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Note

Effect of dietary *chlorella vulgaris* on liver enzymatic profiles of rohu *Labeo rohita* (Hamilton, 1822)

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ABSTRACT

Labeo rohita fingerlings (25 ± 2 g) were fed on diets supplemented with *Chlorella vulgaris* powder at the rate of 0, 0.1, 0.5 and 1.0 g kg^{-1} for a period of 90 days. Blood and serum samples were collected at 30 days interval and assayed for enzymatic parameters *viz.*, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP). After 90 days feeding fish were challenged with a pathogenic strain of *Aeromonas hydrophila* and mortalities were recorded over 10 days post-challenge. Serum AST and ALT activity were significantly ($p \leq 0.05$) different in all the treated groups of fish on day 30 and on day 10 bacterial post-challenge as compared to control. The highest survival on 10^{th} day post-challenge (86.5%) was recorded in group fed with 0.1% *Chlorella* supplemented diets.The intake of *C. vulgaris* at different dosages altered the AST, ALT and ALP activity, but the variation was within the normal ranges that did not hamper the growth of fish. The study indicated that dietary supplementation of *C. vulgaris* did not negatively affect liver function, and was found to have growth promoting activity in rohu.

Keywords**:** *Aeromonas hydrophila*, *Chlorella vulgaris*, *Labeo rohita,* Liver specific enzymes

Introduction

In aquaculture, proper diet has long been recognised as a necessity for maintaining health as well as ability to resist diseases in cultured animals. Use of feed additives in aquaculture has received considerable attention in recent years. In India, most of the farmers rely on mixture of rice bran and mustard oil cake together with natural pond fauna for carp culture. Microalgae have of a broad spectrum of nutritious compounds including proteins, vitamins, essential amino acids, minerals and pigments (Becker, 2007). Among microalgae, *Chlorella* is widely distributed in nature, especially in freshwater bodies. *Chlorella* can survive by photoautotrophy as well as heterotrophy ustilising external carbon source. Hence, *Chlorella* is easily cultured in the laboratory and possesses high applied value (Yamaguchi, 1996). It has been proved that *Chlorella* has high content of proteins, lipids, polysaccharides, vitamins, minerals, and other nutritional substances, including substances of significant bioactivity (Han *et al.,* 2002; Kang *et al.,* 2004). *Chlorella* has a protein content of 51-58% with many essential amino acids and hence could be used as protein source in human and animal diets (Becker, 2007). Current applications of *Chlorella* mainly focus on human food, and limited research has been done in lower vertebrates. There are reports on use of microalgae, such as *Spirulina*, *Euglena* and *Microcystis*, as feed additives to improve growth performance of fish (Das *et al*., 2009; Sirakov *et al*., 2012). It has been reported that feeding of *Chlorella* enhances survival, growth rates and immunity in larval Korean rockfish and juvenile Korean rockfish respectively (Bai *et al.,* 2001; Cho *et al.,* 2001). Radhakrishnan *et al.* (2014) reported on replacement of fish meal with *Chlorella vulgaris* as alternative protein source for sustainable farming of the freshwater prawn *Macrobrachium rosenbergii*. *Chlorella* when used as feed additive in Gibel carp farming improved growth, immunity and activity of digestive enzymes (Xu *et al*., 2014). The carotenoid content of *Chlorella* is reported to improve pigmentation in fishes (Gupta *et al*., 2007).

The effect of algal nutrition on liver enzymes in fish has not been investigated so far in detail, particularly in Indian major carps. The present study evaluated the effect of dietary *C. vulgaris* on activity of specific liver enzymes *viz*., aspartate amino transferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in rohu *Labeo rohita.*

Proximate composition of the basal diet used for the study was 39.4% crude protein, 7.4% lipids, 14.6% ash, 7.1% moisture and 3% fibre. Three experimental diets were formulated by including the required proportions of different feed ingredients such as groundnut oil cake, rice bran, fish meal, soyabean meal, vitamin and mineral mixture (Sahu *et al.,* 2007) along with 1% binder. Powdered form of *C. vulgaris* was added to the above formulation at the rate of 0.1 , 0.5 and 1.0 g kg^{-1} feed. Water was added to the dry ingredients 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.

