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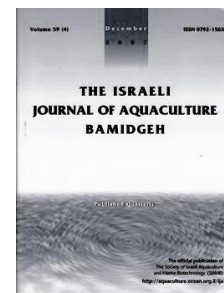
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## Growth and Hemato-Immunological Response to Dietary *i*-Carrageenan in *Labeo rohita* (Hamilton, 1822) Juveniles

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Key words: total erythrocyte count, total leucocyte count, hemoglobin value, thrombocyte count, NBT, myeloperoxidase activity

### Abstract

The study was performed over a period of 60 days to evaluate the effect of dietary carrageenan on growth, hematology, biochemistry, and innate immunity in rohu *Labeo rohita*. A basal diet supplemented with iota (*i*)-Carrageenan at 5, 10 and 20g/kg was fed to three different groups of fish for 60 days. The fish were examined 15, 30, 45, and 60 days after commencement of the study. Parameters for growth (absolute growth, specific growth rate, and percentage weight gain), hematology (total erythrocyte count, total leucocyte count, thrombocyte count and hemoglobin value), biochemistry (total serum protein, albumin, globulin and albumin-globulin ratio), and innate immunity (nitroblue tetrazolium NBT, and myeloperoxidase MPO, activity) were monitored to assess the effect of the *i*-Carrageenan based diet in *L. rohita*. All the parameters examined (growth, hematology, biochemistry, and innate immunity) increased significantly ( $P<0.05$ ) in carrageenan-fed groups compared to the control group. However, the highest values for those parameters were found on the 60<sup>th</sup> day in the group which was fed a 10 g/kg *i*-Carrageenan diet. The study suggests that a 10 g/kg diet of *i*-carrageenan enhances immunity and the overall health status in *L. rohita*.

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## Introduction

Aquaculture is a source of livelihood for a large section of the economically underprivileged population in Asian countries including India. *Labeo rohita* (Hamilton, 1822), an Indian carp, is the major aquaculture species in India as well as in South-east Asian countries (FAO, 2001). It is an economically important aquaculture species in India exceeding 0.95 million tons of production in 2006 (FAO, 2008). Intensive production and rapid development of aquaculture enterprises has increased the susceptibility of fish to infectious diseases resulting in huge economic losses. The main causative agents and associated pathogens are bacteria, viruses and parasites. The use of chemotherapeutics and antibiotics for treatment and control of diseases in aquaculture has great adverse effects. Chemicals, such as trichlorofon and formalin, are far from satisfactory (Goven et al. 1980; Klinger and Floyd 2002). For this reason, effective alternative therapies need to be developed. Efforts have been directed towards the use of different plant products and their extracts as immunostimulants to minimize the adverse effects of antibiotics and various chemotherapeutics (Ganguly et al. 2010).

Carrageenan is a collective term for polysaccharides prepared by alkaline extraction and modification from red seaweeds (*Rhodophyceae*), mostly of the genera *Chondrus*, *Euclima*, *Gigartina* and *Iridaea*. Carrageenan is generally used for thickening, suspending and gelling. K- and *i*-Carrageenans form thermo-reversible gels when cooled in the presence of appropriate counterions. K-carrageenan forms a firm clear gel with poor freeze-thaw stability; the coil-double helix transition being followed by a K<sup>+</sup> (potassium ion) induced aggregation of the helices. K-carrageenan gels may be softened (and are generally regarded to be synergistically strengthened) with locust bean gum. *i*-carrageenan has less specific ionic binding but its increased strength allows helices to form junction zones in soft elastic gels with good freeze-thaw stability. *i*-carrageenan is non-gelling, as the lack of the <sup>1</sup>C<sub>4</sub> 3,6-anhydro-link allows galactose residues to revert to their conformation which does not allow the initial double helix formation required for gelling. Additionally, the high density of charged sulphate groups encourages extensive conformation.

