



Immunostimulatory Effect of Vitamin C on Hemato-biochemical Parameters of *Labeo bata* (Hamilton, 1822)

Lopamudra Sahoo^{1,5}, T. G. Choudhury^{2,3}, C. Debnath¹, J. Parhi^{2*}, M. Datta¹,
C. S. Purusothamman⁴ and K. Paniprasad⁵

¹ ICAR Research Complex for NEH Region, Lembucherra - 799 210, India

² College of Fisheries, Lembucherra - 799 210, India

³ ICAR-Indian Institute of Agricultural Biotechnology, Ranchi - 834 010, India

⁴ ICAR-Central Marine Fisheries Research Institute, Cochin - 682 018, India

⁵ ICAR-Central Institute of Fisheries Education, Mumbai - 400 061, India

Abstract

This study was conducted to evaluate the immunomodulatory effect of vitamin C (Vit. C) on haematological and biochemical parameters in *Labeo bata*. The effect of Vit. C on resistance to challenge with *Edwardsiella tarda* was also investigated. A feeding trial was conducted for a period of 40 days where 240 fishes (10±5 gm) were randomly distributed in to 4 groups in duplicate. Four diets were prepared with different concentration of Vit. C viz., control (0 mg kg⁻¹), low (100 mg kg⁻¹), medium (200 mg kg⁻¹) and high dose (500 mg kg⁻¹). Total erythrocyte count, haemoglobin concentration, total serum protein, albumin and globulin were estimated at 7, 15 and 30 days of feeding and one day and 10 day post challenge with *E. tarda*. It was seen that the RBC count of the group fed medium dose of Vit. C was higher than the rest of the groups after 7 and 15 days of feeding and 10 days post challenge. The haemoglobin concentration of Vit. C treated group was significantly higher than the control. It was also seen that the medium dose of Vit. C (200 mg kg⁻¹ feed) showed better immunity by showing elevated protein, albumin, globulin concentration at higher survival when compared to the control.

Keywords: *Edwardsiella tarda*, *Labeo bata*, immunostimulator, hemato-biochemical

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*E-mail: jparhi@gmail.com

Introduction

Currently aquaculture is witness to the indiscriminate use of a variety of chemotherapeutics including antibiotics and faces a serious threat due to their adverse effects (Iranshahi et al., 2011). Immunostimulants, as alternatives to antibiotics have potential application in aquaculture by improving fish resistance against unfavourable environmental conditions and pathogenic agents compared with other treatment methods (Sakai, 1999).

Ascorbic acid or Vit. C is essential for normal physiological functioning of organisms including fish (Lim & Lovell, 1978) and is a well-known immunostimulator in mammals. Lack of Vit. C in diet increased susceptibility to bacterial diseases in channel catfish (Li & Lovell, 1985). Though most animals can synthesize ascorbic acid from glucuronic acid, fish and crustaceans lack the enzyme gulonolactone oxidase necessary for biosynthesis (Dabrowski, 1990). Vit. E and C play important roles as antioxidants by inactivating damaging free radicals produced through normal cellular activity (Chew, 1995). The antioxidant function helps in enhancing immunity by preserving the functional and structural integrity of important immune cells. Increased levels of Vit. C (mega doses) in the diet have been shown to enhance disease resistance against bacterial pathogens *Edwardsiella ictaluri* and *E. tarda* held in aquaria (Durve & Lovell, 1982) and of salmonids (Blazer, 1982; Navarre, 1985). Specific effects of Vit. C on a variety of non-specific resistance mechanisms and specific immune response has been reported in fish (Hardie et al., 1991; Ortuno et al., 1999).

Labeo bata, is an important cultivable minor carp and is a candidate species meant for diversification north-east states. This species is also vulnerable to diseases as it is a benthopelagic species inhabiting the bottom sediments. It is seen that this species gets infected with fin rot and tail rot, epizootic ulcerative syndrome (EUS) in winter months. Immunomodulation using various agents has been found to improve the immunity of fish. The present study was undertaken to evaluate the immunostimulatory effect of oral administration of Vit. C on the haematological and biochemical parameters of *L. bata*.

