

# Effects of Supplementation Diet Containing *Microcystis aeruginosa* on Haematological and Biochemical Changes in *Labeo rohita* Infected with *Aeromonas hydrophila*

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## Abstract

An experiment of 100 days duration was conducted to test the effect of *Microcystis aeruginosa* in *Labeo rohita* against *Aeromonas hydrophila*. The rohu fingerlings ( $22 \pm 2$  g) were fed with the experimental diets incorporated with different concentrations of *M. aeruginosa* @ 0 g kg<sup>-1</sup>, 0.5 g kg<sup>-1</sup>, 1 g kg<sup>-1</sup> and 5 g kg<sup>-1</sup>. Replicate groups of fish were fed for three months daily @4% body weight. At an interval of 30 days blood and serum samples were assayed for different haematological [total erythrocyte count (TEC), total leucocyte count (TLC), haemoglobin] and biochemical [blood glucose, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP)] parameters. Significantly ( $p \leq 0.05$ ) increased haemoglobin content and TLC was observed in *Microcystis* treated group. It was observed that the serum AST activity was significantly ( $p \leq 0.05$ ) decreased to all the treated groups of fish as compared to control on entire assay period. Serum ALT activity was significantly ( $p \leq 0.05$ ) different to all the treated groups of fish on day 60, on day 90 and on day 10 (except group B) bacterial post challenge as compared to control. After 90 days, fish were challenged with *A. hydrophila* and mortality (%) was recorded up to day 10 post challenge. Highest percentage of survival (72%) was noticed in the group fed 1.0 g *Microcystis* kg<sup>-1</sup> dry diet. The present study suggests that the administration of bluegreen algae, *Microcystis aeruginosa* supplementation diets for 100 days protects the hematological and biochemical parameters in *L. rohita* from *A. hydrophila*.

**Keywords:** *Aeromonas hydrophila*; ALP; AST; Haemoglobin; *Microcystis aeruginosa*; *Labeo rohita*

## Introduction

Nutrient supplementation in fish diets has been an economically promising method for improving the performance of different intensive fish production systems. To enhance a more economically sustainable aquaculture in the current millennium, many feed ingredient alternatives to fish meal at varying levels are now being sought. Thus functional feed additives strategy has recently gained considerable attention. From nutritional point of view, it does not only provide the essential nutrients required for normal physiological functioning, but also serve as a medium by which fish receive other components that may positively affect their health [1]. Several sectors of the aquaculture industry would benefit if cultured organisms were conferred with enhanced feed efficiency, growth performance, and disease resistance without environmental conflicts [2]. The other methods of preventing disease include immunostimulation through alteration of the diet and feeding practices [3]. In aquaculture, there are many studies reporting a variety of substances including bacterial [4], algal [5], animal and plant products [6-9] can be used as immunostimulants to enhance non-specific immune system of cultured fish species. With a detailed understanding of the efficacy and limitations of immunostimulants, they may become powerful tools to control fish diseases.

*Microcystis aeruginosa* (Kützing) is a unicellular, colonial blue-green alga (family Chroococcaceae [10]). Ingestion of waters containing high concentrations of *Microcystis* can cause abdominal stress in humans leading to precautionary beach closures and can kill dogs and farm animals if they drink significant quantities of the bloom waters. However, due to their adverse effects on higher organisms, several cyanobacterial metabolites are regarded as health-threatening toxins and have caused serious concern among water authorities worldwide. But *Microcystis* has been recognized in recent years as a producer

of a high number of secondary metabolites. *Microcystis aeruginosa* shows antibacterial and anti viral activity against the Gram-positive bacterium *Staphylococcus aureus*, some selected Gram-negative fish pathogens and influenza A virus [11-14]. It has been also reported that *Microcystis* stimulates the immunity and makes *L. rohita* more resistant to infection by *A. hydrophila* when fed in dried form in feed [15].

Fish should be fed with a balanced diet as nutritional deficiency can have an adverse impact on disease resistance. The analysis of blood indices has proven to be a valuable approach for analyzing the health status of farmed animals as these indices provide reliable information on metabolic disorders, deficiencies and chronic stress status before they are present in a clinical setting [16]. Haematological study is of immense importance when diagnostically evaluating fish health as in human health [17]. Enzymes are biochemical macromolecules that control metabolic processes of organisms, thus a slight variation in enzyme activities would affect the organism [18]. Thus, by estimating the enzyme activities in an organism, we can easily identify disturbances in its metabolism. In this study we monitored the disturbance of metabolism in fingerlings of *L. rohita* by feeding them

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to *Microcystis* diet. This study was designed to evaluate the effect of feeding of *Microcystis* in the raw forms at different dietary levels on some haematological and biochemical profile of infected fish in *L. rohita* against *A. hydrophila*.