Seaweed polysaccharides like carrageenan, fucoidan and sodium alginate have shown immunostimulatory effects on finfish. Intraperitoneal injection of kappa (K)-carrageenan and sodium alginate provide resistance against pathogens and increase the innate immune response in common carp *Cyprinus carpio* (Fujiki et al. 1994 and 1997; Fujiki and Yano 1997), snakehead *Channa striata* (Miles et al. 2001), rainbow trout *Oncorhynchus mykiss* (Peddie et al. 2002), sea bass *Dicentrarchus labrax* (Bagni et al. 2005) and grouper *Epinephelus coioides* (Cheng et al. 2007). Oral administration of K-carrageenan and sodium alginate enhances the innate immune response of brown-marbled grouper *E. fuscoguttatus* and also provides resistance against *Vibrio alginolyticus* (Cheng et al. 2008). Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis (Siwicki et al. 1994), dietary yeast RNA, ω-3 fatty acid and β-carotene enhances hemato-immunological responses in *Catla catla* juveniles (Jha et al. 2007). Increased hematological and biochemical response in common carp, *Cyprinus carpio*, was observed following herbal treatment for *Aeromonas hydrophila* infection (Harikrishnan et al. 2003) and antimicrobial activity of combined extract from *Azadirachta indica* and *Ocimum sanctum* (Harikrishnan et al. 2010). However, little is known about the protective effect of orally administered seaweed polysaccharide against pathogens on the innate immune response of fish (Bagni et al., 2005).

The present study was designed to evaluate parameters relating to growth, hematology, biochemistry and innate immunity, including nitroblue tetrazolium (NBT) and myeloperoxidase (MPO) activity in rohu *L. rohita*, fed diets containing iota (*i*-Carrageenan at 5, 10 and 20 g/kg respectively.

## Materials and Methods

**Experimental design.** Fingerlings of *L. rohita* (Hamilton, 1822), average length 15±0.85 cm, average weight 19±0.69 g, were obtained from a Government carp farm, Khopoli, Maharashtra, India for this study. After collection, they were immediately taken to the

wet laboratory where they were stocked in 2 fiberglass reinforced plastic (FRP) circular tanks (approx. 2000-l treated and matured ground water) for acclimation. Four treatments (3 experimental and one control) were conducted in twelve 500-l FRP tanks containing 26.4-28.8 °C aerated water. Twenty fish from the stock tank were transferred into each of 500-l FRP tanks and acclimated for two weeks before any assay was conducted.

*Experimental diet and sampling:* Feed was prepared mixing different ingredients such as fish meal, wheat flour, cod liver oil, vitamin and mineral mixture premix, procured from the local market. Four feeds were prepared, three containing Carrageenan (*i*-Carrageenan, type-II, SIGMA-ALDRICH) in different concentrations of T0 (control, without Carrageenan), T1 (5 g/kg Carrageenan), T2 (10 g/kg Carrageenan) and T3 (20 g/kg Carrageenan). The experiment (4 treatments x 3 replications) was carried out using healthy fish. Three fish from each tank were sampled and anesthetized with clove oil (Merck, Germany) 15, 20, 30 and 60 days post feeding. An aliquot of their blood was mixed with EDTA (an anticoagulant); the remainder was used for collecting serum samples which were stored at -20 °C for further analysis of various serum parameters.

*Growth parameters:* Various growth parameters were analyzed using the following formulae:

$$\text{Absolute growth} = \text{Final body weight} - \text{initial body weight}$$

$$\text{Specific growth rate} = 100 \times \frac{\text{Ln Final weight} - \text{Ln Initial weight}}{\text{Number of days}}$$

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

*Hematological parameters:* The blood samples from fish were drawn from the caudal peduncle region using 2 ml sterile glass syringes, rinsed with anticoagulant 2.7% EDTA solution (Qualigens, India). Blood was collected into dry sterilized glass vials containing anticoagulant, EDTA. For 2 ml of blood, 20 µl (2.7% EDTA solution) was used. The hematological studies were conducted for total erythrocyte count (TEC), total leucocyte count (TLC), hemoglobin (Hb) and platelet (thrombocyte) count (PLC). TEC and TLC were determined using Neuber's hemocytometer (Feinoptik, Germany) with RBC diluting fluid (Himedia, India). For TEC and WBC determination, TLC diluting fluid (Himedia, India) was used. The Hb concentration was analyzed following the cyanomethemoglobin method using Drabkins fluid (Qualigens Diagnostics Kit, India). The PLC was carried out by preparing blood smears on grease free clean glass slide, air dried and fixed in methanol. Methanol fixed blood smears were stained with Field stain A and B (Himedia, India). The slides were washed in tap water and allowed to dry before microscopic examination. PLC was determined using a binocular microscope (Olympus, Japan).

*Biochemical parameters:* The collected blood was centrifuged at 3000 x g for 10 min at 28 °C. The separated serum was collected in sterilized Eppendorf tubes and analyzed for various parameters such as serum total protein, measured using the Biuret method (Reinhold 1953) using a diagnostic kit (Qualigen, India), albumin, measured using the bromocresol green binding method (Doumas et al. 1971) and globulin content calculated by subtracting albumin values from total serum protein.

