

# Effect of dietary *Sargassum wightii* and its fucoidan-rich extract on growth, immunity, disease resistance and antimicrobial peptide gene expression in *Labeo rohita*

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**Abstract** This work aimed to appraise growth performance, immune responsiveness, bacterial disease resistance and mRNA expression of two antimicrobial peptides in *Labeo rohita* fingerlings fed diets supplemented with seaweed *Sargassum wightii* and its fucoidan-rich extract. Two hundred and twenty-five fingerlings were divided into five experimental groups in triplicates, each replicate having 15 fish in a 90-L tank. Different groups were fed with isonitrogenous (35% crude protein) and isoenergetic (1408.16 kJ/100 g) diets containing either 0% fucoidan-rich seaweed extract (FRSE), 1% FRSE, 2% FRSE, 3% seaweed powder and 6% seaweed powder for 60 days. Partial nucleotide sequences of the antimicrobial peptides, hepcidin and defensin, were characterized in *Labeo rohita* fingerlings, and gene expression in the different tissues of experimental fish was studied. The fish-fed FRSE-supplemented diets showed significant increase ( $P < 0.05$ ) in the mRNA expression of antimicrobial peptides in liver, skin and intestine tissues. Bacterial challenge study using *Aeromonas hydrophila* showed higher survival in the FRSE-fed groups. Fish-fed FRSE-supplemented diets also showed better non-specific immune response (NBT reduction, serum lysozyme activity, serum albumin: globulin ratio and phagocytic activity) in pre-challenge and post-challenge periods. No significant difference was observed in the growth performance in the different experimental groups. The study indicates that although dietary FRSE is a potential immunostimulant in *Labeo rohita* fingerlings, it does not improve growth performance.

**Keywords** Antimicrobial peptides · Nutraceutical · Immunomodulation · Fish

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## Introduction

Disease outbreak is one of the main limiting factors for sustainable aquaculture production. Tropical countries suffer proportionally greater losses during disease outbreaks (Robar et al. 2010). Motile aeromonas septicaemia, caused by *Aeromonas hydrophila* (Plumb 1999), is one of the most deadly bacterial infections encountered in aquaculture practices. The disease is highly contagious among the fish and results in high mortalities within few days (Roberts 1993) with ulceration, exophthalmia and abdominal distension as symptoms (Camus et al. 1998). There is no commercially available vaccine that is effective against all strains of *A. hydrophila* (Poobalane et al. 2008). Hence, various chemotherapeutants may be used for prevention of this disease in aquaculture. However, their use does not come without the risk of antibiotic resistance in pathogenic bacteria (Huys et al. 2000), tissue antibiotic residues (Angulo et al. 2004), aquatic environment pollution (Hektoen et al. 1995) and transfer of resistance to human pathogenic bacteria (Sørum 2006). Therefore, novel strategies to control bacterial infections are needed (Defoirdt et al. 2011). These alternative measures involve the use of natural bioactive compounds that stimulate the immune system of fish. Antimicrobial peptides (AMPs) are components of the innate immune system of fish that provide protection against bacteria, fungi and virus (Cuesta et al. 2011; Zasloff 2002). Most antimicrobial peptides prevent the colonization of pathogens by lytic or ionophoric mechanism (Smith et al. 2010). Two important and well-known classes of antimicrobial peptides are defensins and hepcidins. Defensins and hepcidins characterized so far in fishes have shown strong antimicrobial activity against pathogens (Falco et al. 2008; Xu and Faisal 2010; Álvarez et al. 2014; Xu et al. 2014). Furthermore, these antimicrobial peptides exhibit increased expression at the transcriptomic level in fishes fed with dietary immunostimulants (Marel et al. 2012; Gong et al. 2014).

An immunostimulant is a chemical, drug, or stressor that enhances the immune response by interacting directly with cells of the system activating them. Non-specific immunostimulants act irrespective of antigenic specificity to augment immune response and stimulate components of the immune system. Seaweeds are cheap and easily available resource of bioactive compounds that can be used as immunostimulants in aquaculture (Deivasigamani and Subramanian 2016). *Sargassum*, a genus of brown seaweed (*Phaeophyceae*), contains a conglomerate of bioactive compounds such as steroids, alkaloids, phenolic compounds, saponins and flavonoids rendering it a number of different bioactive properties (Arunkumar et al. 2005; Zubia and Deslandes 2008). *Sargassum wightii* also contains fucoidan, a water-soluble, sulphated fibrous polysaccharide. Fucoidan is bestowed with many biological properties that include immunostimulation (Kitikiew et al. 2013; Prabu et al. 2016) disease resistance (Chotigeat et al. 2004; Immanuel et al. 2012), growth stimulation in shrimps (Traifalgar et al. 2010) and finfish (Tuller et al. 2014; Prabu et al. 2016). The effect of dietary *Sargassum wightii* and its fucoidan-rich extract on the immuno-physiological responses and growth performance of Indian major carps has not been studied in detail. In this context, the present work was designed with the objective to study the dietary effects of *Sargassum wightii* and its fucoidan-rich seaweed extract (FRSE) on the non-specific immune status, gene expression level of  $\beta$ -defensin 1 and hepcidin and survival of *L. rohita* fingerlings challenged with *A. hydrophila*.

## Materials and methods

### Preparation of fucoidan-rich seaweed extract

The brown seaweed, *Sargassum wightii*, required for the experiment was collected from Mandapam, Tamil Nadu (India) with the help of fishermen. Seaweeds were subsequently sorted out, washed thoroughly with water, shade dried and finally ground into fine powder. This powder was used for feed preparation and extraction of crude fucoidan. The crude FRSE was obtained from seaweed powder by the alcohol-hot water method of Prabu et al. (2016). To quantify the fucoidan content in crude FRSE, it was dialyzed in a cellulose tubing having molecular weight cut off of 5000. The L-Fucose content in the dialyzed sample was calculated according to Dubois et al. (1956) and from this the fucoidan content was obtained by multiplying the fucose values with 1.75.