## Material and Methods

### *Microcystis aeruginosa*

*Microcystis* bloom was collected from Bindusagar, Bhubaneswar, India, with the help of plankton net made of bolting silk cloth (mesh size, 20  $\mu$ ). Collected samples were washed three times in MilliQ (Millipore, USA) water to remove suspended particles adhered to it and finally filtered (Whatman Filter paper Size 40). Harvested *Microcystis* (514 g wet weight) was dried under room temperature for 2-3 days and the dry weight of the algae was 110 g. Then it was powdered. For each experiment, the required quantity of *Microcystis* powder was included in the feed.

### Pathogen

*Aeromonas hydrophila* (ATCC 49140) was cultured in nutrient broth (Himedia) for 24 h at 37°C. The culture broth was centrifuged at 3000xg for 10 min. The supernatant was discarded and the pellet was resuspended in phosphate buffered saline (PBS, pH 7.4), and the OD of the solution was adjusted to 1.5 at 456 nm, which corresponded to  $1 \times 10^7$  cells ml<sup>-1</sup>. These bacterial suspensions were serially diluted using standard dilution technique with PBS and used for the challenge experiment.

### Preparation of fish feed

The proximate composition of the basal diet was 39.4% crude protein, 7.4% lipids, 14.6% ash, 7.1% moisture and 3% fibre as per the

Ingredients	Quantity included (g kg <sup>-1</sup> diet)
Groundnut oil cake	400
Fish meal	250
Rice bran	200
Soya bean meal	120
Vitamins and minerals mixture	20
Starch	10
Composition	Quantity (kg <sup>-1</sup> ) vitamin-mineral mixture
Vitamin A	20 00 000 IU
Vitamin D3	4 00 000 IU
Vitamin B2	0.8 g
Vitamin E	300 IU
Vitamin K	0.4 g
Calcium paththothernate	1 g
Nicotinamide	40 g
Vitamin B12	2.4 g
Choline chloride	60 g
Calcium	300 g
Mangnese	11 g
Iodine	0.4 g
Iron	3.0 g
Zinc	6.0 g
Copper	0.8 g
Cobalt	0.18 g

Calculated crude protein: 40%; estimated crude protein: 39.6%; calculated lipid content: 5%; estimated lipid content: 7.2%.

Supplies the above vitamin–mineral premix (Suplevite M) used in feed formulation per kg of feed were procured from Sarabhai Chemicals, Wadi, Baroda, India.

**Table 1:** Percentage inclusion of ingredients in basal diet with desired crude protein and lipid level [19].

method described by Misra et al. [19] (Table 1). Three experimental diets were formulated by including the required proportions of different feed ingredients such as ground nut oil cake, rice bran, fish meal, soyabean meal, vitamin & mineral mixture [7]. Powdered forms of *M. aeruginosa* were added to the above formulation at the rate of 0.1 g, 0.5 g and 1.0 g Kg<sup>-1</sup> feed. Dry ingredients were mixed thoroughly and 1% binder was added. Water was added and mixed thoroughly in a mixer for 20 min. The resulting dough was pelleted, dried at room temperature for 48 h and then stored in airtight containers until fed.

### Experimental design

For experiment, total 240 numbers of acclimatized rohu fingerlings were taken and divided into four groups (A, B, C and D) with feeding of *Microcystis* at the rate of 0, 0.5, 1.0 and 5.0 g kg<sup>-1</sup> feed for 90 days. Duplicate tanks containing 30 numbers of fish were maintained for each group. After 90 days of feeding trail, 15 fish of duplicate tanks of each group were challenged intraperitoneally with bacterial pathogen, *A. hydrophila* at the rate of  $1 \times 10^5$  CFU per fish. Blood and serum samples were collected from each group and examined for the following parameter, such as total erythrocyte count, total leucocyte count, haemoglobin, glucose, serum aspartate amino transferase, serum alanine amino transferase and alkaline phosphatase.