*Non-specific immune parameters:* Cellular non-specific immune parameter, NBT assay was determined using the Secombes method (1990) as modified by Stasiack and Baumann (1996). To facilitate adhesion of cells, 50 µl of blood was placed into the wells of flat bottom microtiter plates and incubated at 37 °C for 1 h. The supernatant was removed and the loaded wells were washed three times in PBS. After washing, 50 µl of 0.2% NBT was added and then incubated for an additional hour. The cells were fixed with 100% methanol for 2-3 minutes and washed three times with 70% methanol. The plates were then air-dried. In order to dissolve formazon blue precipitate which had formed, 60 µl 2N potassium hydroxide and 70 µl dimethyl sulphoxide were added to each well. The optical density (OD) of the aqueous blue colored solution was read at 620 nm in a

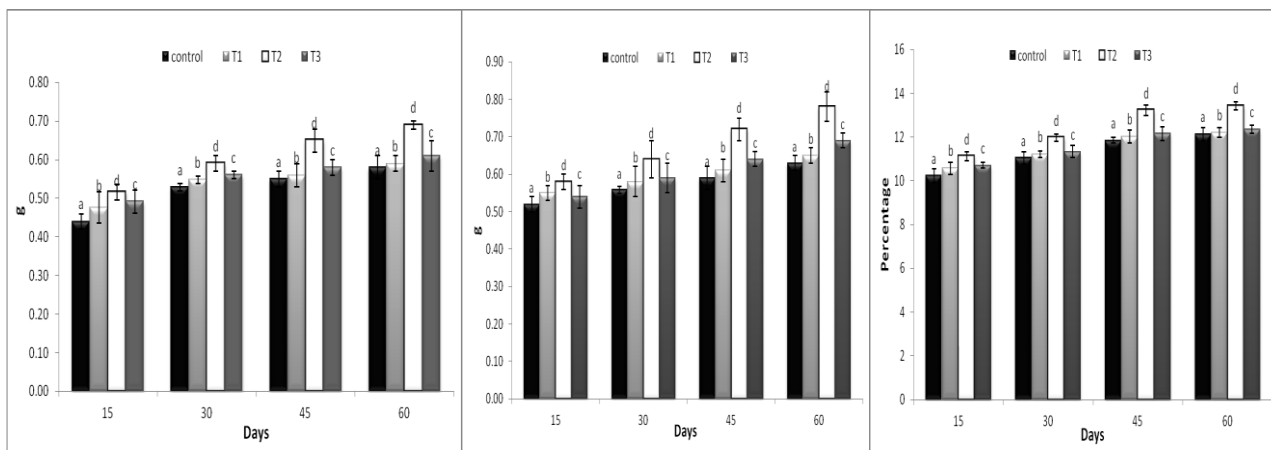
microtiter reader (BioTek, ELISA reader).

Humoral non-specific immune parameter, MPO activity present in serum was measured (Quade and Roth, 1997) with a slight modification (Sahoo et al. 2005). Approximately 10  $\mu$ l of serum was diluted with 90  $\mu$ l of Hank's balanced salt solution (HBSS) without  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  in 96-well plates. Then 35  $\mu$ l of 20 mM „3,3',5,5'-tetramethylbenzidine hydrochloride (TMB) (Himedia, India) and 5 mM  $\text{H}_2\text{O}_2$  (Qualigens, India), both substrates of MPO were prepared and added on the same day. The color change reaction was terminated after 2 minutes by adding 50  $\mu$ l 4 M sulphuric acid ( $\text{H}_2\text{SO}_4$ ). The OD was read at 450 nm in a microtiter reader (BioTek, ELISA reader).

**Statistical analysis:** All the experimental assays were performed in triplicate for each blood/serum sample and the mean  $\pm$  S.E. for hematological, immunological and biochemical parameters were calculated. Statistical analyses were performed using SPSS version 16 and data were subjected to one-way ANOVA followed by Duncan's multiple range test to determine significant difference at 5% ( $P < 0.05$ ) level.

## Results

**Growth parameters:** The absolute growth (Fig. 5), specific growth rate (Fig. 6) and percentage weight gains (Fig. 7) were highest in the treatment groups fed with carrageenan supplemented diet compared to the control group. The maximum value of absolute growth, specific growth rate and percentage weight gain was observed on the 60<sup>th</sup> day post feeding in the T2 (10 g/kg Carrageenan) treatment group.



**Fig 5:** Absolute growth (ABW) during various sampling days (values are mean  $\pm$ SE). Mean values with different superscript within a column for a parameter are significantly different ( $p < 0.05$ ).