## Experimental fish and diets

Three hundred five fingerlings of *L. rohita* with an average weight of  $8.852 \pm 0.2$  g were procured from R.A. Farm, Mumbai, India. Fish were acclimatized for 15 days in laboratory condition in 1000 L tanks at 30 °C, under continuous aeration before starting the experiment. From this stock, eighty fingerlings were used for determination LD<sub>50</sub> prior to bacterial challenge study. Two hundred and twenty-five fingerlings were used for the feeding experiment and bacterial challenge study. Semipurified ingredients were used for feed formulation (Table 1). Five experimental diets (crude protein and lipid levels at 35 and 8%, respectively) were fed to different experimental groups. Continuous aeration was provided to all the tubs throughout the experimental period. The fish were fed to satiation daily at 10.00 h in the morning and 18.00 h in the evening under normal light regime.

## Experimental design and in vivo study

Two hundred and twenty-five fingerlings were randomly transferred to plastic tanks of 100 L capacity with 15 fingerlings per tank in triplicates following a completely randomized design (CRD). The first group was maintained as control in which fish were given no dietary FRSE or seaweed. In second and third groups, fish were given 1 and 2% dietary FRSE, respectively. In the fourth and fifth groups, the fish were provided finely ground seaweed powder at the levels of 3 and 6% of the diet, respectively. After 60 days of feeding trial, five fish from each replicate was selected for blood collection (pre-challenge) by caudal vein puncture using a 1-ml 26G syringe. For analysis, fresh blood was used. For obtaining the serum, part of the blood was allowed to stand for 4 h and then centrifuged at  $1006 \times g$  for 5 min. The straw color serum was pipetted out as supernatant and stored at  $-80$  °C. These fish were killed and liver, gill, spleen, kidney, intestine and skin tissues were

**Table 1** Composition of experimental diets (g%)

Ingredients	Control (C)	1% fucoidan (T1F)	2% fucoidan (T2F)	3% dry seaweed (T3S)	6% dry seaweed (T4S)
Casein	30	30	30	30	30
Gelatin	9.5	9.5	9.5	9.5	9.5
Starch	17	17	17	17	17
Dextrin	20	20	20	20	20
Cellulose	11	10	9	8	5
Cod liver oil	4	4	4	4	4
Sunflower oil	4	4	4	4	4
Vit-min premix	2	2	2	2	2
Carboxymethyl cellulose	2	2	2	2	2
Choline chloride	0.4	0.4	0.4	0.4	0.4
Butylated hydroxy toluene	0.1	0.1	0.1	0.1	0.1
FRSE/seaweed powder	0	1	2	3	6
Total	100	100	100	100	100
Proximate analysis (g%)					
Moisture	7.6 ± 0.30	7.2 ± 0.19	8.0 ± 0.10	7.7 ± 0.10	7.9 ± 0.15
Crude protein	35.7 ± 0.12	34.5 ± 0.03	34.9 ± 0.08	35.0 ± 0.1	35.6 ± 0.15
Crude lipid	7.9 ± 0.03	8.1 ± 0.12	7.8 ± 0.27	7.7 ± 0.02	8.0 ± 0.19
Crude fiber	13.1 ± 0.2	12.8 ± 0.56	13.5 ± 0.34	12.6 ± 1.8	13.6 ± 2.1
Ash	9.3 ± 0.7	9.2 ± 0.2	9.6 ± 0.34	9.8 ± 0.23	10.3 ± 1.2

Composition of vitamin mineral mix (PREMIX 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 µg; 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



taken for mRNA expression studies. All the sampled tissues were kept in RNA later and stored in  $-80\text{ }^{\circ}\text{C}$  for RNA extraction.

#### Median lethal dose (LD<sub>50</sub>)

For determination of (LD<sub>50</sub>) value, 80 fishes from the same stock as the experimental fish were selected which were being reared in similar physicochemical conditions and were fed the experimental control diet (Table 1). A rough idea of the doses to be injected was obtained from the study of Sahu et al. (2011). The bacterial culture was obtained as glycerol and reactivated by immersing in Müller–Hinton Broth and incubation for 24 h at 30 °C. The bacteria were plated on Müller–Hinton Agar by the spread-plate technique to obtain single colony. A single colony was taken and again regrown in Müller–Hinton Broth for 24 h at 30 °C. The culture broth was centrifuged at  $1006\times g$  for 10 min. The supernatant was discarded and the pellets were resuspended in sterile phosphate buffer saline (PBS, pH 7.4) and the OD of the solution was adjusted to 0.50–0.48 nm. This corresponded to a count of  $1.79 \times 10^7$ , confirmed by total plate count. Then the bacterial cells were diluted to get different concentrations required for the experiment. Eighty fish were distributed randomly into eight groups. The fish of seven groups were injected intraperitoneally with 0.1 mL/g of American Type Culture Collection (ATCC) 7966 *Aeromonas hydrophila* bacterial cell suspension at concentrations of  $1.1 \times 10^1$ ,  $1.1 \times 10^2$ ,  $1.1 \times 10^3$ ,  $1.1 \times 10^4$ ,  $1.1 \times 10^5$ ,  $1.1 \times 10^6$  and  $1.1 \times 10^7$  cfu/mL, respectively. The control group was injected 0.1 mL/g of PBS. The fish were maintained for another 96 h and were observed regularly for any overt signs of disease including behavioral abnormalities and mortality. In case of mortality, the causative agent was confirmed by re-isolating *A. hydrophila* from the moribund fish. LD<sub>50</sub> value was calculated as per the trimmed Spearman–Karber method (Hamilton et al. 1977).

#### Bacterial challenge study

Following the feeding trial, 30 fish from each treatment group (10 from each replicate) were anesthetized using 2-phenoxy ethanol and subjected to intraperitoneal injection 0.1 mL/g of PBS containing *A. hydrophila* (ATCC 7966) having  $1.79 \times 10^4$  cfu/mL. The fish were maintained for another 10 days and were observed regularly for any overt signs of disease including behavioral abnormalities and mortality. In case of mortality, the causative agent was confirmed by re-isolating *A. hydrophila* from the moribund fish. Blood was collected after challenge of *A. hydrophila* (post-challenge) for immunological assays.