Materials and Methods

L. bata fingerlings of average body weight 10 ± 5 gm were obtained from farm facilities of ICAR Research Center for NEH region, Tripura Centre and were retained for acclimatization in FRP tanks of 500 l capacity for 30 days. After a month, fishes were randomly distributed to three treatment groups and one control group for administration of Vit. C in feed. Thirty fishes were kept in each of the duplicate tanks designed for each treatment group. A practical diet (obtained from College of Fisheries, Lembucherra, Tripura, India) was supplemented with Vit. C (Himedia, Mumbai) to give 0 (control), 100 (low dose), 200 (medium dose) and 500 mg (high dose) Vit. C kg^{-1} dry pellet. Proximate composition and percent inclusion of the ingredients in experimental diets are given in Table 1. The diets were fed at the rate of 3% of body weight, once daily for 40 days. After 40 days of feeding, fishes from each treatment group were fed with Vit. C free pellets and were then challenged with *E. tarda*. Fish in the different experimental groups were sampled at weekly intervals for 40 days for estimation of haemato-biochemical parameters of the fish.

On every sampling day on 7th, 15th, 30th days and on day one and 10 day post challenge with *E. tarda*, six fishes were sampled at random from each experimental group and were anaesthetized by dipping for 30s in 0.1 ppm of MS 222 solutions. Blood samples were collected from the caudal vein into micro-centrifuge tubes with a 1 ml pre-heparinised syringe from six fish of each sample group. Serum was prepared and stored at -20°C till use. For the immunostimulation studies, blood and serum samples were collected from same fish more than once. After collection of blood or serum, fish were returned to their respective tanks for the remaining period of experiment.

The different sera samples were analysed for total protein following the method of Bradford (1976) using bovine serum albumin (BSA), albumin by Doumas et al. (1971) and globulin (subtracting albumin from total protein).

Red blood cells were counted using a haemocytometer. Haemoglobin content of blood samples was measured by cyanomethemoglobin method as described by Van Kampen & Zijlstra, (1961). Absorbance was measured using a spectrophotometer at 540 nm and the final concentration was calculated by comparing with the standard cyanmethaemoglobin (Qualigen, India). Haemoglobin concentration was then calculated by using the following formula:

$$\text{Haemoglobin (g dl}^{-1}\text{)} = [\text{OD (T)/ OD (S)}] \times [251/1000] \times 60$$

Where OD (T) is the absorbance of the test, OD (S) is the absorbance of the standard.

After feed trials fish in various experimental groups were challenged with the pathogenic isolates of *E. tarda*. For this, the pathogenic isolates of *E. tarda* were grown in nutrient broth for 24 h at 30°C . The cells were harvested and washed twice in sterile PBS and then suspended in PBS at a concentration of 10^6 cells ml^{-1} . The fishes in each experimental group were given intraperitoneal injection of 0.1 ml suspension of *E. tarda* (1.2×10^6 cfu fish^{-1}) in PBS. Cumulative mortality (%) patterns were observed in the challenged fishes.

The relative percent survival (RPS) in different treatment groups were calculated as:

$$\text{RPS} = 1 - (\text{no. of mortality in treatment group/no. of mortality in control group}) \times 100$$

One-way analysis of variance (ANOVA) was used for comparing means and multiple comparisons were carried out using Duncan's new multiple range tests. Statistical significance was determined by setting the aggregate type I error at 5% ($p < 0.05$) for each set of comparisons.

Results and Discussion

According to Raa (2000) immunostimulants are chemical substances that activate immune system and thus render animals more resistant to infections by improving non-specific defence mechanism (Selvaraj et al., 2005a;b). Vit. C as an immunostimulant has been evaluated by Durve &

Lovell (1982 on catfish, by Blazer (1982) on salmonids, groupers (Lin & Shiau, 2005), Rohu (Tewary & Patra, 2008) channel cat fish (Durve & Lovell, 1982).

Total erythrocyte count and haemoglobin concentration are used as health indicator for fishes. In the present study the increase in erythrocyte count in Vit. C treated fish indicates positive health effect on fishes. Previous studies have shown that immunostimulants could increase immune functions by affecting the blood cells (Nya & Austin, 2009; Sahu et al., 2007).