**Fig 6:** Specific growth rate (SGR) during various sampling days (values are mean  $\pm$ SE). Mean values with different superscript within a column for a parameter are significantly different ( $p < 0.05$ ).

**Fig 7:** Percentage weight gain (PWG) during various sampling days (values are mean  $\pm$ SE). Mean values with different superscript within a column for a parameter are significantly different ( $p < 0.05$ ).

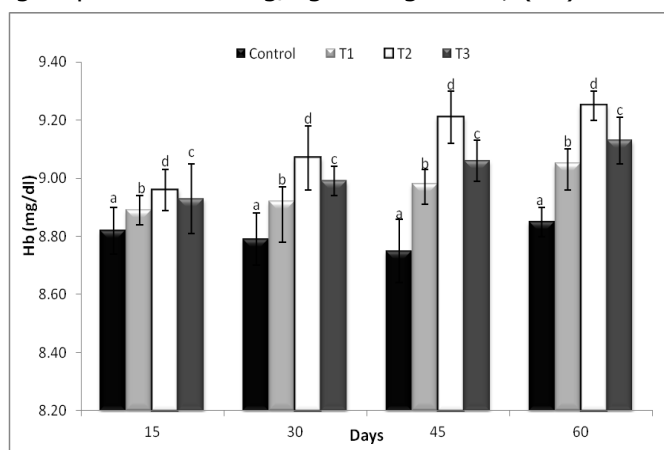
**Hematological parameters:** see Table 1.

Table 1: Hematological parameters (TEC, TLC) of *Labeo rohita*

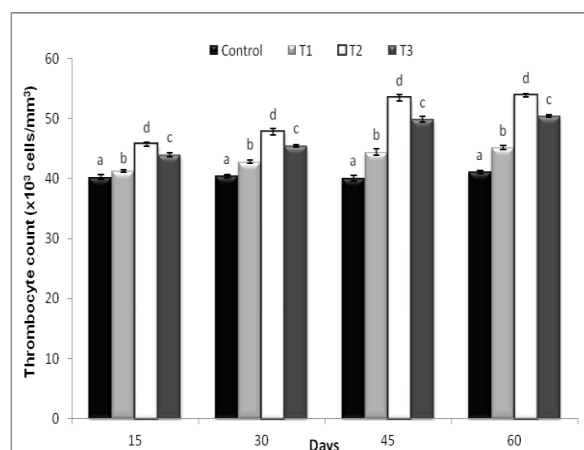
Days	Treatments				
		Control	T1	T2	T3
15	TEC	2.27 $\pm$ 0.02 <sup>a</sup>	*2.31 $\pm$ 0.03 <sup>b</sup>	*2.51 $\pm$ 0.01 <sup>d</sup>	*2.42 $\pm$ 0.01 <sup>c</sup>
	TLC	31.91 $\pm$ 0.34 <sup>a</sup>	*32.29 $\pm$ 0.20 <sup>b</sup>	*35.77 $\pm$ 0.17 <sup>d</sup>	*34.04 $\pm$ 0.14 <sup>c</sup>
30	TEC	2.28 $\pm$ 0.11 <sup>a</sup>	*2.39 $\pm$ 0.24 <sup>b</sup>	*2.58 $\pm$ 0.33 <sup>d</sup>	*2.51 $\pm$ 0.26 <sup>c</sup>
	TLC	31.59 $\pm$ 0.25 <sup>a</sup>	*33.45 $\pm$ 0.31 <sup>b</sup>	*36.24 $\pm$ 0.21 <sup>d</sup>	*35.86 $\pm$ 0.42 <sup>c</sup>
45	TEC	2.24 $\pm$ 0.07 <sup>a</sup>	*2.49 $\pm$ 0.04 <sup>b</sup>	*2.69 $\pm$ 0.02 <sup>d</sup>	*2.58 $\pm$ 0.01 <sup>c</sup>
	TLC	31.11 $\pm$ 0.50 <sup>a</sup>	*35.86 $\pm$ 0.29 <sup>b</sup>	*43.93 $\pm$ 0.19 <sup>d</sup>	*39.21 $\pm$ 0.26 <sup>c</sup>
60	TEC	2.19 $\pm$ 0.28 <sup>a</sup>	*2.52 $\pm$ 0.35 <sup>b</sup>	*2.71 $\pm$ 0.18 <sup>d</sup>	*2.62 $\pm$ 0.22 <sup>c</sup>
	TLC	32.04 $\pm$ 0.15 <sup>a</sup>	*36.24 $\pm$ 0.21 <sup>b</sup>	*44.18 $\pm$ 0.29 <sup>d</sup>	*40.27 $\pm$ 0.15 <sup>c</sup>