### Sampling

Feed was suspended from fish for 24h before blood samples were collected. From randomly picked fish (n=20 from each subgroup) at 30-day intervals, after anaesthetizing with 0.1 ppm MS-222. Blood was collected from the caudal vein with a 1-mL plastic syringe ringed with heparin and stored at 4°C and used the same day. Blood samples were also collected without heparin, allowed to clot, centrifuged at 7000 g and sera collected and refrigerated. From each group twelve and eight fishes were sampled for serum and blood, respectively, and kept in a separate tank. Sera and blood were pooled into six groups, depending upon volume, for estimation of immunological and biochemical parameters [15].

### Determination of haematological parameters

The collected blood samples were immediately subjected to haematological analysis. The haemoglobin content of different blood samples collected was measured by cyanomethemoglobin method as per Van Kampen and Zijlstra [20]. Reagent solution (5 ml) containing potassium hexacyanoferrate (III) solution (potassium hexacyanoferrate III 0.6 mmol/l, potassium phosphate buffer 0.5 mmol/l; pH 7.2), potassium cyanide solution (potassium cyanide 0.75 mmol/lit, potassium phosphate buffer 2.5 mmol/l; pH 7.2 and detergent 0.1 g/l) was taken in a cleaned and dry test tube and then 0.002 ml of blood was added to it. Simultaneously a blank reading was taken. It was mixed thoroughly and incubated at 20-25°C. Absorbance was taken at an optical density 546 nm using the Bio-Rad Spectrophotometer. The haemoglobin was expressed as g%. The blood was diluted with appropriated diluting fluids for total erythrocyte count and total leucocyte counts were determined using improved Neubauer haemocytometer and calculated [21]. Replicated counts were made for each blood samples. The TEC and TLC count was expressed as cells per mm<sup>3</sup>.

### Determination of blood/serum biochemical Parameters

The different sera samples collected earlier were analysed for AST and ALT following the procedure of Wallnofer et al. [22] using diagnostic kits (Bayer Diagnostics, Baroda, India). Serum ALP was

determined by the procedure of Rosalki et al. [23]. Blood glucose content was estimated following the procedure of Schmidt [24] using standard kits (as per the manufacture’s instructions; Roche diagnostic).

### Challenge of fish

After 90 days of feeding, 15 fish from each subgroup was challenged intraperitoneally with a lethal dose of *A. hydrophila* ( $1 \times 10^5$  CFU per fish) and observed for a 10-day period for mortality. Cumulative mortality percentage was determined over 10 days. Haematological and biochemical parameters were assayed in post challenged groups of survived fish as per the methods described earlier.

### Statistical analysis

All experiments were performed in duplicate. All statistical analyses were performed by the Statistical Analysis System (SAS) program package. Data were expressed as mean  $\pm$  standard error (S.E). The data were analyzed by the one-way analysis of variance (ANOVA) and mean differences among experimental groups were evaluated using Duncan’s multiple range tests (DMRT) at the  $p \leq 0.05$  significance level [25].

## Results

### Haematological indices

Significantly ( $p \leq 0.05$ ) different haemoglobin content was observed in group of fish fed with  $5 \text{ g kg}^{-1}$  *Microcystis* (D) on day 60, B on day 90 and C on day 10 bacterial post challenge period. However, the group of fish fed with *Microcystis* powder showed insignificant ( $p > 0.05$ ) difference haemoglobin level as compared to control on day 30 day of exposure period.

Insignificant difference ( $p > 0.05$ ) of TLC was found in *Microcystis* fed fish on day 30 of exposure period. The entire treatment group showed significantly ( $p \leq 0.05$ ) different TLC as compared to control on day 90 and on day 100 (i.e. day 10 post challenge) exposure period. Similarly the significant ( $p \leq 0.05$ ) difference was observed in B and D as compared to control on day 60 of observation period.

The group of fish fed with *Microcystis* showed significantly increased ( $p \leq 0.05$ ) TEC in C on day 90 and on day 10 post challenge period as compared to their respective control. An insignificant ( $p > 0.05$ ) difference TEC was found in the entire treatment groups as compared to control on day 30 and on day 60 day of exposure period (Table 2).

### Serum/blood biochemical indices

The group of fish fed with *Microcystis* was found a significantly ( $p \leq 0.05$ ) decrease of blood glucose level in the treatment groups as compared to control at all the day of exposure period (except B on day 60 and day 90). There is no significant change of blood glucose level was observed in the entire experimental group in comparison to control on day 90 (Figure 1).