#### Growth study

On the 1st and 60th day of feeding trial, weight measurement was taken using an electronic balance (Citizen, India). The growth performance of fish was evaluated in terms of percentage weight gain (%), feed conversion ratio (FCR), feed efficiency ratio (FER), specific growth rate (SGR), and protein efficiency ratio (PER) using the following formulae:

$$\begin{aligned}\text{Weight gain (\%)} &= [(\text{FW} - \text{IW}) \times 100]/\text{IW}, \\ \text{FCR} &= \text{Feed given (DW)}/\text{body weight gain (WW)}, \\ \text{SGR (\%)} &= \{[\ln(\text{FW}) - \ln(\text{IW})]/N\} \times 100, \\ \text{PER} &= \text{body weight gain (WW)}/\text{crude protein fed (DW)},\end{aligned}$$

(where FW is the final weight, IW is the initial weight, DW is the dry weight, WW is the wet weight gain, ln is the natural log, and *N* is the number of cultural days).

#### RNA extraction and preparation of cDNA

Total RNA from the tissues was extracted using Trizol™ reagent (Invitrogen Waltham, USA). DNaseI treatment was given to extract RNA using DNaseI, RNase-free kit (Thermo scientific, Waltham, MA, USA) to remove any co-purified genomic DNA. The DNase I treated RNA isolated from different tissues of *L. rohita*





1 h to facilitate adhesion of cells. Then the supernatant was removed and the loaded wells were washed thrice in phosphate buffer saline (PBS). After washing, 50  $\mu\text{L}$  of 0.2% NBT was added and was incubated for 1 h. The cell was then fixed with 100% methanol for 2–3 min and was again washed thrice with 30% methanol. The plates were then air dried. Sixty microlitres of 2 N potassium hydroxide and 70  $\mu\text{L}$  dimethyl sulphoxide were added into each well to dissolve the formazan blue precipitate formed. The OD of the turquoise blue colored solution was then read in ELISA reader (BioTek Power Wave 340, India) at 620 nm. Serum protein was estimated by biuret and BCG dye binding method (Reinhold 1953) using total protein kit (Merck, India). Albumin was estimated by bromocresol green binding method (Doumas et al. 1971) using albumin kit (Merck, India). Globulin was calculated by subtracting albumin values from total serum protein values. A:G ratio was calculated by dividing albumin values by globulin values. Phagocytic activity was determined following the method of Anderson and Siwicki (1995). A sample of 0.1 mL of serum was taken in wells of microtiter plate and 0.1 mL of *Staphylococcus aureus* ( $1 \times 10^7$  cells) suspended in phosphate buffer saline (pH 7.2), was added and shaken sufficiently. This bacterial serum mixture was incubated at room temperature for 20 min. From this mixture, 5  $\mu\text{L}$  was taken on a clean glass slide and a smear was prepared. The smear was air dried and fixed with 95% of ethanol for 5 min and air dried once again. The smear was stained with Giemsa stain for 10 min. A total of two hundred haemocytes from each smear were observed under the light microscope and the number of phagocytizing cells and bacteria engulfed by the phagocytes were counted. Phagocytic activity was expressed using the formula:

$$\text{Phagocytic activity} = (\text{Number of phagocytizing cells} / \text{Total number of phagocyte cells counted}) \times 100.$$

#### Gene expression study using qPCR

Real-time PCR was conducted to determine the mRNA expression levels of  $\beta$ -defensin 1 and hepcidin. For gene expression studies, tissue samples from three fish from each replicate were selected and pooled. Investigation of gene expression was done using qRT-PCR gene-specific primers with  $\beta$ -actin gene used as an internal control. Real-time PCR amplifications were carried out in LightCycler<sup>®</sup> Real-time PCR detection system (Roche, USA). The 25  $\mu\text{L}$  reaction mix volume was prepared containing 12.5  $\mu\text{L}$  of Maxima<sup>™</sup> SYBR Green qPCR master mix (Thermo scientific, USA), 0.5  $\mu\text{L}$  of (0.3 pM) each gene-specific primer and 2  $\mu\text{L}$  (20 ng) of cDNA and 9.5  $\mu\text{L}$  of nuclease-free water. The default thermal profile was used for PCR amplification and it consisted of initial denaturation at 95  $^{\circ}\text{C}$  for 10 min, followed by 40 cycles of denaturation at 95  $^{\circ}\text{C}$  for 15 s and annealing and extension at 60  $^{\circ}\text{C}$  for 1 min. Cycle threshold ( $C_t$ ) values were obtained from the exponential phase of PCR amplification, and the expressions of genes were normalized to the expression of  $\beta$ -actin generating a  $\Delta C_t$  value [ $C_t$  of target gene) – ( $C_t$  of  $\beta$ -actin)]. The relative expression level of the target gene in the treatment group to target gene in the control group was determined using  $2^{-\Delta\Delta C_t}$  method proposed by Livak and Schmittgen (2001), which gives the value that corresponds to the  $n$ -fold difference relative to the control.

#### Calculation of relative percentage survival

Relative percentage of survival (RPS) versus control was calculated at the end of 14 days of infection using the following formula (Amend 1981):

$$\text{RPS} = 1 - (S_T/S_C) \times 100,$$

where  $S_T$  is the percentage of surviving fish in treatment group and  $S_C$  is the percentage of surviving fish in control group.

#### Statistical analysis

The results of immunological parameters, growth performance and gene expression studies obtained from treatments were tested for statistical significance using one-way analysis of variance (ANOVA) followed by



Duncan's new multiple range tests using the statistical package, SPSS 16.0.  $P < 0.05$  was considered as statistically significant.

## Results

### Quantification of fucoidan

The L-fucose content of the dialyzed extract was 32 mg/g dried seaweed powder. This was multiplied by a factor of 1.75 to get the total fucoidan content of 5.6 g/100 g dried seaweed powder.

### Growth parameters

There was no significant difference in the percentage weight gain, specific growth rate, protein efficiency ratio and feed conversion ratio among the different treatment groups (Table 3).

