced by the addition of FRSE and seaweed powder in the diet which exhibited better PER achieved by FRSE diet fed groups than the control group. Though the growth promoting effect of fucoidan has not been reported, but indirect role in mitigating stress to growth rate may not be ruled out. Similar result was supported by (Traifalgar *et al.* 2010) in *Marsupenaeus japonicus* fed diets supplemented with graded levels of *Undaria pinnatifida* fucoidan.

A variety of immunostimulants including fucoidan (Chotigeat *et al.* 2004) stimulate the fish phagocytes such as macrophages and neutrophils. The superoxide anions (O_2^-) and its reactive derivatives hydrogen peroxide and free radicals like hydroxyl radicals are produced by fish phagocytes at times of intense oxygen consumption that is known as the respiratory burst activity (Secombes & Fletcher 1992; Secombes 1996). These generated reactive oxygen species (ROS) are considered to be harmful for fish pathogenic bacteria (Hardie, Ellis & Secombes 1996; Itou, Lida & Kawatsu 1996). Thus, phagocytes play a vital role in limiting the dispersion of infectious agents and are responsible for the eventual destruction of phagocytosed pathogens by evolving elaborate killing mechanisms such as degranulation and

production of reactive oxygen and nitrogen intermediates (Neumann, Stafford, Barreda, Ainsworth & Belosevic 2001). The degranulation is necessary for the release of Myeloperoxidase (MPO) and diverse antimicrobial enzymes (Quade & Roth 1997). Hence, increased respiratory burst activity can be positively correlated with the increased bacterial pathogen killing activity of fish phagocytes (Sharp & Secombes 1993). The respiratory burst activity of phagocytes was measured by reduction in nitroblue tetrezolium (NBT) by intracellular superoxide radicals produced by the fish leucocytes (Siwicki & Studnica 1987; Sahu, Das, Pradhan *et al.* 2007; Ardo *et al.* 2008). In the present study, an increasing trend of NBT was observed before and after challenge study with increasing level of FRSE fed groups. Higher NBT value was registered in TF_3 group supplemented with 3% of FRSE suggesting the immuno-protecting role of fucoidan. This is in agreement with the enhanced NBT activity in *Labeo rohita* fed with varying levels of garlic incorporated feed (Sahu, Das, Misra, Pradhan & Sarangi 2007).

Resistance of fish to infection by microorganisms relies mostly on an immediate response carried out by cells and serum soluble molecules. Phagocytic activity is strongly influenced by immunostimulants, possibly due to their high demand for antioxidative substances necessary for preventing oxidative damage induced by free radicals produced to counteract pathogen aggression (Wahli, Verlhac, Gabaudan, Schuep & Meier 1998). Phagocytosis and other killing mechanism of phagocytic cells are an important defence mechanism against pathogenic bacteria in fish (Rao *et al.* 2006). In the present study, phagocytic activity increased with the increasing level of dietary FRSE. The highest phagocytic activity was found in the groups fed with 3% FRSE and the lowest was noticed in the control group. After infection with *A. hydrophila,* the phagocytic activity was again increased in all the treatments with maximum hike in the TF_3 groups as like the pre-challenge condition. Phagocytic index also followed the same trend as like the phagocytic activity. The increased phagocytic activity also proved by increase in the NBT activity. This result is similar to the phagocytic activity of vitamin C (Tewary & Patra 2008) and mango kernel (Sahu, Das, Pradhan *et al.* 2007) on *Labeo rohita* and diet containing active component of *Ocimum sanctum*, *Withania somnifera* and *Myristica fragrans* on *Epinephelus tauvina* juveniles (Sivaram, Babu, Immanul, Murugadass, Citarasu & Petermarian 2004).

Head kidney is essential for erythropoietin production (Gordon, Goper & Zaryani 1967) and any damage in it cause decrease in erythropoietin level, which in turn decreases RBC production and Hb synthesis even under hypoxic conditions (Reddy, Vijayakumari, Kalarani & Davies 1992). Immunostimulants have the potential to improve the function of head kidney meanwhile augmenting the erythropoiesis and haematosynthesis. In the present study, increased FRSE fed groups exhibited higher ($P < 0.05$) erythrocyte count than the control group. Erythrocyte number was significantly higher in fish fed with 3.0% FRSE incorporated diet. Since, the TEC value increased, haematocrit value was also increased gradually with the increasing concentration of FRSE in the diet. This is agreement with the previous findings (Sahu, Das, Pradhan *et al.* 2007; Mohamad & Abasali 2010). Erythrocyte count and haemoglobin level significantly decreased ($P < 0.05$) in the post-challenge period which is observed in the previous studies in Nile Tilapia (Ranzani-Paiva, Ishikawa, Eiras & Silveira 2004) and Rohu (Misra *et al.* 2006). During post-challenge, the decrease in the haemoglobin content in present study is correlates with the study in which furunculosis caused by *A. salmonicida* in Atlantic salmon (Foda 1973). The same kinds of results were also reported in *Etroplus suratensis* (Pathiratne & Rajapakshe 1998) and *Cyprinus carpio* (Harikrishnan, Nisha & Balasundaram 2003). The decrease in Hct and haemoglobin concentration of fish exposed to *A. hydrophila* indicates that the RBCs are being destroyed by the leucocytosis activity in an erythrocytic anaemia with subsequent erythroblastosis on erythropoietic organs (Haney, Hursh, Mix & Winton 1992).