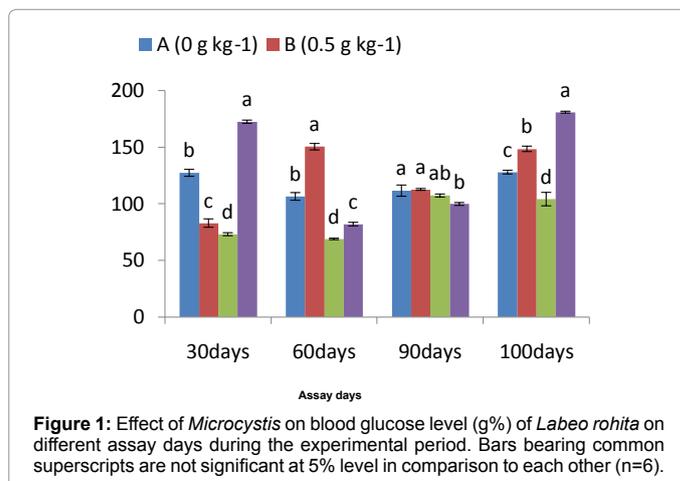
The level of AST was significantly ( $p \leq 0.05$ ) different to all the treated groups of fish on day 60 (except group D), day 90 and after bacterial post challenge as compared to control. But no significant difference was found within treatment groups (except B) on day 30 of observation period (Figure 2).

The group of fish fed with *Microcystis* showed a significantly ( $p \leq 0.05$ ) different in ALT activity on day 60, day 90 and on day 10 post challenge (except group of fish fed  $0.5 \text{ g kg}^{-1}$ ) as compared to their respective control. But there was no significant difference was noticed between treated groups and control on day 30 of exposure period

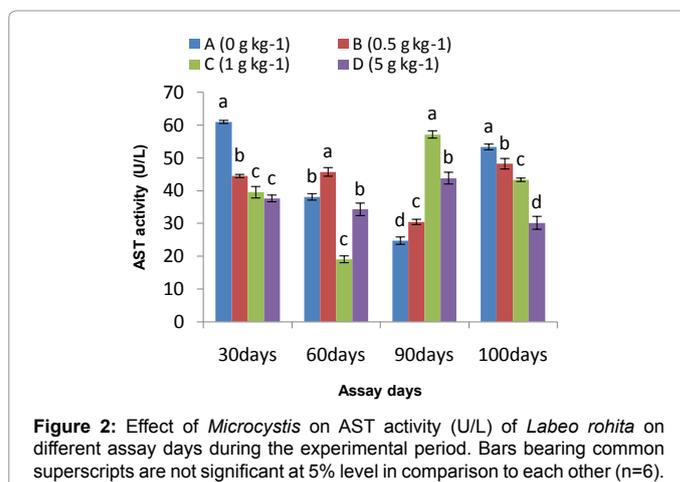
Parameters	Groups	Pre challenge				Post challenge
		30 days	60 days	90 days	100 days	
HB (g%)	A	8.20 $\pm$ 0.63 <sup>a</sup>	7.00 $\pm$ 0.69 <sup>b</sup>	7.55 $\pm$ 0.95 <sup>b</sup>	8.01 $\pm$ 0.22 <sup>b</sup>	
	B	7.65 $\pm$ 0.32 <sup>a</sup>	8.24 $\pm$ 0.46 <sup>ab</sup>	10.25 $\pm$ 0.3 <sup>a</sup>	7.45 $\pm$ 0.60 <sup>b</sup>	
	C	9.22 $\pm$ 0.46 <sup>a</sup>	8.35 $\pm$ 0.52 <sup>ab</sup>	9.22 $\pm$ 0.41 <sup>ab</sup>	9.99 $\pm$ 0.38 <sup>a</sup>	
	D	8.02 $\pm$ 0.62 <sup>a</sup>	9.65 $\pm$ 0.27 <sup>a</sup>	8.002 $\pm$ 0.3 <sup>b</sup>	8.25 $\pm$ 0.42 <sup>b</sup>	
TLC	A	14.02 $\pm$ 0.60 <sup>a</sup>	13.22 $\pm$ 0.57 <sup>c</sup>	14.57 $\pm$ 0.56 <sup>b</sup>	15.96 $\pm$ 0.55 <sup>c</sup>	
	B	13.98 $\pm$ 1.07 <sup>a</sup>	19.25 $\pm$ 0.59 <sup>a</sup>	20.00 $\pm$ 0.67 <sup>a</sup>	19.22 $\pm$ 0.40 <sup>b</sup>	
	C	15.21 $\pm$ 0.47 <sup>a</sup>	15.02 $\pm$ 0.41 <sup>bc</sup>	19.11 $\pm$ 1.00 <sup>a</sup>	18.19 $\pm$ 0.73 <sup>b</sup>	
	D	14.52 $\pm$ 0.72 <sup>a</sup>	16.66 $\pm$ 0.72 <sup>b</sup>	20.21 $\pm$ 0.39 <sup>a</sup>	22.33 $\pm$ 0.69 <sup>a</sup>	
TEC	A	0.71 $\pm$ 0.12 <sup>a</sup>	0.81 $\pm$ 0.05 <sup>a</sup>	0.73 $\pm$ 0.08 <sup>b</sup>	0.94 $\pm$ 0.09 <sup>b</sup>	
	B	0.82 $\pm$ 0.05 <sup>a</sup>	0.92 $\pm$ 0.06 <sup>a</sup>	0.82 $\pm$ 0.04 <sup>b</sup>	0.90 $\pm$ 0.03 <sup>b</sup>	
	C	0.73 $\pm$ 0.04 <sup>a</sup>	0.99 $\pm$ 0.08 <sup>a</sup>	1.22 $\pm$ 0.15 <sup>a</sup>	1.50 $\pm$ 0.05 <sup>a</sup>	
	D	0.78 $\pm$ 0.09 <sup>a</sup>	1.23 $\pm$ 0.21 <sup>a</sup>	0.87 $\pm$ 0.08 <sup>b</sup>	0.92 $\pm$ 0.19 <sup>b</sup>	