### Non-specific immune parameters

In the pre-challenge period, the 2% FRSE (T2F) group revealed a significantly higher serum lysozyme activity (Fig. 1) compared to other experimental groups, which showed no significant difference among them. The same trend was followed in the post-challenge period. In the pre-challenge study, the control group exhibited the least NBT activity, which was statistically similar to the groups T3S and T4S (Fig. 2). The highest and significantly different activity was found in the T2F group. In the post-challenge study, the highest was found in the T2F group. All other groups showed no significant difference in the NBT activity. In both pre- and post-challenge periods the lowest A:G ratio was observed in the T2F group, whereas the highest A:G ratio was observed in the control group (Table 4). In the pre-challenge period, the highest phagocytic activity was found in the T2F group, whereas the least was found in the control group. The same trend was also followed in the post-challenge period (Fig. 3).

### Sequence analysis partial $\beta$ -defensin 1 and hepcidin genes

The sequence for  $\beta$ -defensin 1 received was 148 bp long and the sequence of hepcidin was 269 bp long. The nBLAST search in NCBI GenBank database with *L. rohita* partial  $\beta$ -defensin-1 showed sequence similarity of 100% with *Cyprinus carpio* beta defensin-like protein (JF343439.1) and *Megalobrama amblycephala*  $\beta$ -defensin 1 mRNA (KC351182.1) followed by 98% similarity with *Danio rerio* defensin, beta-like 1 (NM\_001081553.1). *L. rohita* partial hepcidin mRNA sequence showed sequence similarity of 98% with *Cyprinus carpio* hepcidin (JX855261.1) and *Schizothorax richardsonii* hepcidin-like antimicrobial peptide mRNA (KC894741.1) followed by 96% similarity with *Puntius sarana* mRNA for hepcidin (FN429020.1). *L. rohita* partial hepcidin and defensin gene sequence was submitted to NCBI GenBank vide accession number KM 040782 and LC 259205, respectively.

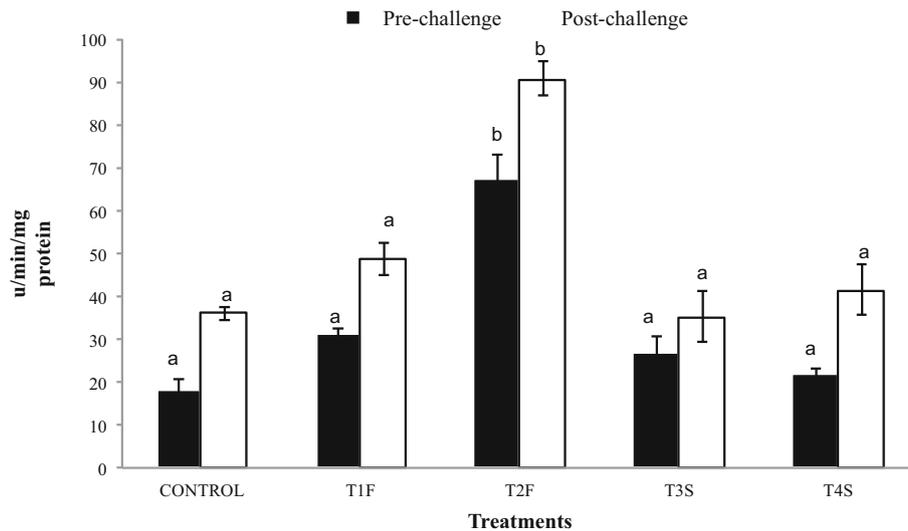
**Table 3** Growth parameters of different experimental groups fed different experimental diets at the end of the experiment

Treatments	Wt. gain %	SGR	FCR	PER
Control	43.68 $\pm$ 1.37	0.26 $\pm$ 0.01	2.75 $\pm$ 0.08	0.96 $\pm$ 0.02
T1F	46.39 $\pm$ 0.68	0.27 $\pm$ 0.03	2.58 $\pm$ 0.04	0.96 $\pm$ 0.01
T2F	41.89 $\pm$ 2.04	0.25 $\pm$ 0.01	2.87 $\pm$ 0.14	1.00 $\pm$ 0.05
T3S	45.10 $\pm$ 1.02	0.26 $\pm$ 0.001	2.66 $\pm$ 0.06	0.93 $\pm$ 0.02
T4S	45.15 $\pm$ 2.00	0.26 $\pm$ 0.01	2.66 $\pm$ 0.12	0.93 $\pm$ 0.04

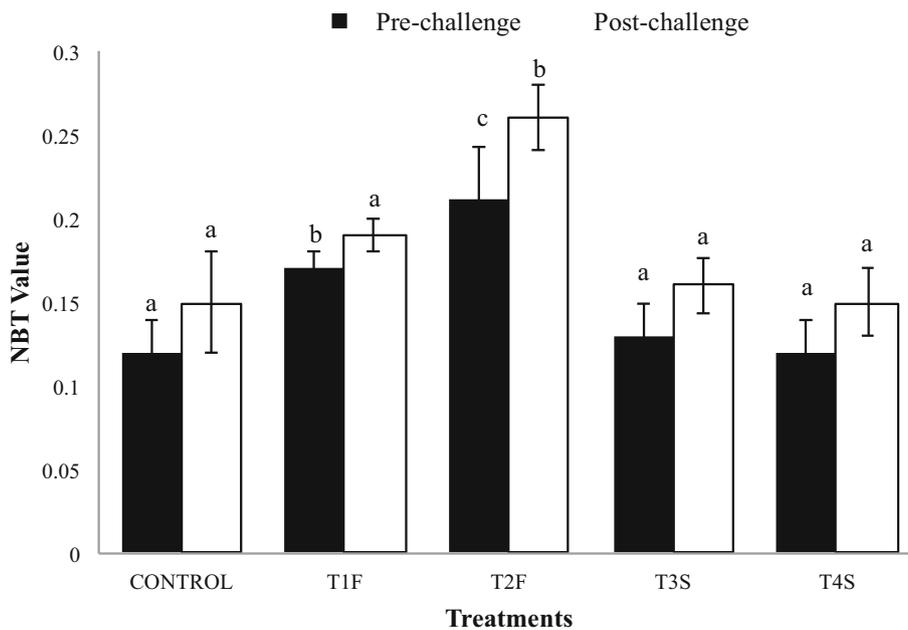
Mean values ( $n = 3$ ) in the same column with no superscript do not differ significantly ( $P < 0.05$ ). Data were expressed as mean  $\pm$  SE