\*Significant difference between control and treatment group during various sampling days (values are mean  $\pm$ SE). Mean values with different superscript within a column for a parameter are significantly different ( $p < 0.05$ ). TEC (Total erythrocyte count) ( $\times 10^6$  cells/ $\text{mm}^3$ ) and TLC (Total leukocyte count) ( $\times 10^3$  cells/ $\text{mm}^3$ )

TEC and TLC and Hb content (Fig. 3) were significantly higher ( $P < 0.05$ ) in the carrageenan incorporated groups in comparison to the control. The TLC increased significantly from the 15<sup>th</sup> day onwards and reached its peak on day 60 post feeding. TEC and PLC (Fig. 4) increased steadily throughout the experiment. The highest TEC, TLC, hemoglobin and platelet (thrombocyte) count (PLC) were recorded on day 60 in the group fed with 10 g/kg Carrageenan, (T2) diet.



**Fig 3:** Total haemoglobin (Hb) value during various sampling days (values are mean  $\pm$ SE). Mean values with different superscript within a column for a parameter are significantly different ( $p < 0.05$ ).



**Fig 4:** Total thrombocyte count during various sampling days (values are mean  $\pm$ SE). Mean values with different superscript within a column for a parameter are significantly different ( $p < 0.05$ ).

**Biochemical parameters:** The total protein, albumin, and globulin in serum increased significantly ( $p < 0.05$ ) in the treatment groups fed with *i*-carrageenan supplemented diet and the highest value was observed on day 60 of the experiment. The albumin/globulin ratio (A/G) in the treatment groups (representing higher globulin concentration in serum) decreased significantly ( $P < 0.05$ ) compared to the control group. The highest protein, albumin and globulin level and the lowest A/G ratio was observed on day 60 in the treatment groups fed the T2 diet (Table 2).

Table 2: The total protein, albumin and globulin (g/dl) and A/G ratio (%) values in *Labeo rohita*

Days		Treatments			
		Control	T1	T2	T3
15	Total protein	1.84 $\pm$ 0.05 <sup>a</sup>	*1.86 $\pm$ 0.07 <sup>b</sup>	*2.31 $\pm$ 0.12 <sup>d</sup>	*2.24 $\pm$ 0.05 <sup>c</sup>
	Albumin	1.08 $\pm$ 0.06 <sup>a</sup>	*1.09 $\pm$ 0.02 <sup>b</sup>	*1.19 $\pm$ 0.17 <sup>d</sup>	*1.18 $\pm$ 0.14 <sup>c</sup>
	Globulin	0.76 $\pm$ 0.05 <sup>a</sup>	*0.79 $\pm$ 0.08 <sup>b</sup>	*1.12 $\pm$ 0.11 <sup>d</sup>	*1.06 $\pm$ 0.07 <sup>c</sup>
	A/G Ratio	1.42 $\pm$ 0.13 <sup>d</sup>	*1.23 $\pm$ 0.45 <sup>c</sup>	*1.06 $\pm$ 0.68 <sup>a</sup>	*1.11 $\pm$ 0.44 <sup>b</sup>
30	Total protein	1.79 $\pm$ 0.15 <sup>a</sup>	*2.18 $\pm$ 0.08 <sup>b</sup>	*2.54 $\pm$ 0.22 <sup>d</sup>	*2.38 $\pm$ 0.25 <sup>c</sup>
	Albumin	1.06 $\pm$ 0.09 <sup>a</sup>	*1.16 $\pm$ 0.12 <sup>b</sup>	*1.28 $\pm$ 0.07 <sup>d</sup>	*1.22 $\pm$ 0.07 <sup>c</sup>
	Globulin	0.73 $\pm$ 0.21 <sup>a</sup>	*1.02 $\pm$ 0.28 <sup>b</sup>	*1.26 $\pm$ 0.19 <sup>d</sup>	*1.16 $\pm$ 0.31 <sup>c</sup>
	A/G Ratio	1.45 $\pm$ 0.07 <sup>d</sup>	*1.13 $\pm$ 0.05 <sup>c</sup>	*1.01 $\pm$ 0.38 <sup>a</sup>	*1.05 $\pm$ 0.14 <sup>b</sup>
45	Total protein	1.82 $\pm$ 0.02 <sup>a</sup>	*2.41 $\pm$ 0.07 <sup>b</sup>	*2.95 $\pm$ 0.12 <sup>d</sup>	*2.79 $\pm$ 0.13 <sup>c</sup>
	Albumin	1.11 $\pm$ 0.50 <sup>a</sup>	*1.21 $\pm$ 0.14 <sup>b</sup>	*1.36 <sup>d</sup> $\pm$ 0.05	*1.33 $\pm$ 0.11 <sup>c</sup>
	Globulin	0.71 $\pm$ 0.06 <sup>a</sup>	*1.20 $\pm$ 0.08 <sup>b</sup>	*1.59 $\pm$ 0.12 <sup>d</sup>	*1.46 $\pm$ 0.13 <sup>c</sup>
	A/G Ratio	1.56 $\pm$ 0.23 <sup>d</sup>	*1.01 $\pm$ 0.52 <sup>c</sup>	*0.85 $\pm$ 0.17 <sup>a</sup>	*0.91 $\pm$ 0.05 <sup>b</sup>
60	Total protein	1.90 $\pm$ 0.42 <sup>a</sup>	*2.48 $\pm$ 0.35 <sup>b</sup>	*3.02 $\pm$ 0.49 <sup>d</sup>	*2.86 $\pm$ 0.28 <sup>c</sup>
	Albumin	1.15 $\pm$ 0.19 <sup>a</sup>	*1.22 $\pm$ 0.14 <sup>b</sup>	*1.38 $\pm$ 0.31 <sup>d</sup>	*1.35 $\pm$ 0.19 <sup>c</sup>
	Globulin	0.75 $\pm$ 0.11 <sup>a</sup>	*1.26 $\pm$ 0.26 <sup>b</sup>	*1.64 $\pm$ 0.16 <sup>d</sup>	*1.50 $\pm$ 0.31 <sup>c</sup>
	A/G Ratio	1.53 $\pm$ 0.26 <sup>d</sup>	*0.96 $\pm$ 0.19 <sup>c</sup>	*0.84 $\pm$ 0.22 <sup>a</sup>	*0.90 $\pm$ 0.27 <sup>b</sup>