Note: Data are expressed as mean  $\pm$  S.E. Superscript column wise on right hand side for particular treatment group are significantly ( $P < 0.05$ ) different from the control group. MaC-Control group, Ma1-0.5 gkg<sup>-1</sup>, Ma2-1.0 gkg<sup>-1</sup> and Ma3-5.0 gkg<sup>-1</sup> *Microcystis aeruginosa* fed group.

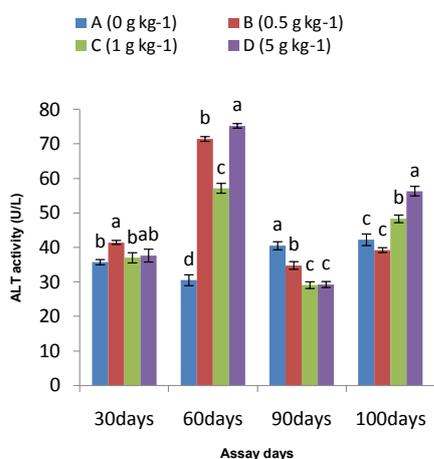
**Table 2:** Effect of oral feeding of *Microcystis* powder on haematological parameters of *L. rohita* followed by i. p. challenge of *A. hydrophila* after 90 days.



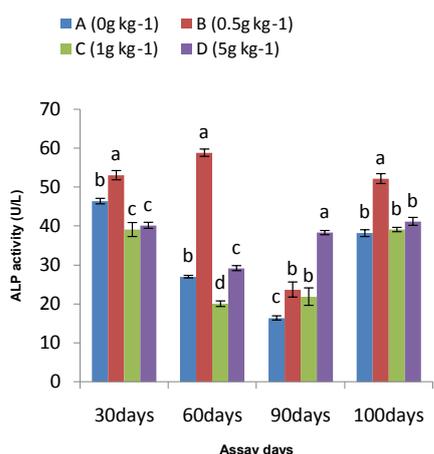
**Figure 1:** Effect of *Microcystis* on blood glucose level (g%) of *Labeo rohita* on different assay days during the experimental period. Bars bearing common superscripts are not significant at 5% level in comparison to each other (n=6).



**Figure 2:** Effect of *Microcystis* on AST activity (U/L) of *Labeo rohita* on different assay days during the experimental period. Bars bearing common superscripts are not significant at 5% level in comparison to each other (n=6).



**Figure 3:** Effect of *Microcystis* on ALT activity (U/L) of *Labeo rohita* on different assay days during the experimental period. Bars bearing common superscripts are not significant at 5% level in comparison to each other (n=6).