Wt. gain % weight gain percentage, SGR specific growth rate, FCR feed conversion ratio, PER protein efficiency ratio





**Fig. 1** Pre- and post-challenge serum lysozyme activities of different experimental groups. Mean values ( $n = 3$ ) in the experimental group with different superscripts, (a, b) differ significantly ( $P < 0.05$ ). Data were expressed as mean  $\pm$  SE. Control—0% FRSE, T1F—1% FRSE, T2F—2% FRSE, T3S—3% seaweed powder, T4S—6% seaweed powder



**Fig. 2** Pre- and post-challenge NBT values of different experimental groups. Mean values ( $n = 3$ ) in the experimental group with different superscripts (a, b, c) differ significantly ( $P < 0.05$ ). Data were expressed as mean  $\pm$  SE. Control—0% FRSE; T1F—1% FRSE; T2F—2% FRSE; T3S—3% seaweed powder; T4S—6% seaweed powder

#### Tissue distribution and real-time PCR analysis of the expression levels of $\beta$ -defensin 1 and hepcidin

There was a variation in the tissue level expression of  $\beta$ -defensin 1 and hepcidin in *Labeo rohita*. Defensin mRNA expression was observed in gill, skin, liver and intestine whereas hepcidin was expressed in liver, kidney, spleen and intestine (Fig. 4). On the 60th day of feeding of experimental diets, the skin tissue of T2F group showed a 7.9-fold increase ( $P > 0.05$ ) in  $\beta$ -defensin 1 expression compared to the control group (Fig. 5). The expression in the liver was increased 2.1-fold in the T2F group as compared to the control group. A slight but significant ( $P > 0.05$ ) increase of 1.8-fold was found in the  $\beta$ -defensin 1 expression of the

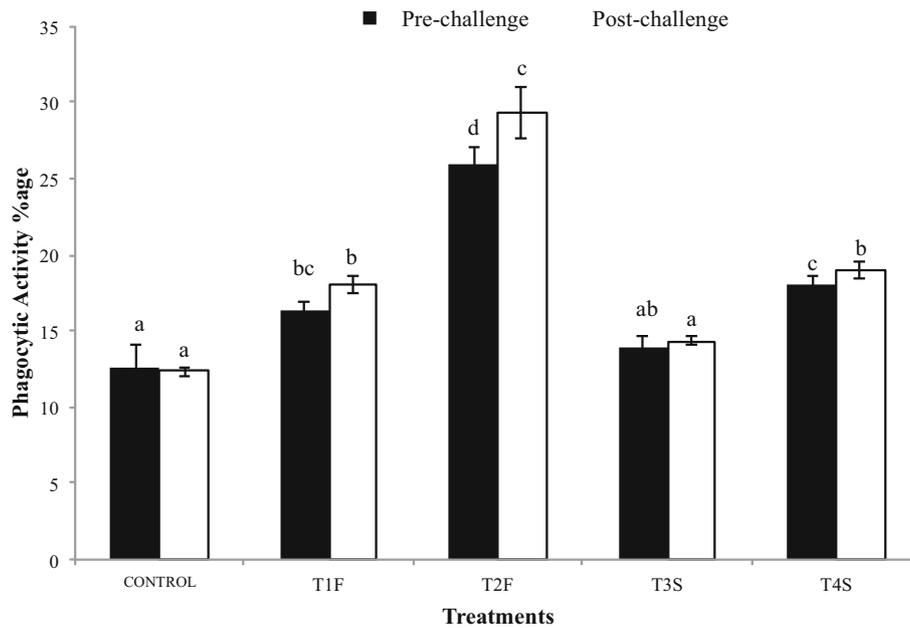
**Table 4** Serum total protein, albumin, globulin and A:G ratio of different experimental groups

	Pre-challenge			Post-challenge				
	Total protein	Albumin	Globulin	A:G Ratio	Total protein	Albumin	Globulin	A:G
Control	1.82 <sup>a</sup> ± 0.01	1.21 <sup>b</sup> ± 0.01	0.61 <sup>a</sup> ± 0.01	1.97 <sup>d</sup> ± 0.03	1.63 <sup>a</sup> ± 0.03	1.23 <sup>a</sup> ± 0.01	0.40 <sup>a</sup> ± 0.020	3.03 <sup>a</sup> ± 0.12
T1F	2.38 <sup>c</sup> ± 0.02	1.16 <sup>ab</sup> ± 0.01	1.22 <sup>c</sup> ± 0.03	0.95 <sup>b</sup> ± 0.020	2.06 <sup>b</sup> ± 0.71	1.18 <sup>ab</sup> ± 0.01	0.88 <sup>ab</sup> ± 0.71	1.33 <sup>b</sup> ± 0.34
T2F	2.58 <sup>d</sup> ± 0.04	0.99 <sup>a</sup> ± 0.01	1.58 <sup>d</sup> ± 0.03	0.62 <sup>a</sup> ± 0.013	2.38 <sup>ab</sup> ± 0.04	1.13 <sup>ab</sup> ± 0.03	1.24 <sup>ab</sup> ± 0.04	0.91 <sup>b</sup> ± 0.051
T3S	1.66 <sup>a</sup> ± 0.08	0.99 <sup>a</sup> ± 0.06	0.67 <sup>a</sup> ± 0.02	1.46 <sup>c</sup> ± 0.05	1.36 <sup>a</sup> ± 0.10	0.99 <sup>b</sup> ± 0.07	0.36 <sup>a</sup> ± 0.06	2.92 <sup>a</sup> ± 0.57
T4S	2.13 <sup>b</sup> ± 0.08	1.12 <sup>ab</sup> ± 0.01	1.00 <sup>b</sup> ± 0.05	1.13 <sup>b</sup> ± 0.15	1.8 <sup>ab</sup> ± 0.11	1.13 <sup>ab</sup> ± 0.12	0.66 <sup>ab</sup> ± 0.08	1.79 <sup>b</sup> ± 0.40