The RBC count of the group fed 200 mg Vit. C kg⁻¹ feed was higher than other groups after 7 and 15 days of feeding and 10 days post challenge (Table 2). Haemoglobin in blood plays a vital role and carries oxygen to tissues. Haemoglobin concentration of Vit. C treated group was significantly higher than the control group (Table 2). This primarily indicates that Vit. C increases the oxygen supply in the blood of the fishes reflecting beneficial effect on the health of the fishes. The significant decrease of haemoglobin concentration post challenge may be due to swelling of RBC as well as poor mobilisation of haemoglobin from spleen (Scott & Rogers, 1981). It could also be as result of hemolysin produced by *E. tarda*

Infected fish fed with control diet had decreased RBC count and haemoglobin levels, which may be a result of erythroblastosis. Decreased RBC and haemoglobin were also seen in *L. rohita* fed control diet without supplementation of chitin or chitosan (Aathi et al., 2013).

There was a significant increase in total serum protein after 7 days of feeding in different groups

Table 1. Proximate composition and percent inclusion of the ingredients in experimental diets

Proximate Component	Percent Composition
Moisture	5.5
Crude Protein	21.0
Crude Lipid	2.6
Crude Fiber	15.7
Ash	10.6
Nitrogen free extract/ Digestible carbohydrate	45

receiving Vit. C compared to the control groups (Table 2). On subsequent assays there was no significant difference between the treated and control groups although the value was always higher in the Vit. C fed groups. It was seen that serum protein of the control group was significantly less than the protein of the immunostimulated group, one day post challenge with *E. tarda*. After 10 days post challenge there was no significant difference between the control and treated group but the values were higher in the treated groups.

Increase in serum protein, albumin and globulin is associated with stronger innate immune response in fish (Wiegertjes et al., 1996). Serum albumin and globulin values are always higher in the fish treated with different immunostimulants (Choudhury et al., 2005). In this study it was seen that the serum protein of the Vit. C fed group was higher than the control after 7 days of feeding. Although after 15 and 30 days of feeding significant difference was not seen, yet the values of the Vit. C fed group were higher than the control group.

The same holds true for albumin concentration of the fish where it was found that although there was no significant difference between treatment and control groups, the albumin concentration of the Vit. C fed group was higher than the control group as observed from Table 2. Globulins like gamma globulins are essential for maintaining a healthy immune system. Increase in globulin fraction in medium dose of Vit. C (Table 2) indicates that Vit. C has enhanced the immunity status of *L. bata* to combat infections. Serum proteins include various humoral elements of the non-specific immune system and high concentrations of total serum protein, albumin and globulin indicate enhancement of non-specific immune response of fishes (Abasali & Mohamad, 2010). Concentration of albumin and globulin in fishes fed with 200 mg Vit. C kg⁻¹ feed were also higher. Medium dose of Vit. C showed better immunity indicated by elevated protein levels compared to the control and other dose-groups. Concentration of albumin and globulin in fishes fed with 200 mg Vit. C kg⁻¹ feed was also higher. This indicates that Vit. C incorporated at the rate of 200 mg kg⁻¹ feed is optimum for *L. bata*

The relative per cent survivability of treatment groups fed with Vit. C at 100, 200 and 500 mg kg⁻¹

Table 2. Serum biochemical and haematological parameters observed on various assay days during the dietary immunostimulation trial with Vitamin C