The leucocytes play an essential role in non-specific immunity of fish and that can be evident from their count which can be considered as a good indicator of the health status of any fish along with other immunological parameters (Roberts 1978). During inflammation leucocytes possess a significant role. At times of infection, the increase in leucocytes probably represents the inflammatory response against the bacteria (Roberts 1978). In the present study, the leucocytes count was higher in TF_3 group which was significantly higher than the control group, which may be due to immune-modulatory response of dietary FRSE. Similar observation was made in rohu fingerlings

when fed with yeast RNA incorporated diet (Choudhury, Pal, Sahu, Kumar, Das & Mukherjee 2005) and dietary carbohydrate as well as n-3 PUFA (Misra *et al.* 2006) and catla fingerlings fed dietary yeast RNA, ω-3 fatty acid and β-carotene as immunostimulants (Jha, Pal, Sahu, Kumar & Mukherjee 2007).

Fish serum lysozyme is produced by leukocytes including neutrophils and macrophages. A total lysozyme level is a measurable humoral component of the non-specific defence mechanism which has the potential to prevent the growth of pathogenic bacteria by splitting beta-1, 4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell walls and hence stimulating the phagocytosis of pathogenic bacteria (Ellis 1990; Alexander & Ingram 1992; Gopalakannan & Arul 2006; Choi, Park, Yoon, Kim, Jang & Choe 2008). Increased levels of lysozyme activity are known to possess natural protective mechanism in fish (Ingram 1980). Umpteen numbers of reports are available in which immunostimulants can enhance the lysozyme activity (Chen, Wu, Yin & Li 2003; Puangkaew, Kiran, Somsmoto, Okamoto, Satoh & Takeuchi 2004; Hanif, Bakopoulos, Leonardos & Dimitriadis 2005). In the present study, serum lysozyme activity was also found an increasing trend as that of NBT which is in agreement with *Fenneropenaeus chinensis* fed with *Sargassum fusiforme* polysaccharide extracts (Huang *et al.* 2006) and *Labeo rohita* fed with levamisole (Wijendra & Pathiratne 2007).

After challenged with *A. hydrophila* the serum lysozyme activity was increased in the experimental groups as the concentration of FRSE level increased in the diet. The present result is correlated with the observations made in common carp, *C. carpio* that exhibited an increased serum lysozyme activity after challenge with *A. punctata* (Vladimirov 1968; Siwicki & Studnica 1987) and in Atlantic salmon which had challenged with *A. salmonicida* (Møyner, Røed, Sevtdal & Heum 1993). The level of serum lysozyme was enhanced in *Labeo rohita* and *Anguilla japonica* after feeding with *Achyranthes aspera* seed (Rao *et al.* 2006) and extract of *Viscum album* (Choi *et al.* 2008) respectively. The *Paralichthys olivaceous* challenged with *Edwardsiella tarda,* exhibits increased synthesis of serum lysozyme (Hilima, Hirono & Aoki 1997). The production of serum lysozyme by Atlantic salmon macrophages *in vitro* is enhanced in the presence of yeast glucan or bacterial lipopolysaccharide as an immunostimulant (Lamas & Ellis 1994). After pathogenic bacterial challenge, in most of the fish a significant increase in the numbers of leukocytes could supports the results of present study and that is the indication of activation of the non-specific immune system in the infected fish.