**Figure 4:** Effect of *Microcystis* on ALP activity (U/L) of *Labeo rohita* on different assay days during the experimental period. Bars bearing common superscripts are not significant at 5% level in comparison to each other (n=6).

(Figure 3). A significant ( $p \leq 0.05$ ) difference in ALP activity was found in all pre challenge treatment groups as compared to control in each assayed period as shown in Figure 4. But there was insignificant ( $p > 0.05$ ) difference of ALP level was found on day 10 bacterial post challenge (except B) as compared to control.

### Disease resistance

After challenging the fish with *A. hydrophila* the mortality was recorded up to 10th day. There was no mortality of fish up to 18 h post infection. The maximum survival was shown in group of fish fed 0.5g kg<sup>-1</sup> followed by 1.0g kg<sup>-1</sup>.

### Discussion

*Microcystis* besides producing toxins like Microcystin LR [26] it was found that it has potential use in the control of fish and shellfish diseases and also as immunostimulant [13,15]. Haematological and biochemical parameters have been acknowledged as valuable tools for monitoring fish health. So it is important to monitor the health status of fish after administration of *Microcystis* as feed additive in basic diet of fish.

Results of the haematological parameters of rohu fingerlings in this study showed that there were significant differences ( $p \leq 0.05$ ) among different dietary groups. It has been shown that variables such as age, sex, dietary state and stress alter blood values [27,28]. In present study the total leucocyte count significantly ( $p \leq 0.05$ ) increased in *Microcystis* fed group. This result is supported by another study [18], which found that there was an increase in the WBC count when *L. rohita* fingerlings were fed with *Mango kernel*. The increase in total leucocyte, neutrophils, lymphocytes and monocytes following feeding of algal and herbal diets supports the notion of antimicrobial properties of the algae [14,29] and traditional herbal medicine [17,30]. The white blood cells of rohu are known to increase following immunostimulation [31,32] and feeding with algal diets for 90 days [5]. The result of present investigation shows that the total erythrocyte counts increased in group of fish fed with 1g kg<sup>-1</sup> *Microcystis* on day 90 and on day 10 after postchallenge can be correlated with the observation in Nile tilapia, *O. niloticus* that showed decreased RBC number after bacterial inoculation [33]. No significant difference of total erythrocyte count among the groups. The erythrocyte count increased with the administration of *Microcystis*, which might indicate an immunostimulant effect. The findings conform to those by Duncan and Klesius [34], who reported that the number of erythrocytes was significantly ( $p \leq 0.05$ ) greater in channel catfish fed with a diet containing  $\beta$ -glucan.

Glucose is one of the most important sources of energy for the animals. It has been reported as an indicator of stress caused by physical factors [35]. Blood glucose level in all the group of fish treated with *M. aeruginosa* supplemented diet showed no significant variation with the treatment groups on day 90 and after post challenge on day 10 (except group B and D). It shows that the fish were not under stress when fed with optimum level of *Microcystis* supplemented diets. Similar types of observation with different types of dietary supplementation were observed [36].

The present study was targeted to find out its impact on the fish by evaluating the major stress enzyme to liver taking a model fish rohu which contributes about more than 80% to the freshwater aquaculture production in India. The ALT and AST are indicators for the diagnosis of liver function [37] and damage [38]. Decreased AST and ALT activity in serum showed that oxaloacetate and glutamate are not available to Krebs cycle through this route of transmission [39]. Microcystins have been reported to impact the liver of fish [40,41]. ALT activity in the liver of fish fed the high algae meal diet was lower than that of the fish fed the control diet in the present study. Rabergh et al. [40] and Navratil et al. [42] reported that ALT and AST activities in blood plasma increased after common carp (*Cyprinus carpio L.*) received intraperitoneal injections of Microcystin-LR. However in our present study, dietary *Microcystis* did not increase the liver enzymes e.g. AST and ALT which might be attributed either to absence or less availability of Microcystin-LR. Increased activity of ALP was marked in group of fishes fed with *Microcystis* over the days and a significantly ( $p \leq 0.05$ ) higher ALP activity was observed in group of fish fed with 0.5 g kg<sup>-1</sup> dose for 60 days. Increased phosphatase activity indicates higher breakdown of energy reserved which are utilized for growth and survival of fishes. All these results indicate that *Microcystis* increases the resistance of *L. rohita* so that it can withstand the adverse conditions of a challenge. However, appropriate field trials remain necessary before using *Microcystis* as a feed additive in aquaculture farm.

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