Parameters	Groups	7 th Day	15 th Day	30 th Day	1DPC	10DPC
RBC (millions/cumm)	Control	1.30±0.08 ^a	2.55±0.06 ^c	1.08±0.07 ^a	1.28±0.12 ^a	1.33±0.17 ^a
	100 mg kg ⁻¹	1.47±0.07 ^b	1.78±0.28 ^b	1.62±0.06^c	1.74±0.19^b	1.37±0.10 ^a
	200 mg kg ⁻¹	1.70±0.08^c	2.67±0.35^c	1.14±0.07 ^{ab}	1.40±0.08 ^a	1.42±0.17^a
	500 mg kg ⁻¹	1.16±0.11 ^a	1.13±0.06 ^a	1.27±0.18 ^b	1.54±0.28 ^{ab}	1.25±0.06 ^a
Haemoglobin (g dl ⁻¹)	Control	8.39±1.26 ^a	7.86±1.01 ^a	7.82±0.73 ^a	8.43±0.30 ^a	8.06±0.67 ^a
	100 mg kg ⁻¹	10.42±0.34 ^b	10.10±0.23 ^{bc}	10.36±0.40 ^b	9.85±0.75 ^{bc}	9.83±0.43 ^b
	200 mg kg ⁻¹	10.10±0.55 ^b	10.00±0.56 ^b	9.97±0.70 ^b	9.49±0.85 ^{ab}	9.94±0.52^b
	500 mg kg ⁻¹	10.77±0.52^b	11.09±0.50^c	10.63±0.72^b	10.78±0.70^c	9.79±0.71 ^b
Total Serum Protein (g dl ⁻¹)	Control	3.12±0.11 ^a	3.96±0.54 ^{ab}	3.58±0.36 ^a	3.61±0.34 ^a	3.61±0.67 ^a
	100 mg kg ⁻¹	5.91±0.57^b	4.20±1.29 ^{ab}	3.59±0.35 ^a	4.71±0.32 ^b	3.38±0.50 ^a
	200 mg kg ⁻¹	5.56±0.55 ^b	4.61±0.39^b	5.13±0.58^b	5.00±0.31^b	4.32±0.86^a
	500 mg kg ⁻¹	5.63±0.42 ^b	3.03±0.67 ^a	4.03±0.33 ^a	4.21±1.12 ^{ab}	3.74±0.43 ^a
Albumin (g dl ⁻¹)	Control	1.54±0.26 ^a	1.48±0.22 ^a	0.86±0.19 ^a	0.97±0.05 ^a	0.94±0.08 ^a
	100 mg kg ⁻¹	2.65±0.37^b	1.17±0.13 ^a	1.23±0.39 ^{ab}	1.57±0.44 ^{ab}	1.26±0.28 ^a
	200 mg kg ⁻¹	1.81±0.22 ^a	1.30±0.38^a	1.66±0.24^b	2.41±0.29^c	2.36±0.56^b
	500 mg kg ⁻¹	1.89±0.22 ^a	1.27±0.43 ^a	1.56±0.28 ^b	1.88±0.64 ^{bc}	1.04±0.17 ^a
Globulin (g dl ⁻¹)	Control	1.58±0.31 ^a	2.71±1.22 ^{ab}	2.07±0.61 ^a	1.73±0.32 ^a	1.66±0.63 ^a
	100 mg kg ⁻¹	3.26±0.51 ^b	2.62±0.82 ^{ab}	2.36±0.64 ^b	2.04±0.55 ^a	2.12±0.63 ^{ab}
	200 mg kg ⁻¹	3.76±0.57^b	3.44±0.38^b	2.37±0.46^b	2.59±0.33^b	2.69±0.54^b
	500 mg kg ⁻¹	3.73±0.23 ^b	1.76±0.73 ^a	2.01±0.28 ^a	2.33±1.74 ^{ab}	1.96±0.54 ^a

The observed values were expressed as mean ± standard deviation. Mean values with different superscripts are significantly (pd≤0.05) different from each other

DPC: Days Post Challenge with bacteria *Edwardsiella tarda*

feed were found to be 74, 79.5 and 74 respectively. Highest relative percent survival was seen in the medium dose group. Low dose and high dose groups had the same relative percent survivability. This study shows that supplementation of Vit. C had a positive influence on the survival of *L. bata* by resisting the *E. tarda* infection.

From the present study it can be concluded that fishes fed with Vit. C (200 mg kg⁻¹ feed) for a short term period of 30 days fight infections more effectively than the fishes which are not fed Vit. C. Vit. C exhibits a beneficial effect on the health of *L. bata* by enhancing the haematological and protein parameters of the fish. This in turn aids the fish to resist infection. Medium dose of Vit. C (200 mg kg⁻¹) showed better results as compared to low and high doses which were tried in the experiment.

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