The serum proteins includes various humoral elements of the non-specific immune system, measurable total protein, albumin and globulin levels suggests that high concentrations are likely to be a result of the enhancement of the non-specific immune response of fish. In the present study, highest plasma protein, albumin and globulin content were found in 3% FRSE fed groups during pre-challenge. It was observed that serum protein, albumin and globulin level was increased steadily with the increasing FRSE level in the diet than the control group fish. The higher serum globulin level during the pre-challenge period was reported in *L. rohita* (Kumar, Sahu, Pal, Choudhury, Yengkokpam & Mukherjee 2005; Misra *et al.* 2006) since, globulins are vital for maintaining a healthy immune system because of the presence of immune-globulin in the blood. Lower albumin/globulin ratio indicates the presence of more amounts of globulin which was found in TF_3 group, indicating more globulin fraction may be induced by FRSE, which can be correlated with other immunological indices like increased phagocytic activity and leucocytes count in this experiment. After challenge with *A. hydrophila* the serum protein and globulin level was decreased than their pre-challenged counterpart but the albumin fraction was comparatively higher than the pre-challenged condition. After challenge with pathogenic bacteria, reduction in serum protein level might be due to leakage of serum protein through the walls of vascular tissues because of its highly enhanced permeability (Green 1978; Ellis, Hastings & Munro 1981) even though the destruction of RBCs and the resultant release of cell contents into the blood stream (Haney *et al.* 1992) and also together with improper synthesis of serum protein and its non-specific proteolysis. (Ellis *et al.* 1981).

The determination of blood glucose level is considered as an efficient method to evaluate the stress caused by variety of stressors including physical factors (Manush, Pal, Das & Mukherjee 2005) and pollutants (Svobodova 1971). Umpteen

numbers of literatures are available on the level of glucose associated with different stressors including ablation of claws in prawns (Manush *et al.* 2005), harsh handling (Wedemeyer 1972; Carey & McCormick 1998) adverse effect of transportation (Barton & Schreck 1987) and increased packing density (Chatterjee, Pal, Das, Manush, Sarma, Venkateshwarlu & Mukherjee 2006). All types of stress elevated the secretion of catecholamines which in turn increases the breakdown of glycogen and enhanced the level of blood glucose (Nakano & Tomlinson 1967). The elevated glucose level in the infected or stressed animals is known to ward off the infection or stress (Citarasu, Sivaram, Immanuel, Rout & Murugan 2006). In the present study, an inverse relationship between glucose level and increasing level of FRSE in the diet was observed. As the level of FRSE increased in the diet, the level of glucose decreased. This might be due to the capability of FRSE to reduce the effects of stressors. The highest blood glucose level was found in the control group than the FRSE diet fed groups. Though blood glucose content increased in all the treatments during post challenge period but FRSE diet fed groups exhibited the less glucose level than the control group. Lower blood glucose content in the treatment groups compare with the control after challenged with *A. hydrophila*, appears to be FRSE effects in reducing the stress of *P. hypophthalmus* fingerlings.

The ability of different substances to increase macrophage activity is known in fish but far less is known about the potential interaction of such signals. These interactions can be possible in fish as seen in mammals, in the case of interferon-γ (IFN-γ) and LPS which are required to induce macrophage activation. In the present study, the expression level of IFN-γ2a gene before the challenge with bacteria was increased with increasing level of FRSE in the diet. A notable increase was observed in the expression level of IFN-γ2a gene after *A. hydrophila* infection than the pre-challenged condition but there were not much difference among the different experimental groups. This is in agreement with, the head kidney tissue exhibited better expression profiles of the IFN-γ gene in healthy and viral haemorrhagic septicaemia virus (VHSV) challenged Japanese flounder fish which result in 24 h duration shows maximum expression in VHSV challenged kidney tissue (Matsuyama, Fujiwara, Sakai & Nakayasu 2008). But there is no work with reference to bacterial infection. Further study is necessary for better understanding of expression of IFN-γ2a gene with relates to bacterial infection.

In this study, the highest relative percentage of survival was recorded in TF_3 and TF_2 group followed by TF_1 and TS_6 group which indicates that supplementation of FRSE had a positive influence on the survival of *P. hypophthalmus* fingerlings by resisting the *A. hydrophila* infection. It has been previously reported by many authors that fucoidan benefit many elements in the immune system. In the present study, it has been found that after challenge with *A. hydrophila,* serum lysozyme activity, phagocytic activity and expression of interferon-γ gene of different treatment groups were higher than the pre-challenge treatment groups (Chotigeat *et al.* 2004; Huang *et al.* 2006; Matsuyama *et al.* 2008).

Conclusion

The present study reveals that the administration of fucoidan rich seaweed extract enhanced the non-specific immune parameters and disease resistance against *A. hydrophila* in *P. hypophthalmus*. Hence, it can be concluded that, the sulphur esters exists in the FRSE may enhance the production of macrophages and cytokines through non-specific defence mechanism along with the interlinked antibodies production through the specific defence mechanism for the response of *A. hydrophila* infection to protect the host against red disease. Although the inclusion of seaweed powder exhibit moderately good immunostimulatory activity due to the presence of considerable amount of alginic acid (a potential immunostimulant) along with the fucoidan content but the inclusion of high quantity of seaweed powder affects the colour and texture of the commercial feed due to the pigments and colloidal property of seaweed respectively. Hence, FRSE can be effectively and extensively used as an immunostimulants in fish feed.

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