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# Synergistic effect of L-methionine and fucoidan rich extract in eliciting growth and non-specific immune response of *Labeo rohita* fingerlings against *Aeromonas hydrophila*

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ABSTRACT

An experiment of 60 days was conducted to study the immunomodulatory response and growth performance of *Labeo rohita* fingerlings fed with fucoidan rich seaweed extract (FRSE) and methionine. Four hundred and twenty fingerlings were randomly distributed into seven different experimental groups in triplicates following completely randomised design (CRD). Fishes were fed to satiation with purified diets containing either 0% FRSE with 0.3% methionine (control), 0% FRSE with low dose (0.9%) methionine ($T_1$), 0% FRSE with high dose (1.5%) methionine ($T_2$), 1% FRSE with 0.3% methionine ($T_3$), 2% FRSE with 0.3% methionine ($T_4$), 1% FRSE with high dose methionine ($T_5$) or 2% FRSE with low dose methionine ($T_6$) in the feed. There was a significant ($P < 0.05$) effect of dietary fucoidan and methionine on the weight gain %, specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) with highest weight gain % observed in the $T_6$ group. At the end of the experimental trial fishes were challenged with *Aeromonas hydrophila* and the highest relative survival percentage was recorded in the $T_6$ group, which was not significantly different from $T_4$ group ($P > 0.05$). Immunological parameters: respiratory burst activity, myeloperoxidase activity, lysozyme activity, total immunoglobulin, phagocytic activity and total leukocyte count (TLC) were increased with the 2% level of dietary FRSE along with low dose methionine, whereas serum albumin/globulin (A/G) ratio and blood glucose level showed a decreasing trend ($P < 0.05$). These results suggest the synergistic effect of FRSE and methionine for growth and immunity in contrast to the only immune boosting capacity of fucoidan. Thus, 2% FRSE along with low dose methionine can act as immunostimulant and growth promoter in the diets of *L. rohita* fingerlings.

## 1. Introduction

The aquaculture production is globally dominated by freshwater fish species, especially the carps. In Indian aquaculture, the major production is primarily contributed by three carp species, *Labeo rohita* (Rohu), *Catla catla*, and *Cirrhinus mrigala*, commonly known as Indian major carp (IMC) (FAO, 2012). Among the three species, *L. rohita* contributes the major part of the culture due to its delicious taste that confers it a good market demand and high consumer preference. This has led to the intensive farming of this species.

Intensive aquaculture causes stress to fish, increases susceptibility to many diseases and immune suppression (Wang et al., 2015). Hence, immunomodulation has been proposed as an ideal prophylactic method to protect the cultured fish from pathogens by up-regulating their innate immune system (Aoki, 1992). The use of antibiotics and other chemotherapeutic drugs has been criticized in fish culture due to certain issues like development of antibiotic resistant bacteria, environmental pollution and the accumulation of antibiotic residues in animals including fish (Citarasu et al., 2002).To safe guard the fish from the diseases and to increase the aquaculture production, feeding of immunostimulants are in practice to improve their non-specific defence mechanism.

Many phytochemicals or nutraceuticals have been used as modulators of the immune system in livestock feeds, but their use is still scanty in aquaculture. In this context, Fucoidan, a polysaccharide containing substantial percentages of L-fucose and sulphate ester groups is one such immunostimulant present in brown seaweeds and some marine invertebrates. Fucoidan also demonstrates a variety of pharmaceutically relevant biological activities, including anticoagulant and antithrombotic (Zhu et al., 2010), antiviral (Makarenkova et al., 2010), antitumor (Alekseyenko et al., 2007), anti-inflammatory (Semenov et al., 1998), and antioxidant properties (Wang et al., 2010). Fucoidan

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has been repeatedly proved to be immunostimulant in the diets of fishes (Prabu et al., 2016). Fucoidan also enhances the growth of shrimps (Traifalgar et al., 2010) but the growth promoting effect of fucoidan has not been reported in Indian major carp. Besides this, the immunostimulating potential of FRSE has been found to be effective at 3% level of inclusion in the diet of *Pangasiodon hypophthalmus* (Prabu et al., 2016). Also, the economics of fucoidan depends upon its minimum inclusion level in the diet, as maximum 5.5% recovery of fucoidan has been reported (Prabu et al., 2016). Fucoidan is widely available from various low-cost resource brown seaweeds. Hence, fucoidan has been one of focused area of research to develop the nutraceuticals for both in human and animal use.

Several additives in the fish feeds have been proved to be the modulators of the immune system. Additives like amino acids act as the precursors of endogenous protein synthesis meant for growth and metabolic functions (Fafournoux et al., 2000). Besides, methionine is usually the first limiting and important essential amino acid required by fishes for normal growth and other important functions in the body (Mai et al., 2006). Several studies confirmed that methionine exhibits the immunostimulating activity, and improved the innate immune responses by proliferation and differentiation of T cells (Kinscherf et al., 1994; Grimble and Grimble, 1998).

Dietary intervention to increase the growth of fish with FRSE, which is a good immunostimulant, may be an ideal approach to make a balance between growth and immunity of fish. This will open a gateway to develop a fucoidan based feed additive to enhance both growth and immunity of fish. Therefore, it was hypothesised that the supplementation of methionine will enhance the immunomodulatory response of FRSE synergistically, as it has been reported that the over-sulphation of fucoidan enhances its antioxidant activity (Wang et al., 2009). In this context, present work was designed to study the synergistic effects of dietary supplementation of fucoidan and methionine on growth and non-specific immune responses of *L. rohita* challenged with *Aeromonas hydrophila*. As per our knowledge present study is the first report on the synergistic effect of FRSE along with methionine supplementation in animal nutrition.

## 2. Material and methods

### 2.1. Seaweed collection and fucoidan rich seaweed extract (FRSE) preparation

Indian brown seaweed, *Sargassum wightii*, used for the present experiment was collected from Mandapam, Tamil Nadu, India. Freshly collected, healthy and disease free *S. wightii* seaweeds were thoroughly washed with freshwater to remove any extraneous and dirty materials adhering to them followed by shade drying for about 3 days and finally pulverization to a fine powder.