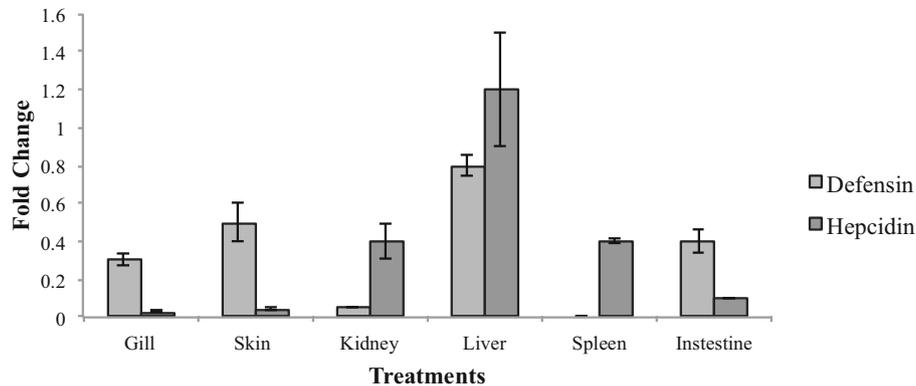
Data were expressed as mean ± SE

Mean values ( $n = 3$ ) in the same column with different superscript (a, b, c, d, ab) differ significantly ( $P < 0.05$ )





**Fig. 3** Pre- and post-challenge phagocytic activity of different experimental groups. Mean values with different superscript (a, b, c, d, ab) differ significantly ( $P < 0.05$ ). Data were expressed as mean  $\pm$  SE,  $n = 3$

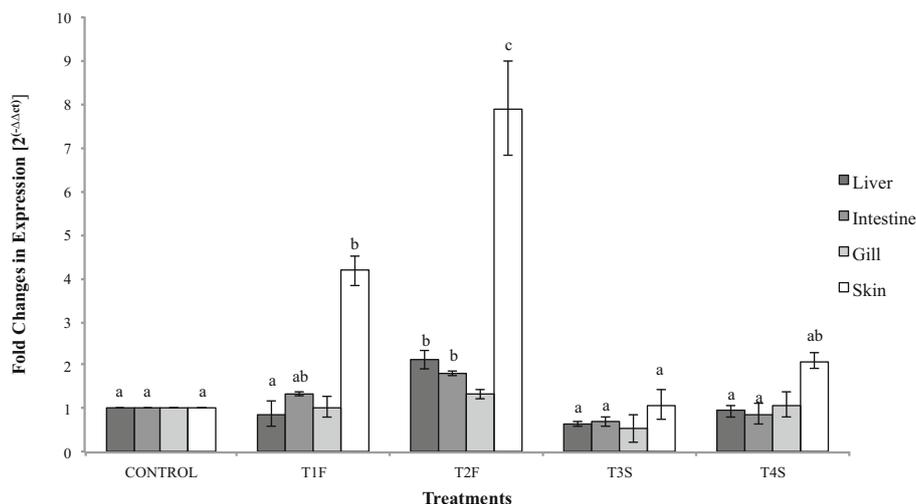


**Fig. 4** Distribution of mRNA expression of antimicrobial peptides in different tissues of juvenile *Labeo rohita*. The relative expression of defensin and hepcidin normalized with beta-actin in samples was calculated using comparative  $C_t$  method. The fold change was compared with beta-actin of muscle tissue in which the copy number was considered as onefold. Data for real-time PCR were expressed as mean  $\pm$  SE ( $n = 3$ )

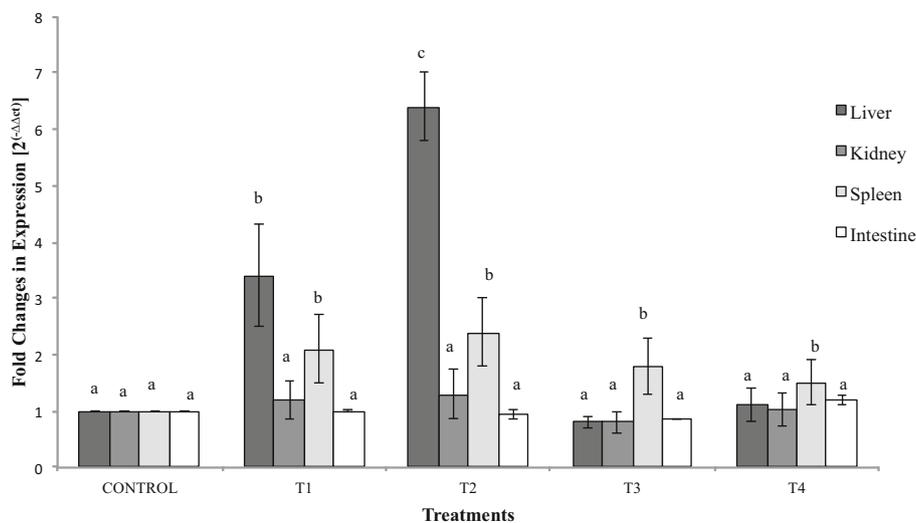
intestine, whereas no significant increase was found in the gill. The crude fucoidan extract fed at dietary level of 1 and 2% showed a 3.4- and 6.4-fold higher expression of hepcidin in the liver (Fig. 6). Compared to the control group, the expression of hepcidin in the spleen was slightly, but significantly, higher in all the other groups.