\*Significant difference between control and treatment group during various sampling days (values are mean  $\pm$ SE). Mean values with different superscript within a column for a parameter are significantly different ( $p < 0.05$ ).

**Innate immune response:** The respiratory burst activity, the NBT assays (Fig. 1) to show the phagocytic activity of mononuclear (monocyte) and polymorphonuclear (PMN) (neutrophils) cells] were analyzed. They were significantly higher ( $p < 0.05$ ) in the *i*-carrageenan diets than in the control throughout the experiment (Fig 1). The MPO enzyme with anti-microbial activity, (Fig 2) had significantly higher ( $p < 0.05$ ) values (Fig 2). The highest NBT and MPO activity was observed in the T2 group (fed the 10 g/kg carrageenan supplemented diet) on 60<sup>th</sup> day of the treatment compared to the T3, T1 and control groups.

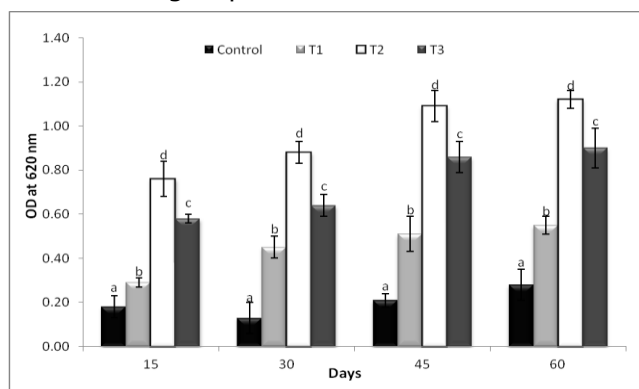


Fig 1: Total NBT (Nitroblue Tetrazolium) values during various sampling days (values are mean  $\pm$  SE). Mean values with different superscript within a column for a parameter are significantly different ( $p < 0.05$ )

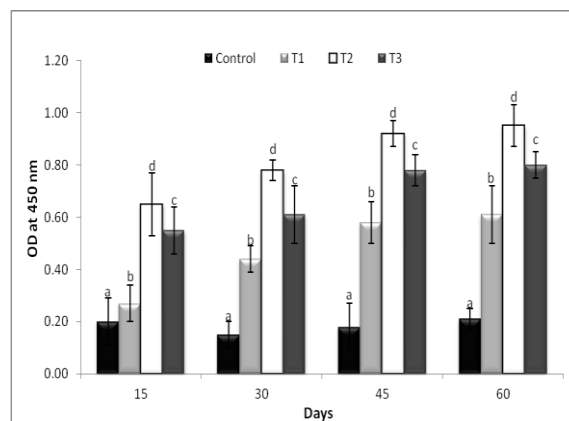


Fig 2: Total Myeloperoxidase activity during various sampling days (values are mean  $\pm$  SE). Mean values with different superscript within a column for a parameter are significantly different ( $p < 0.05$ ).