The extraction of fucoidan was carried out as described by Yang et al. (2008) with slight modifications. The fine seaweed powder weighing 100 g was then taken in an appropriately sized vessel to which 1000 mL of 85% ethanol was added, followed by overnight stirring at room temperature. The next day, the alcohol was decanted and the residual seaweed was washed with acetone followed by centrifugation at 29030*g* for 15 min and the supernatant obtained was decanted. The residual seaweed was dried at room temperature overnight to remove the residual acetone. Then, seaweed was mixed with 1000 mL of distilled water and stirred for 7 h at 70 °C. The hot mixture was filtered through a muslin cloth and the residual seaweed was again mixed with 500 mL of water followed by stirring for 7 h at 70 °C. Again the water was filtered out with a muslin cloth. Both extracts were mixed together and centrifuged at 29030*g* for 15 min. The supernatant was collected and calcium chloride pellets were then added to the supernatant to make the concentration to 1% of the extract, followed by vigorous mixing. The extract was stored at 4 °C overnight. The next day, the calcium salt of alginates was filtered out and the dark orange brown aliquot was obtained which was crude fucoidan rich seaweed extract (FRSE).

**Table 1**
Composition of the experimental diets (% DM basis).

| Ingredients | Control | $T_1$ | $T_2$ | $T_3$ | $T_4$ | $T_5$ | $T_6$ |
|---|---|---|---|---|---|---|---|
| Casein | 33 | 32 | 31 | 33 | 33 | 31 | 32 |
| Gelatin | 7.25 | 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 | 16.75 |
| Starch | 19.5 | 19.5 | 19.5 | 19.5 | 19.5 | 19.5 | 19.5 |
| Cellulose | 9.84 | 10.24 | 10.64 | 8.84 | 7.84 | 9.64 | 8.24 |
| CMC | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Cod liver oil | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Sunflower oil | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Vitamin-mineral mix | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| BHT | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Choline chloride | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 |
| L-cysteine | 0.86 | 0.86 | 0.86 | 0.86 | 0.86 | 0.86 | 0.86 |
| L-methionine | 0.3 | 0.9 | 1.5 | 0.3 | 0.3 | 1.5 | 0.9 |
| Fucoidan | 0 | 0 | 0 | 1 | 2 | 1 | 2 |
| | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

### 2.2. Quantification of FRSE

The FRSE sample was quantified after dialysing for 24 h and the L-fucose content was analysed according to the method of Dubois et al. (1956). The absorbance was measured at 480 nm with a characteristic yellow-orange solution. From the L-fucose content, the fucoidan yield was calculated empirically that 1 μg of fucoidan is equivalent to 1.75 × fucose (μg) (Doner and Whistler, 1973; Choosawad et al., 2005).

### 2.3. Experimental diets

The diets were prepared with the purified ingredients as indicated in Table 1. The requirement of dietary methionine for Indian major carps was reported to be 1.2% of the diet at 1% constant dietary cysteine level (Imtiaz and Jafri, 2003). L-methionine was added above the requirement of fish in two different levels at constant 1% cysteine level while taking the sulphur containing amino acid profile of casein and gelatin (0.9% methionine and 0.14% cysteine) into consideration. The methionine content of casein and gelatin together was 0.9%, so in order to fulfil the basic requirement (1.2%) of the particular amino acid, a 0.3% extra L-methionine was supplemented in all the treatments including control. Therefore, low dose and high dose methionine were added at 1.5 times (1.8%) and 2 times (2.4%) of the fish's methionine requirement. Seven diets with identical composition except the levels of cellulose, methionine and FRSE to keep the crude protein and lipid level at 35% and 8%, respectively, were prepared in the Fish Nutrition Laboratory of CIFE. All the ingredients were properly mixed with a requisite amount of water to make the dough except the BHT, oil and vitamin-mineral mix. Then, the dough was steam cooked for 15 min in an autoclave. After cooling, oil, BHT and vitamin-mineral mix were mixed thoroughly to get the even distribution of vitamin-mineral premix in the dough. Then, the pellets of the feed were prepared by pressing the dough through an extruder of 2 mm diameter, which was thoroughly dried in the moisture-free environment at 60 °C in a hot air oven and finally packed.

### 2.4. Analytical methods

Feed and carcass samples were analysed for proximate composition employing 'AOAC' procedures (AOAC, 1975). The water sample was analysed for parameters like temperature, dissolved oxygen, pH, free carbon dioxide, total hardness, ammonia, nitrite and nitrate levels following 'APHA' procedures (APHA, 1985).

**Table 2**
Experimental design.

| Treatments | Condition |
|---|---|
| Control (C) | Basal diet (No fucoidan & 0.3% methionine) |
| Treatment ($T_1$) | Basal diet + methionine (Low dose-0.9%) |
| Treatment ($T_2$) | Basal diet + methionine (High dose-1.5%) |
| Treatment ($T_3$) | Basal diet + 1% fucoidan + 0.3% methionine |
| Treatment ($T_4$) | Basal diet + 2% fucoidan + 0.3% methionine |
| Treatment ($T_5$) | Basal diet + 1% fucoidan + methionine (High dose-1.5%) |
| Treatment ($T_6$) | Basal diet + 2% fucoidan + methionine (Low dose-0.9%) |

### 2.5. Fish and experimental design

*L. rohita* fingerlings used for the present study were procured from Hans Aquaculture, Raigarh, Mumbai India and were acclimatised for 2 weeks in the Wet Laboratory Central Institute of Fisheries Education (CIFE). During this period fish were fed twice daily with control diet to satiation under a natural photoperiod and continuous aeration.

The experiment was carried for a period of 60 days in 21 plastic tanks (150 L capacity) covered with plastic lids. At the start of the experiment fish were fasted for 24 h and weighed. Fish (20 per tank) of initial weight 8 to 8.5 g were randomly distributed in the seven distinct treatment groups each with three replicates following CRD and were subjected with seven different experimental diets (Table 2). The fishes were fed to satiation in two daily feedings (08:00 and 18:00 h) for 60 days. Throughout the experimental period, water temperature remained between 28 and 32 °C; pH was between 7.2 and 8.4; dissolved oxygen was > 5.0 mg/L; ammonia-nitrogen was < 0.1 mg/L and nitrite was < 0.1 mg/L.