#### LD<sub>50</sub> value and challenge study

The LD<sub>50</sub> value obtained after 5 days of observation period for the viable cells of *A. hydrophila* (ATCC 7986) injected to *Labeo rohita* fingerlings was found to be  $1.79 \times 10^4$  cfu/mL. After injecting 0.1 mL/g of the same concentration bacteria in the experimental fish, the first mortality was observed at 11 h. At the end of the 14 days challenge period, the highest relative percentage of survival was found in the T2F group, fed 2%



**Fig. 5** Relative expression of *Labeo rohita* defensin mRNA in the liver, intestine, gill and skin after 60 days of experimental feeding regimen. Beta-actin was used as an internal control to calibrate the cDNA template for all samples. The data are presented as a fold increase of normalized mRNA copies in tissues of fish fed with different experimental diets to normalized mRNA copies in the tissues of fish fed with control diet. Data were expressed as mean  $\pm$  SE,  $n = 3$ . Mean values of the same tissue with different superscripts (a, b, ab and c) differ significantly ( $P < 0.05$ ). Mean values of the same tissue with no superscript do not differ significantly ( $P < 0.05$ )



**Fig. 6** Relative expression of *Labeo rohita* hepcidin mRNA in the different tissues after 60 days of experimental feeding regimen. Beta-actin was used as an internal control to calibrate the cDNA template for all samples. The data are presented as a fold increase of normalized mRNA copies in liver of fish fed with different experimental diets to normalized mRNA copies in the liver of fish fed with control diet. Data are expressed as mean  $\pm$  SE,  $n = 3$ . Mean values with different superscripts (a, b and c) differ significantly ( $P < 0.05$ ). Mean values of the same tissue with no superscript do not differ significantly

dietary fucoidan (Table 5). The groups fed 3 and 6% seaweed powder, T3S and T4S, exhibited the lowest relative percentage survival.

## Discussion

The culture conditions, which deviate from the natural habitat, induce stress in fish (Barton and Iwama 1991). Stress increases susceptibility to diseases, possibly due to chronic elevated cortisol levels, causing

**Table 5** Percentage mortality and relative percentage of survival of the different treatment groups after challenge with *Aeromonas hydrophila* at the end of the experimental trial of 60 days

Treatments	Percentage mortality	Relative percentage of survival
Control	55.55 <sup>a</sup> ± 6.4	00.00 ± 00.00
T1F	40.74 <sup>b</sup> ± 3.7	26.66 <sup>b</sup> ± 6.66
T2F	29.63 <sup>c</sup> ± 3.7	46.66 <sup>c</sup> ± 6.66
T3S	50.00 <sup>a</sup> ± 3.2	10 <sup>a</sup> ± 5.77
T4S	53.70 <sup>a</sup> ± 1.8	3.33 <sup>a</sup> ± 3.33

Data of percentage mortality expressed as mean ± SE ( $n = 3$ ). Relative percentage of survival compared against mean value of percentage mortality of control group (55.55%) which is assumed to be 100%. Mean values in the same column with different superscripts (a, b, c) differ significantly ( $P < 0.05$ )

immunosuppressive and catabolic actions (Refstie 1977). Although the use of prophylactic or metaphylactic antibiotics is a logical approach to avoid infections, it suffers from certain inherent drawbacks (Hektoen et al. 1995). Immune system of fish can be stimulated through the diet either by providing certain dietary nutrients in excess than the requirements (Pohlenz et al. 2012) or by administration of certain immunostimulant non-nutritive ingredients (Rieder and Samuelsen 2012). However, cost is the major deciding factor for including those ingredients in the feed. In the present study, emphasis was laid on the feasibility and cost effectiveness of the extraction process of fucoidan and the yield was calculated to be 5.6% in the crude extract. The yield of fucoidan varies widely depending upon the method of extraction employed and also the species of seaweeds (Ale et al. 2012). The present inexpensive method of alcohol water extraction does not involve the use of acid and can be employed for large-scale preparation of fucoidan-rich seaweed extract.

The effect of fucoidan-rich seaweed extract on growth performance of carps has not been studied yet. Fucoidan has shown significant increase in growth performance in aquatic animals (Traifalgar et al. 2010; Immanuel et al. 2012; Tuller et al. 2014; Prabu et al. 2016). We found no significant difference in growth performance of the experimental groups. There are number of reports which suggested that fucoidan is a potential inhibitor of cellular division (Religa et al. 2000; Yoshimoto et al. 2015) and cellular growth in various organisms (Aisa et al. 2004; Haneji et al. 2005; Maruyama et al. 2006; Alekseyenko et al. 2007). These studies indicate that fucoidan may not induce growth performance and hence support our findings. The growth promoting effect of fucoidan in some organisms may be an indirect effect through stress mitigation. However, it remains to be confirmed and needs further research.

The non-specific immune system of fish can respond to external factors (Tort et al. 2003) including dietary compounds (Magnadottir 2010). Respiratory burst (NBT reduction) is an important component of the non-specific immune system involved in the early eradication of pathogen (Bellavite 1988). Respiratory burst activity in fish may be modulated through production of reactive oxygen species in response to immunostimulants (Liu et al. 2014). Fucoidan-fed fish groups exhibited higher NBT reduction activity of the phagocytes compared to the control and the seaweed fed groups. This indicates that fucoidan boosts the immune system and health status of fish (Yang et al. 2014; Prabu et al. 2016). Phagocytic activity represents an immediate response carried out by the phagocytes to kill the pathogenic bacteria as a part of their defense mechanism (Neumann et al. 2001). It is a key element in host defenses against bacterial infections (Kantari et al. 2008). We found a dose-dependent increase in the phagocytic activity of the macrophages with the highest phagocytic activity observed in the 2% FRSE treatment, which has also been observed previously by El-Boshy et al. (2014). Lysozyme has a bactericidal effect by destroying cellular walls of bacteria. Lysozyme also stimulates phagocytic activity and participates in the regulation of immune cell differentiation and proliferation (Ghiasi et al. 2010). The dietary FRSE at 2% dietary level could increase the lysozyme activity of the fish compared to the control groups. Serum lysozyme activity has been found to be responsive to dietary fucoidan (El-Boshy et al. 2014; Prabu et al. 2016). At 2% dietary FRSE, the A:G ratio in the serum of fish reduced significantly compared to the control. The results indicate that the non-specific immune parameters are stimulated by dietary fucoidan-rich seaweed extract in a dose-dependent manner. It will be interesting to study the possible level of inclusion of fucoidan in the diet of carps providing immunostimulation at the least cost.