## Discussion

As fish depend on innate immunity for protection against disease, the effect of a diet incorporating carrageenan on the innate immune response was studied. We found that the hematological, biochemical, and innate immune parameters of rohu which were fed a diet supplemented with carrageenan increased. The overall health status and immune response were not concentration-dependent of carrageenan in the diets. All health status parameters in the fish, (increased total leukocyte count, total erythrocyte count, hemoglobin content, thrombocyte count, total protein content, albumin content, globulin concentration, NBT value and MPO activity) were best in fish fed the T2 diet (containing 10 g/kg Carrageenan). Very little is known about the innate immune response of the fish to oral administration of seaweed polysaccharide.

In this study, dietary administration of *i*-carrageenan significantly improved growth parameters (absolute growth, specific growth rate and percentage weight gain) in the fish fed a supplemented diet. Growth factors were more favorable in the fish fed diets T1, T2, and T3 than the fish fed diet T0 (control). Of the diets containing *i*-carrageenan, diet T2 (10 g/kg) had the optimal impact on growth factors. A similar effect has been reported using "kelp" which includes brown algae *Ascophyllum nodosum*, *Sargassum* spp and *Laminaria digitata*, as dietary supplements (Cruz-Suarez et al. 2000). Dietary administration of alginic acid increased growth and survival of juvenile Chinook salmon *Onchorhynchus tshawytscha*, (Peddie et al. 2005). Oral administration of  $\beta$ -glucan, and EcoActivaTM, as feed supplements enhanced non-specific immune parameters and growth rate of snapper, *Pagrus auratus*, (Cook et al. 2002). Dietary Beta-1,3/1,6 glucans enhanced growth performance and non-specific defense mechanisms in juvenile Dentex, *Dentex dentex*, (Efthimiou, 1996).

Hematological parameters are used as an index of fish health status in a number of fish species to detect physiological changes following different stress conditions (Agrawal and Mahajan 1980). Total erythrocyte count which varies from species to species is also affected by stress and environmental temperature, but usually ranges between 1.05 to  $3.0 \times 10^6/\text{mm}^3$ . The total RBC counts were highest in the T2 group followed by T3 and T1. Similarly, higher TER was reported in azadirachtin fed gold fish (Kumar et al. 2013), *Allium sativum* (Sahu et al. 2004) and n-3 PUFA (Misra et al. 2006) fed *L. rohita* respectively. Similar trends were reported in channel catfish *Ictalurus punctatus* fed a

diet containing *Azadirachta indica* and  $\beta$ -glucan respectively (Scott and Rogers, 1981; Duncan and Klesius, 1996). Total leucocyte cells are known as the first line of defense (Alberts et al. 2006) and play a major role in innate immunity. Increases in TLC along with other immunological parameters are indicators of health status in fish. In our study, increased WBC count was more significant in the T2 group than in the T1, T3, and T0 groups. Similarly the leukocyte count in goldfish fed a diet containing Azadirachtin increased significantly compared to the control group (Kumar et al. 2013). Significantly higher TLC was also reported in treatment groups of *L. rohita* fed yeast RNA (Choudhury et al. 2005) and n-3 PUFA (Misra et al. 2006). Hemoglobin carries dissolved gases, a waste product of metabolism, which is excreted. Hemoglobin content increased along with RBC count. Hemoglobin concentration also increased in goldfish fed Azadirachtin (Kumar et al. 2013). Thrombocytes have an immune function in the body and play a role in phagocytic activity. Although they do not belong to the leukocyte group, the presence of thrombocytes in phagocytic activity are reported in various species of birds (Grecchi et al. 1980; Kajigaya et al. 1985), amphibians (Dias and Sinihorini 1991) and fish (Suzuki 1986; Matushima and Mariano 1996). Thrombocytes increased in the treatment groups during our experiment. The highest value was observed in T2 followed by T3 and T1. Respiratory burst activity generating reactive oxygen radicals is well characterized in macrophages/monocytes and granulocytes (Dalmo et al. 1997). The NBT reduction product, obtained after reaction with superoxide, indicates health status and the immune status in fish (Anderson et al. 1992). After intense activity, fish phagocytes are able to generate superoxide anion ( $O_2^-$ ) and its reactive derivatives (i.e. hydrogen peroxide and hydroxyl radicals) during a period of intense oxygen consumption, called the respiratory burst (Sercombes et al., 1992). A variety of agents, including bacterial products (Lamas et al. 1994), glucans (Misra et al. 2006), yeast RNA (Yang et al. 2013); Sakai et al. 2001), garlic, turmeric powder (Sahu et al. 2004 and 2006), and azadirachtin (Kumar et al. 2012, 2013) are known to stimulate phagocytes. In the present study, higher respiratory activity (NBT value) was observed in T2 group followed by T3 and T1 groups.