### 2.6. Growth parameters

The experimental animals were weighed using an electronic balance at every 15 days interval. Before weighing fishes were starved overnight. The growth performance was assessed using the following formulae:

$$\text{Weight gain}(\%) = (\text{FW} - \text{IW}) \times 100/\text{IW},$$

$$\text{FCR} = \text{Feed given}(\text{DW})/\text{body weight gain}(\text{WW}),$$

$$\text{SGR}(\%) = [\ln(\text{FW}) - \ln(\text{IW})]/[\text{N}] \times 100.$$

$$\text{PER} = \text{body weight gain}(\text{WW})/\text{protein fed}$$

Where FW = final weight, IW = initial weight, DW = dry weight, WW = wet weight, ln = natural log and N = number of culture days.

### 2.7. Sample collection

At the end of the feeding trial blood samples were collected from six fish per tank using heparinized syringes for determination of respiratory burst (NBT), hematology and phagocytic activity. Another set of blood samples of the same fish was taken without heparin and allowed to clot for 30 min. Then by centrifugation at 5000 × *g* for 10 min, serum was separated and stored at − 70 °C for the analysis of the non-specific immune responses.

### 2.8. Hematology and blood cell determination

The total red blood cells (RBC: $10^6$ mm$^{-3}$) and white blood cells (WBC: $10^3$ mm$^{-3}$) were enumerated in an improved Neubauer hemocytometer using Hayem and Turck diluting fluids (Blaxhall and Daisley, 1973). Hematocrit (Ht %) was determined by the standard micro-hematocrit method after centrifugation (D-7200 Tutilige 7) at 2000*g* for 5 min and expressed as a percentage. The haemoglobin (Hb, g dl$^{-1}$) level was determined according to cyanmethemoglobin procedure by following the method of Van Kampen and Zijlstra (1961).

### 2.9. Immunological parameters assay

#### 2.9.1. Respiratory burst activity (NBT) and myeloperoxidase activity (MPO)

Nitrobluetetrazolium [NBT] assay method of Secombes (1990) modified by Stasiack and Bauman (1996) was used for the estimation of NBT activity of the phagocytes. The absorbance at 620 nm was measured and the activity was expressed as NBT-reduction in 100 μL of cell suspension. Myeloperoxidase content present in serum was measured according to Quade and Roth (1997). The OD was read at 450 nm in an ELISA reader.

#### 2.9.2. Total immunoglobulin

Serum total immunoglobulin content was measured according to the method described by Siwicki and Anderson (2000).

#### 2.9.3. Serum lysozyme activity

Serum lysozyme was assessed by method of Anderson and Siwicki (1995).

#### 2.9.4. Phagocytic activity and phagocytic index

The phagocytic cells were detected with *Staphylococcus aureus* (Bangalore Genei, Hyderabad, India) by the method of Anderson and Siwicki (1995) with slight modification. A sample (0.1 mL) of blood was placed in a microtiter plate well, 0.1 mL of *S. aureus* 1 × $10^7$ cells suspended in phosphate buffered saline pH 7.2 was added and then mixed well. Five microlitre of the blood suspension was taken onto a clean glass slide to prepare a smear and the smear was fixed with 95% ethanol followed by staining with Giemsa stain. The sum of 100 phagocytic cells from each smear was observed under the light microscope and the number of neutrophils and monocytes and the bacteria engulfed by each phagocyte were counted.

The phagocytic activity expressed as the number of cells phagocytosing divided by the total number of phagocytes counted. The phagocytic index is a measure of phagocytic activity determined by counting the number of bacteria engulfed per phagocyte during a limited period of incubation of a suspension of bacteria and phagocytes in blood.

### 2.10. Serum biochemical parameters

#### 2.10.1. Serum glucose

Serum glucose was estimated by Nelson and Somogyi method (Oser, 1944). The absorbance was recorded at 540 nm against blank.

#### 2.10.2. Serum total protein, albumin and globulin

Serum total protein was evaluated by the Biuret method (Reinhold, 1953) using an Innoline Total protein kit (Merck India). Albumin was estimated by the bromocresol green binding method (Doumas and Biggs, 1972) using Innoline albumin kit (Merck India). The OD of the standard and test against a blank was recorded in a spectrophotometer at 550 nm and 578 nm, for total serum protein and albumin, respectively. The values of total globulin content 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.

### 2.11. Challenge study

*A. hydrophila*, a pathogenic bacterium was obtained from the Aquatic Animal Health and Management Division CIFE Mumbai. The bacteria were grown on nutrient broth (Himedia, Mumbai, India) for 24 h at 28 °C. The broth contained culture was centrifuged at 3000*g* for 10 min. The supernatant was discarded and the pellet was again

suspended in sterile phosphate buffer saline (pH 7.4). The OD value of the solution was adjusted to 0.5–0.6 at 456 nm by serial dilution to obtain the final bacterial cell concentration of $1.8 \times 10^7$ CFU $mL^{-1}$ which was used for challenge study (Misra et al., 2006; Mohamad and Abasali, 2010).

At the end of feeding experiment (60 days), 10 fish from each replicate were intraperitoneally injected into the abdominal cavity, posterior to the pelvic girdle with 200 μL of a bacterial suspension with a bacterial cell concentration of $1.8 \times 10^7$ CFU $mL^{-1}$. Mortality was recorded on the second day onwards until the 10th day of challenge in all the experimental groups. Post-challenge sampling of the fishes survived was carried out on the 10th day for post-challenge analysis. *A. hydrophila* was confirmed by isolating it from the dead fish. Relative percentage of survival (RPS) was calculated according the formula proposed by Amend (1981).

$$\text{Relative survival (\%)} = 1 - \frac{\text{mortality of treatment}}{\text{mortality of control}} \times 100$$

### 2.12. Statistical analysis

The statistical analysis of the data was carried out by using statistical software package SPSS (ver. 22, USA) in which data were subjected to one way ANOVA and Duncan's multiple range tests were used to determine the significant differences between the means. Paired *t*-test was also used to determine the significant difference between the mean of pre- and post-challenge condition. Comparisons were made at the 5% probability level.