Antimicrobial compounds are important components of the innate immune system and are crucial to host defense. Out of the 2159 antimicrobial peptides identified from animals, 114 have been isolated from fish (The Antimicrobial Peptide Database 2017). Two antimicrobial peptides,  $\beta$ -defensin 1 and hepcidin were partially characterized from *Labeo rohita* with sequences of 148 and 269 bp, respectively. No study has been carried out to study the tissue level expression of antimicrobial peptides in response to feeding FRSE in fish. Antimicrobial peptides are the primitive components of the innate immune system, known to express in liver skin, gills and gut, (Marel et al. 2012) and act as the first line of defense against the bacterial pathogens. Therefore, expression study of the antimicrobial peptides in such tissues may provide significant information regarding the stimulation of peptide expression by immunostimulants and involvement in defense against bacterial pathogens. We found highest expression of  $\beta$ -defensin 1 in the skin, followed by the liver, whereas no significant upregulation was observed in the gill. The mechanism of immunostimulation by fucoidan in fish is not clearly known. However, the increased expression of  $\beta$ -defensin 1 in the skin may be explained on the basis of water-soluble nature of fucoidan and the possibility of interaction of fish with the pellets or with the leachate (Casadei et al. 2013). The expression of  $\beta$ -defensin 1 in the skin of fish has been induced by pathogenic challenge (Chen et al. 2013) as well as by immunostimulants in fish (Marel et al. 2012). Oral administration of FRSE had significant effect on the  $\beta$ -defensin 1 mRNA expression in the gut of the fish. The fish gut is a complex of endogenous probiotic bacteria, pathogenic bacteria, mucus, enzyme and bile secretions and the semidigested food. The endogenous probiotic bacteria and the gut mucosa prevent the colonization of the pathogenic bacteria and could also exert immunostimulatory effect on the gut-associated lymphoid system (Picchiatti et al. 2007). Thus, the use of immunostimulant may alter the eubiosis of the gut or may stimulate the immune system. The effect of fucoidan on the gut microbiota of teleost fish has not been studied so far. Fucoidan is unique in the sense that it is an immunostimulant-possessing antibacterial property (Zapozhzhets et al. 1995). Indeed, it will be interesting to study the interaction of fucoidan on the gut microbiota and its effect on immune system of the fish. Besides  $\beta$ -defensin 1, hepcidin is another most studied antimicrobial peptide (Shi and Camus 2006) that has been established as an iron-regulating hormone. For the expression study of hepcidin, the liver tissue was chosen, as it is the main site of expression of hepcidin (Krause et al. 2000). The expression of hepcidin gene in the liver was upregulated almost 6.5 times on the 60th day by feeding 2% FRSE. Hepcidin expression has been found responsive in different fish species when challenged with lipopolysaccharides (Hirono et al. 2005) and pathogenic bacteria (Wang et al. 2009). Hepcidin also shows inhibiting effect on *A. hydrophila* (Cai et al. 2012). However, only a limited number of studies have sought to investigate the effect of dietary immunostimulants on the gene expression of hepcidin in teleost fishes (Skov et al. 2012).

*A. hydrophila* is a Gram-negative, rod-shaped bacteria with single flagellum. Antimicrobial peptides have been shown to be active against Gram-negative bacteria (Hsieh et al. 2010). The mechanisms of multiple antimicrobial peptides have been analyzed and a model, termed the Shai–Matsuzaki–Huang model, has been proposed in which cationic antimicrobial peptides may use their positive charge to bind to the surface anionic membrane components of bacterial cells (Zaslhoff 2002). This binding is suggested to result in the leakage of bacterial cellular components, thereby killing the bacterial cells. Higher expression of antimicrobial peptides was recorded in the skin, liver and the intestine of *L. rohita*. These tissues are known to be the portal of entry and predilection sites of *A. hydrophila* in the fish. Therefore, the reduced post-challenge mortality of the fucoidan-fed fish may be attributed to the protection provided by increased expression of antimicrobial peptides and also stimulation of innate immune system.

The activation of antimicrobial peptides gene expression in particular, and the non-specific immune system in general, by feeding sulphated polysaccharides (FRSE) is thought to be mediated primarily through their recognition by specific receptors. The interaction of immunostimulatory polysaccharides with cell receptors may trigger signaling pathways, ultimately leading to induction of gene transcription (Xu et al. 2014). Therefore, it may be assumed that immunostimulatory action of fucoidan in the fish follows the same mechanism as other polysaccharides. However, at present, it remains to be confirmed and requires further research.

Seaweed powder is obtained from drying and pulverizing of the seaweed *Sargassum wightii*. The seaweed powder is the base material for the extraction of crude fucoidan. In the T3S and T4S group, seaweed powder was mixed with the feed at the rate 3 and 4%, respectively. Syad et al. (2013) found that *Sargassum wightii* has a fiber content of 17% dry weight and crude protein content of 1.4 mg/g dry weight. This means that the

chance of any nutritional supplementation of the fish by feeding dry seaweed powder is very meager. Seaweed was included in T3S and T4S to evaluate any effect of dietary fucoidan from the seaweed powder on the fish. The growth performance and immune status and gene expression of antimicrobial peptides in these treatments had no significant difference from the control group. We observed that only fucoidan extracted from the seaweed powder has beneficial effect on fish immunity. Although there is considerable amount of fucoidan in T3S and T4S feeds coming from the seaweed powder, it is possible that seaweed bound fucoidan is in some inactive form and hence unable to incite any response in the fish.

## Conclusion

Careful examination of the changes in genes expression and other immune parameters could reveal the processes that are involved in triggering the immune system of the fish by seaweed compounds. In the present study, we report characterization of two antimicrobial peptide genes  $\beta$ -defensin 1 and hepcidin with 148 and 269 bp, respectively, in *L. rohita*. Although there was no improvement in the growth of fish due to feeding fucoidan-rich seaweed extract, the expression of hepcidin was significantly upregulated in the liver.  $\beta$ -defensin 1 expression was also upregulated in the liver, skin and intestine. The non-specific immune parameters also showed response when the fish were fed with fucoidan rich seaweed extract. The post-challenge survival was highest in the FRSE fed groups. It concludes that fucoidan at the dietary level of 2% is a potential immunostimulant for *L. rohita*, although it does not have any growth promoting effect. The information generated through this study can be useful for designing studies on farmed fish to demonstrate the suitability of seaweed polysaccharides in preventive health care.

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## Compliance with ethical standards

**Conflict of interest** All authors report no conflict of interest.

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