Myeloperoxidase is an important enzyme having anti-microbial activity. It utilizes hydrogen peroxidase during the respiratory burst to produce hypochlorous acid (Dalmo et al. 1997). Myeloperoxidase is present in the azurophilic granules of polymorphonuclear (PMN) leukocytes and monocytes at the center of the neutrophils armamentarium (Beutler 2004). The *i*-carrageenan T2 treated group showed increased MPO activity, with higher activity recorded on 45<sup>th</sup> and 60<sup>th</sup> days compared to the other treatment (and control) groups. Oral administration of K-carrageenan and sodium alginate enhances the innate immune response of brown-marbled grouper *E. fuscoguttatus* and also provides resistance against *Vibrio alginolyticus* (Cheng et al., 2008).

Intraperitoneal injection of K-carrageenan and sodium alginate provides resistance against pathogens and increases the innate immune response in common carp *C. carpio* (Fujiki et al. 1994 and 1997; Fujiki and Yano 1997), snakehead *Channa striata* (Miles et al. 2001), rainbow trout *Oncorhynchus mykiss* (Peddie et al. 2002), sea bass *Dicentrarchus labrax* (Begni et al. 2005) and grouper *E. coioides* (Cheng et al. 2007). Goldfish fed with a diet containing Azadirachtin showed higher phagocytic activity and myeloperoxidase activity compared to the control group (Kumar et al., 2013). *L. rohita* fed with mango kernel and *L. rohita* fed with levamisole supported our results (Sahu et al., 2007; Wijendra et al., 2007) however our findings are not confirmed in *L. rohita* fed with diet containing n-3 PUFA (Misra et al., 2006).

The total protein represents the sum of albumins and globulins. Albumin is synthesized by the liver using dietary proteins and its presence in the plasma creates an osmotic force that maintains fluid volume within the vascular space. The globulins are the proteins that include gamma globulins (antibodies) and a variety of enzymes and carrier/transport proteins. Since, gamma fraction usually makes up the largest portion of the globulins, low globulin level is often an indicator of antibody deficiency. Serum albumin and globulin values in fish treated with different immunostimulants were significantly higher than the control (Choudhury et al. 2005). The highest protein and globulin concentration was found in the T2 (10 g/kg carrageenan incorporated) diet group



followed by T3 and T1, on the 45<sup>th</sup> and 60<sup>th</sup> days respectively compared to the control group. In our study, the lowest A/G ratio was found in the T2 group. Since the gamma fraction makes up the largest portion of globulin, it can be inferred this treatment may have enhanced the immune response in *L. rohita*. This result is supported by increased phagocytic activity, myeloperoxidase activity, and leukocyte count. After long term feeding with mango kernel, the serum total protein increased compared with the control diets (Sahu et al. 2007). The present study confirms the findings of previous studies. (Choudhury et al., 2005; Sahu et al., 2007; Wijendra et al., 2007; Kumar et al., 2012, 2013).

Our findings indicate that a diet containing *i*-carrageenan plays an important role in enhancing overall health status and innate immunity in *L. rohita*. The greatest increases in total leukocyte count (TLC), total erythrocyte count (TEC), hemoglobin content (Hb), platelet (thrombocyte) count (PLC), total protein content, albumin content, globulin concentration, NBT (Nitroblue Tetrazolium) activity and Myeloperoxidase (MPO) activity were found in rohu, fed a diet containing *i*-carrageenan (10 g/kg). There was overall health, and immune response improvement in rohu fed diets containing *i*-carrageenan higher or lower than the 10 g/kg (T2) however the optimal dose of *i*-carrageenan was found to be 10 g/kg. Further research is needed to determine the effects on growth and health status of *i*-carrageenan in other major cultured species. Our results add information on the role of immunostimulants in increasing growth and overall health status and provide a basic experimental model for use of an immunostimulant as a growth promoter and immunity enhancer.

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