## 3. Results

### 3.1. Quantification of fucoidan rich seaweed extract (FRSE)

The L-fucose content of the concentrated and dialysed extract was 32 mg/g dried seaweed powder. Hence, the fucoidan yield was 5.6 g/100 g dried seaweed powder.

### 3.2. Physicochemical parameters of water

The different physicochemical parameters of water such as temperature (28–32 °C), pH (7.2–8.4), dissolved oxygen (5.2–7 $mgmL^{-1}$), ammonia (0.06–0.1 $mgmL^{-1}$), Nitrite-N (0.06–0.1 $mgL^{-1}$), Nitrate-N (0.05–1 $mgL^{-1}$) were recorded and were found in the normal ranges.

### 3.3. Feeding response to diets

Before the start of experiment in the acclimatization period all fish were fed with the purified control diet. The feed intake was low for the initial two days after which it was normalized. No behavioural or health changes was noticed in any of the treatments including control group.

### 3.4. Growth parameters

The percentage weight gain was found to be significantly different ($P < 0.05$) among the various treatment groups (Table 3). The highest SGR was found in $T_6$ group which was significantly higher ($P < 0.05$) than all other groups. $T_6$ group also registered lowest FCR and highest PER.

### 3.5. Hematology and blood cell determination

Feeding of fucoidan-based nutraceutical diet significantly ($P < 0.05$) affected the haemoglobin, total erythrocyte count (TEC) and total leukocyte count (TLC) with the highest value in $T_6$ group and the lowest in the control group (Table 4). The same trend was followed in the post-challenge period. But the TEC values in post-challenge condition were less than their pre-challenged counterpart, in all the treatments groups. The highest hematocrit value was observed in the $T_6$ group both in pre and post-challenge conditions, which were significantly different from all the other treatment groups ($P < 0.05$).

**Table 3**
Growth parameters of different dietary groups at the end of the experiment.

| Treatments | Wt. gain % | SGR | FCR | PER |
|---|---|---|---|---|
| Control | $32.79^{d} \pm 1.78$ | $0.47^{e} \pm 0.02$ | $6.45^{a} \pm 0.35$ | $0.43^{d} \pm 0.03$ |
| $T_1$ | $39.64^{c} \pm 0.72$ | $0.55^{d} \pm 0.008$ | $5.41^{b} \pm 0.12$ | $0.51^{c} \pm 0.01$ |
| $T_2$ | $46.09b^{c} \pm 0.81$ | $0.63^{c} \pm 0.009$ | $4.78^{b} \pm 0.06$ | $0.56^{c} \pm 0.01$ |
| $T_3$ | $43.58^{c} \pm 2.74$ | $0.60^{cd} \pm 0.03$ | $5.03^{b} \pm 0.24$ | $0.56^{c} \pm 0.02$ |
| $T_4$ | $43.01^{c} \pm 3.22$ | $0.59^{cd} \pm 0.03$ | $5.12^{b} \pm 0.33$ | $0.54^{c} \pm 0.03$ |
| $T_5$ | $56.47^{b} \pm 1.45$ | $0.74^{b} \pm 0.01$ | $3.95^{c} \pm 0.11$ | $0.68^{b} \pm 0.01$ |
| $T_6$ | $95.84^{a} \pm 1.91$ | $1.12^{a} \pm 0.016$ | $2.66^{d} \pm 0.02$ | $1.04^{a} \pm 0.01$ |

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

### 3.6. Respiratory burst activity and myeloperoxidase activity

The NBT and MPO activity were found to differ significantly ($P < 0.05$) among the different experimental groups. The control group exhibited the least NBT activity whereas $T_6$ group recorded the highest activity followed by $T_4$ and $T_5$ groups during the pre-challenge period. NBT and MPO activities were increased in the post-challenge period than their pre-challenge counterparts (Figs. 1 and 2). Similarly, in the post-challenge period lowest NBT and MPO activity were found in the control group, whereas the highest was found in the $T_6$ group. However, there were no significant ($P > 0.5$) differences in NBT and MPO values between pre- and post-challenge counterparts for each treatment.

### 3.7. Total immunoglobulin

During the pre-challenge period the total immunoglobulin values were observed to be higher in $T_6$ group followed by $T_4$, $T_5$ and lower in the control group. In the post-challenge period, the total immunoglobulin values were higher than the pre-challenged counterparts but not significantly different. Also, in the post-challenge period, same trend as that of pre-challenge was observed in which the $T_6$ group was found to have highest immunoglobulin content (Fig. 3).

### 3.8. Serum lysozyme and phagocytic activity

In the pre-challenge phase, the $T_6$ treatment group showed significantly higher serum lysozyme activity (Fig. 4). The same trend was also observed in the post-challenge period. A significant variation ($P < 0.05$) was found between pre- and post-challenge serum lysozyme activities of $T_3$, $T_4$ and $T_6$ experimental groups.

There was a significant difference in the phagocytic activity and phagocytic index of the T6 treatment group from the rest of treatments both in the pre-challenge as well as post-challenge periods (Table 5). Post-challenge period exhibited a significant increase in the phagocytic activity and the phagocytic index of the different treatment groups. In the pre-challenge period, the highest phagocytic activity and index was found in the $T_6$ group, whereas the least was found in the control group. The same trend was recorded in the post-challenge period.

### 3.9. Serum biochemical parameters

#### 3.9.1. Serum glucose

There was a significant difference observed in the serum glucose levels of the various experimental groups both in the pre-challenge and post-challenge period. In the pre-challenge period low serum glucose

**Table 4**
Pre- and post-challenge blood parameters of different experimental groups.

| Treatments | Hb (%) | | TEC ($10^6$/mm$^3$) | | TLC ($10^3$/mm$^3$) | | HCT (%) | |
|---|---|---|---|---|---|---|---|---|
| | Pre | Post | Pre | Post | Pre | Post | Pre | Post |
| Control | $4.80^{eA} \pm 0.25$ | $4.16^{dA} \pm 0.53$ | $1.49^{cA} \pm 0.06$ | $1.40^{cA} \pm 0.19$ | $158.9^{dA} \pm 2.36$ | $160.96^{dA} \pm 6.03$ | $25.36^{cA} \pm 3.01$ | $21.4^{bA} \pm 2.49$ |
| $T_1$ | $6.56^{dA} \pm 0.29$ | $5.81^{bcA} \pm 0.37$ | $2.04^{bA} \pm 0.05$ | $1.81^{bA} \pm 0.23$ | $184.53^{cA} \pm 5.88$ | $196.16^{cB} \pm 3.72$ | $26.43^{bcA} \pm 1.64$ | $22.9^{bA} \pm 2.03$ |
| $T_2$ | $5.24^{eA} \pm 0.20$ | $4.49^{cdA} \pm 0.38$ | $1.63^{cA} \pm 0.10$ | $1.44^{cA} \pm 0.24$ | $161.4^{dA} \pm 5.83$ | $172.2^{dA} \pm 6.01$ | $29.46^{bcA} \pm 1.90$ | $27.3^{abA} \pm 2.01$ |
| $T_3$ | $7.14^{cdA} \pm 0.24$ | $6.24^{bB} \pm 0.25$ | $2.04^{bA} \pm 0.06$ | $1.83^{bA} \pm 0.23$ | $200.83^{bA} \pm 5.89$ | $214.5^{bA} \pm 5.17$ | $29.4^{bcA} \pm 2.25$ | $27.23^{abA} \pm 2.39$ |
| $T_4$ | $7.80^{bA} \pm 0.17$ | $6.81^{bA} \pm 0.41$ | $2.23^{bA} \pm 0.04$ | $1.97^{abA} \pm 0.31$ | $211.96^{abA} \pm 5.57$ | $222.8^{bA} \pm 3.98$ | $33.33^{bA} \pm 1.64$ | $30.7^{abA} \pm 1.95$ |
| $T_5$ | $7.51^{bcA} \pm 0.25$ | $6.25^{bA} \pm 0.58$ | $2.13^{bA} \pm 0.04$ | $1.90^{bA} \pm 0.45$ | $212.46^{abA} \pm 3.37$ | $219.7^{bA} \pm 6.09$ | $32.5^{bA} \pm 1.87$ | $27.93^{abB} \pm 1.06$ |
| $T_6$ | $10.27^{aA} \pm 0.26$ | $9.17^{aA} \pm 0.64$ | $2.54^{aA} \pm 0.04$ | $2.31^{aA} \pm 0.17$ | $221.66^{aA} \pm 4.12$ | $241.86^{aA} \pm 5.45$ | $40.2^{aA} \pm 1.90$ | $35.96^{aA} \pm 2.08$ |

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

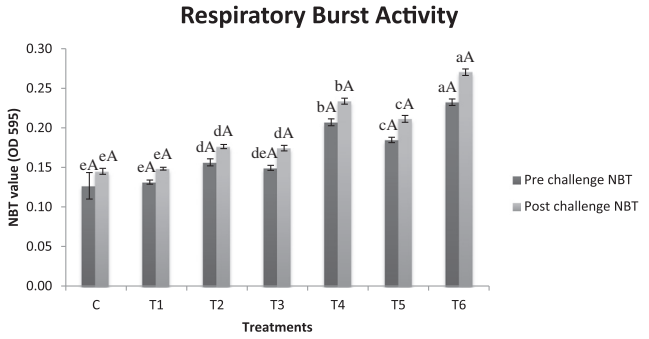


**Fig. 1.** Pre- and post-challenge NBT activity in the blood of *Labeo rohita* fingerlings fed with different experimental diets. Mean values in the experimental group with different superscript (a,b,c) differ significantly ($P < 0.05$). Mean values in the experimental groups with same superscript (A,A) between pre and post challenge group under each treatment do not differ significantly ($P > 0.05$). Data were expressed as Mean ± SE n = 3.

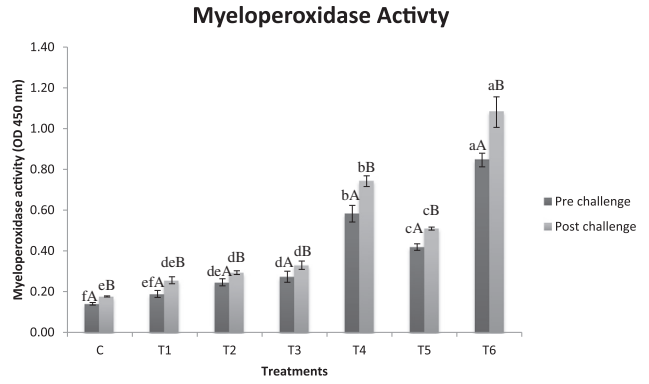


**Fig. 2.** Pre- and post-challenge Myeloperoxidase activity in the serum of *Labeo rohita* fingerlings fed with different experimental diets. Mean values in the experimental group with different superscript (a,b,c) differ significantly ($P < 0.05$). Mean values in the experimental groups with different superscript (A,B) between pre and post challenge group under each treatment vary significantly ($P < 0.05$). Data were expressed as Mean ± SE n = 3.

concentration was recorded in the $T_6$ group followed by $T_4$ group, however, both were not significantly ($P > 0.05$) different (Fig. 5). The same trend was observed in the post-challenge period with significantly ($P < 0.05$) high serum glucose level in control.

### 3.9.2. Serum total protein, albumin and globulin

In both pre- and post-challenge conditions, the serum protein level exhibited the increasing trend in the $T_6$ group (Table 6). But during the post-challenge condition serum protein level was lower than the pre-challenge counterpart. There was significant variation ($P < 0.05$) between pre and post-challenge serum protein in all treatments except control and $T_2$ experimental groups. Similarly, for the content of serum

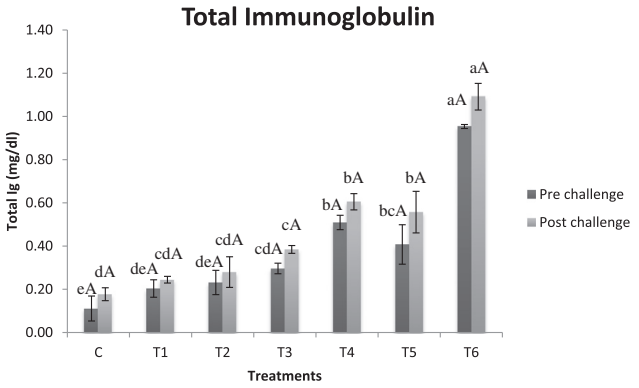


**Fig. 3.** Pre- and post-challenge Total Immunoglobulin activity in the serum of *Labeo rohita* fingerlings fed with different experimental diets. Mean values in the experimental group with different superscript (a,b,c) differ significantly ($P < 0.05$). Mean values in the experimental groups with same superscript (A,A) between pre and post challenge group under each treatment do not differ significantly ($P > 0.05$). Data were expressed as Mean ± SE n = 3.

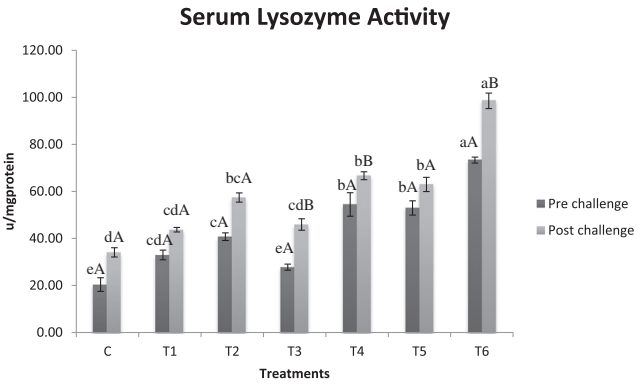


**Fig. 4.** Pre- and post-challenge lysozyme activity in the serum of *Labeo rohita* fingerlings fed with different experimental diets. Mean values in the experimental group with different superscript (a,b,c) differ significantly ($P < 0.05$). Mean values in the experimental groups with different superscript (A,B) between pre and post challenge group under each treatment vary significantly ($P < 0.05$). Data were expressed as Mean ± SE n = 3.

globulin, the same trend during pre and post-challenge condition was recorded as like serum protein. Highest serum globulin content was found in the $T_6$ group. The significant difference ($P < 0.05$) between pre and post-challenge globulin content was found in all treatments except the control and $T_6$ groups. The pre and post-challenge serum albumin content of different experimental groups significantly differ among the various treatments ($P < 0.05$).

A significant difference existed between pre and post-challenge serum albumin level only in $T_1$ ($P < 0.05$). The least value of A/G ratio was found in the $T_6$ group of both pre and post-challenge conditions, whereas highest value was recorded in the control group. There was no

**Table 5**
Pre and Post-challenge Phagocytic activity and Phagocytic index of the different experimental groups.

| Treatments | Pre-challenge | | Post-challenge | |
|---|---|---|---|---|
| | Phagocytic activity | Phagocytic index | Phagocytic activity | Phagocytic index |
| Control | $14.33^{cA} \pm 1.76$ | $1.38^{bA} \pm 0.07$ | $15.33^{bA} \pm 2.40$ | $1.40^{bA} \pm 0.05$ |
| $T_1$ | $16.33^{bcA} \pm 2.60$ | $1.46^{abA} \pm 0.06$ | $17.66^{bA} \pm 2.40$ | $1.52^{abA} \pm 0.25$ |
| $T_2$ | $17.66^{bcA} \pm 2.33$ | $1.58^{abA} \pm 0.13$ | $19.33^{bA} \pm 3.92$ | $1.67^{abA} \pm 0.14$ |
| $T_3$ | $17.33^{bcA} \pm 2.96$ | $1.47^{abA} \pm 0.21$ | $18.66^{bA} \pm 3.84$ | $1.54^{abA} \pm 0.21$ |
| $T_4$ | $24.33^{abA} \pm 3.17$ | $1.62^{abA} \pm 0.11$ | $26.66a^{bA} \pm 4.33$ | $1.70^{abA} \pm 0.17$ |
| $T_5$ | $20.66^{bcA} \pm 3.17$ | $1.56^{abA} \pm 0.07$ | $23.33^{abA} \pm 3.48$ | $1.67^{abA} \pm 0.07$ |
| $T_6$ | $29.66^{aA} \pm 2.33$ | $1.84^{aA} \pm 0.068$ | $32.66^{aA} \pm 4.97$ | $1.95^{aA} \pm 0.04$ |

Mean values in the same column with different superscript (a,b) in pre- and post-challenge group under each treatment vary significantly ($P < 0.05$). Mean value in the same row with same superscript (A,A) do not differ significantly ($P > 0.05$). Data expressed as Mean ± SE. n = 3.

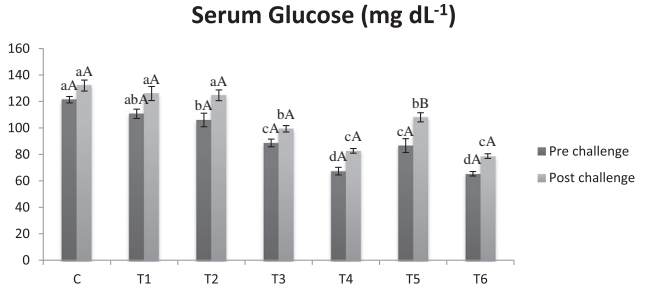


**Fig. 5.** Pre- and post-challenge blood glucose (mg $dL^{-1}$) of different experimental groups. Mean values in the experimental group with different superscript (a,b,c) differ significantly ($P < 0.05$). Mean values in the experimental groups with different superscript (A,B) between pre and post challenge group under each treatment vary significantly ($P < 0.05$). Data expressed as Mean ± SE n = 3.

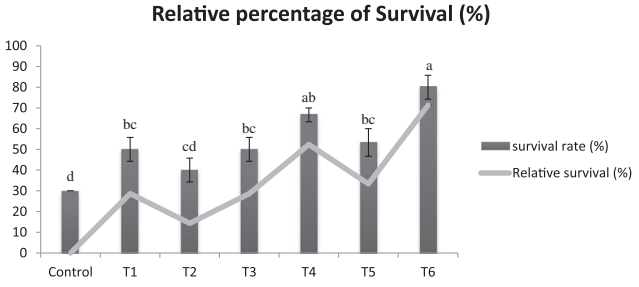


**Fig. 6.** Relative percentage of Survival of *Labeo rohita* fingerlings fed with different experimental diets. Mean values in the experimental group with different superscript (a,b,c) differ significantly ($P < 0.05$). Data were expressed as Mean ± SE n = 3.

significant ($P > 0.05$) change existing between pre and post challenge A/G ratio of different experimental groups except the $T_4$ and $T_5$ groups ($P < 0.05$).

### 3.10. Relative percentage of survival

The highest relative percentage of survival was observed in $T_6$ group, whereas the lowest value was observed in the control group (Fig. 6).

## 4. Discussion

Fucoidan is a complex sulphated polysaccharide, derived from marine brown seaweeds and some invertebrates, usually containing large proportions of L-fucose and sulphate (Duarte et al., 2001; Bilan et al., 2006). This is recognized as a potent immunostimulatory compound in a variety of animals including fish and shrimp (Prabu et al., 2016; Traifalgar et al., 2010). Several studies have shown that fucoidan is a well-known growth promoter in the diets of shrimps and some fishes (Traifalgar et al., 2010; Tuller et al., 2012). But so far it has not been confirmed in the Indian Major Carps. The current study found that the yield of fucoidan was 5.6%, which is in agreement with the Prabu et al. (2016) findings who observed 5.5% yield when fucoidan was extracted from *S. wightii*.

It was hypothesised that the supplementation of methionine will enhance the immunomodulatory response of FRSE synergistically, as it has been reported that the oversulphation of fucoidan enhances its antioxidant activity (Wang et al., 2009). Previous studies have observed the importance of methionine and fucoidan individually on growth promotion as well as immunomodulation in animals like fish (Mai et al., 2006; Kinscherf et al., 1994; Tuller et al., 2012). The results of this study showed that 2% FRSE and low dose methionine when supplemented together, the weight gain percent was significantly higher ($P < 0.05$) than other groups. A similar trend was observed for SGR, FCR, and PER. The outcome of the previous studies, *Lates calcarifer* showed significant growth at higher levels of fucoidan (Tuller et al., 2012) corroborates our results. *Marsupenaeus japonicas* when fed with the fucoidan-supplemented diets, showed enhanced performance in growth compared to control without fucoidan (Traifalgar et al., 2010).

The efficient method of evaluating the stress caused by a variety of

**Table 6**
Pre and Post-challenge Serum protein, albumin, Globulin and A:G ratio of the different experimental groups.

| Treatments | Pre-challenge | | | | Post-challenge | | | |
|---|---|---|---|---|---|---|---|---|
| | Total protein | Albumin | Globulin | A:G ratio | Total protein | Albumin | Globulin | A:G ratio |
| Control | $1.70^{fA} \pm 0.04$ | $0.97^{dA} \pm 0.02$ | $0.72^{dA} \pm 0.05$ | $1.34^{aA} \pm 0.17$ | $1.58^{eA} \pm 0.06$ | $1.01^{bA} \pm 0.07$ | $0.57^{eA} \pm 0.06$ | $2.85^{aA} \pm 0.46$ |
| $T_1$ | $1.98^{eA} \pm 0.04$ | $0.99^{cdA} \pm 0.04$ | $0.98^{bcA} \pm 0.05$ | $1.01^{bA} \pm 0.16$ | $1.86^{dB} \pm 0.05$ | $1.05^{abB} \pm 0.05$ | $0.80^{cdB} \pm 0.09$ | $1.33^{bcA} \pm 0.25$ |
| $T_2$ | $2.19^{cdA} \pm 0.03$ | $1.06^{bcA} \pm 0.03$ | $1.12^{bA} \pm 0.02$ | $0.94^{bcA} \pm 0.05$ | $2.04^{cA} \pm 0.03$ | $1.07^{abA} \pm 0.04$ | $0.97^{bcB} \pm 0.06$ | $1.10^{cA} \pm 0.19$ |
| $T_3$ | $2.09^{deA} \pm 0.06$ | $1.16^{aA} \pm 0.05$ | $0.92^{cA} \pm 0.05$ | $1.26^{aA} \pm 0.07$ | $1.89^{dB} \pm 0.07$ | $1.17^{aA} \pm 0.07$ | $0.72^{deB} \pm 0.08$ | $1.65^{abA} \pm 0.19$ |
| $T_4$ | $2.44^{bA} \pm 0.05$ | $1.03^{cdA} \pm 0.05$ | $1.41^{aA} \pm 0.07$ | $0.73^{dA} \pm 0.04$ | $2.26^{bB} \pm 0.07$ | $1.12^{abA} \pm 0.05$ | $1.13^{abB} \pm 0.05$ | $0.99^{cB} \pm 0.16$ |
| $T_5$ | $2.26^{cA} \pm 0.04$ | $1.12^{abA} \pm 0.04$ | $1.14^{bA} \pm 0.03$ | $0.99^{bcA} \pm 0.05$ | $2.07^{cB} \pm 0.03$ | $1.14^{aA} \pm 0.06$ | $0.92^{cB} \pm 0.04$ | $1.24^{bcB} \pm 0.10$ |
| $T_6$ | $2.67^{aA} \pm 0.05$ | $1.20^{aA} \pm 0.05$ | $1.47^{aA} \pm 0.07$ | $0.81^{cdA} \pm 0.06$ | $2.41^{aB} \pm 0.04$ | $1.18^{aA} \pm 0.08$ | $1.23^{aA} \pm 0.08$ | $0.97^{cA} \pm 0.12$ |

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

stressors is determined by estimating the level of blood glucose (Manush et al., 2005). The elevated glucose level in the infected or stressed animals is known to ward off the infection or stress (Citarasu et al., 2006). In the present study, the lower serum glucose is indicated by higher glucose utilizing the capacity of fucoidan, as it increases the transport of glucose from blood while acting as anti-diabetic (Kim et al., 2012; Wang et al., 2014). These results corroborate the ideas of Yang et al. (2014), who suggested that the glucose level of the fish significantly decreased when fed with fucoidan-rich diet.

A variety of immunostimulants including fucoidan (Chotigeat et al., 2004) stimulate the fish phagocytes such as macrophages and neutrophils. Fish phagocytes can generate superoxide anions ($O_2^-$) and its reactive derivatives (i.e. hydrogen peroxide and free radicals like hydroxyl radicals) during a period of intense oxygen consumption called the respiratory burst. These reactive oxygen species are considered to be toxic to bacterial fish pathogens (Itou et al., 1996) and are generated by phagocytes after stimulation. The stimulation of the phagocytic cell membrane leads to increased consumption of oxygen, the reduction of which is catalyzed by a membrane-bound enzyme, NADPH-oxidase gives rise to $O_2^-$ (Lee and Shiau, 2004). It is evident that increased respiratory burst activity can be correlated with the increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes, 1993). Myeloperoxidase (MPO) on the other hand produces hypochlorous acid (HOCl) from hydrogen peroxide ($H_2O_2$) and chloride anion ($Cl^-$) (or the equivalent from a non-chlorine halide) during the neutrophil's respiratory burst. In the present study, the NBT and MPO showed some impressive results in treatment fortified with high-level FRSE and low dose methionine both in pre- and post-challenge periods. The results of an increase in NBT with FRSE are in agreement with the studies of Chotigeat et al. (2004). Similarly, the increased trend of MPO activity supports the data in which Myeloperoxidase activity in levamisole treated *Catla catla* was found to be significantly higher compared to the control (Perera and Pathiratne, 2008). The high NBT and MPO activity of $T_6$ can be assumed due to oversulphation of fucoidan due to methionine supplementation that results in enhancement of scavenging activity (Wang et al., 2009).

Leukocytes play an important role in the innate or non-specific immunity of the fish. In the current study, all the blood parameters indicate an enhancement in the immune response in the $T_6$ group. These results seem to be consistent with other researches which reported that the Hb%, RBCs, total WBCs count and HCT% increased in the freshwater fish when fed diets supplemented with either methionine alone or in combination with lysine (Derballa et al., 2010). Erythrocyte count, haemoglobin level and HCT% significantly decreased in the post-challenge period, which can be compared with Ranzani-Pavia et al. (2004) who observed a decreased erythrocyte number after bacterial challenge in Nile Tilapia.

Among the serum proteins the globulins are the major humoral elements, which play an important role in the immune response. The liver is responsible for synthesizing the albumin proteins, which creates an osmotic force that maintains the fluids volume of the vascular space. In the present study, the serum total protein, albumin, globulin and total immunoglobulin were analysed both in the pre-challenge as well as the post-challenge period, which showed better results in the $T_6$ group than others due to the synergistic effect of 2% FRSE with low dose methionine. A lower A:G ratio indicates a superior immune status of the fish that was observed in the $T_6$ group, both in the pre- as well as the post-challenge periods. After challenge with the *A. hydrophila*, the serum protein level decreased as compared to their pre-challenge counterparts. This may be due to vascular leaking of the protein because of increased permeability of the membrane (Green and Rice, 1979). Immunoglobulins form an important humoral component of the specific immune system. The results of the current study showed that the total immunoglobulin level significantly increased in 2% FRSE with low methionine group. This was supported by Takai et al. (2014) who found that enhancement of immunoglobulin production occurred by fucoidan from Mekabu (*Udaria pinnantifida*) in mouse spleen lymphocytes. Kuang et al. (2012) also observed an increase in IgM levels with different dietary methionine hydroxy analogue (MHA) levels in juvenile Jian carp.

Phagocytosis eliminates pathogens in the early phase of infection, measured in terms of phagocytic activity (Neumann et al., 2001). These engulfing and other killing mechanisms of phagocytic cells are an important defence mechanism against pathogenic bacteria in fish (Rao et al., 2006) and immunostimulants strongly influence this activity. In the present study, the phagocytic activity increased in response to the dietary fucoidan-based nutraceuticals. A possible explanation for this might be that the supplementation of FRSE and low dose methionine together activates the phagocytic cells in order to get rid of infection like any other immunostimulant. These findings are in agreement with Prabu et al. (2016), who reported an increase in the phagocytic activity of *P. hypophthalmus* fed fucoidan-based diets. Similarly, the levels of phagocytic activity and phagocytic index of freshwater fish was found to be high in methionine or lysine supplemented feed (Derballa et al., 2010).

The serum lysozyme is used as an indicator of innate immune response in fish (Tort et al., 2003). The leukocytes like neutrophils and macrophages produce the serum lysozyme in fish which is a humoral component of the non-specific defence mechanism. The findings of the current study corroborate with the reports of Rao et al. (2006) in *L. rohita* fed the diet containing *Achyranthes aspera* and Jian and Wu (2004) in *Cyprinus carpio* fed with traditional Chinese medicine, who observed a high lysozyme activity. Kuang et al. (2012) found that serum lysozyme activity significantly increased with optimal synthetic methionine (MHA) supplement during post-challenge study. In the post-challenge period, the serum lysozyme activity was observed to increase in all the treatments, which is supported by the findings of Siwicki and Studnica (1987), in which *C. carpio* showed enhancement in serum lysozyme activity after challenged with *Aeromonas punctata*.

## 5. Conclusion

It can thus be suggested that methionine supplementation at lower dose along with 2% FRSE synergistically increases the growth performance as well as the non-specific immune response of *L. rohita*. Further research might be undertaken to explore some more nutraceuticals to reduce further the FRSE level in diets. Nanoparticle delivery of FRSE may be an alternative approach to reduce the level of FRSE in the diets for economical aquaculture.

## Conflict of interest

The authors report no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aquaculture.2017.06.001.

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