A total of 360 numbers of rohu fingerlings were acclimatised to the experimental conditions and divided into four groups (A, B, C and D), each group having duplicate tanks with 45 fishes per tank. Group A was fed with basal diet and acted as the control and the remaining groups were fed with *Chlorella* supplemented diets at levels of 0.1 g kg^{-1} feed (Group B), 0.5 g kg^{-1} feed (Group C) and 1.0 g kg^{-1} feed (Group D) for 90 days. Fish were fed with their respective diets at the rate of 4% of their body weight per day throughout the experiment. The daily ration was divided into two and fed at 09.00 and 17.00 hrs (Das *et al.,* 2009).

Pathogenic strain of *Aeromonas hydrophila* (ATCC 49140) was cultured in nutrient broth (Himedia) for 24 h at 37°C. The culture broth was centrifuged at 3000 *g* for 10 min. The supernatant was discarded and the pellet was resuspended in phosphate buffered saline (PBS, pH 7.4), and the cell density was adjusted to to $1x10^7$ cells ml^{-1}. The bacterial suspension was serially diluted using standard dilution technique with PBS and used for the challenge experiment.

After 3 months of feeding, 20 fish from each group in duplicate were challenged with a lethal dose of *A. hydrophila* ($1X10^5$ CFU per fish) by intraperitoneal injection of 100 µl of the bacterial suspension. The fishes were observed for mortality for a period of 10 days.

Fishes were sampled at 30 days intervals during the experimental feeding and also after 10 days post-challenge with *A. hydrophila*, to collect blood for enzyme analysis. Feeding was suspended for 24 h, prior to collection of blood samples from fish. From randomly picked fish (n=30 from each subgroup) at 30 day intervals, blood samples were collected without anticoagulant, after anaesthetising with 0.1 ppm MS-222. Blood samples were allowed to clot for 1 h at room temperature, centrifuged (3000 *g* for 5min) and sera collected and stored at -80°C until use (Das *et al.*, 2009). Sera were pooled from each subgroup depending upon volume, for estimation of enzymatic parameters.

Serum AST was determined following Wallnofer *et al*. (1974). Serum (200 µl) was added to 2 ml of the test reagent, mixed thoroughly and incubated for 1 min at 37°C. Subsequently, 200 µl of α-oxoglutarate was added. The solution was mixed thoroughly and the initial absorbance was recorded at 340 nm. Three subsequent readings were taken at 1 min intervals. AST activity was expressed as U l^{-1} of serum. The final concentration was calculated as:

AST activity (U l^{-1}) = 1905 x ΔA 340 nm min^{-1}

where 1U = 16.67 x 10^{-3} µ kat

For estimation of serum ALT (Wallnofer *et al*., 1974) serum (200 µl) was added to 2 ml of test reagent, mixed thoroughly and incubated at 25°C for 1 min. Subsequently, 200 µl of α-oxoglutarate was added and the solution was mixed thoroughly. Initial absorbance was recorded at 340 nm. Three subsequent readings were taken at 1 min intervals as above. The ALT activity was expressed as U l^{-1} of serum. The final concentration was calculated as:

ALT Activity (U l^{-1}) = 1905 x ΔA at 340 nm min^{-1}

where 1U=16.67 x 10^{-3} µ kat

Serum ALP was determined as per Rosalki *et al*. (1993). Serum (50 µl) was added to 3 ml of test reagent containing diethanolamine buffer, $MgCl_2$ and p-nitrophenyl phosphate. This was mixed thoroughly and the initial absorbance was recorded at 405 nm. Three subsequent readings were taken at 1 min intervals at 405 nm. ALP activity was expressed as U l^{-1} of serum. The final concentration was calculated as:

ALP Activity (U l^{-1}) = 3300 x $ΔA_{sample}$ at 405 nm min l^{-1}

1U = 16.67 x 10^{-3} µ kat

where ΔA = change in Absorbance per min

The data were analysed by one way analysis of variance (ANOVA) and mean differences among experimental groups were evaluated using Duncan's multiple range tests (DMRT) (Duncan, 1955) at $p<0.05$ significance level.

Modulation in enzymatic parameters of fish fed with the experimental *Chlorella* diets are depicted in Fig. 1 to 3. A *hydrophila* compared to group of fish fed with 1 g kg^{-1} *Chlorella*. The level of AST significantly increased ($p≤0.05$) in the control group after challenge with the pathogenic bacteria, Fish fed *Chlorella* supplemented diets showed significantly ($p≤0.05$) different AST activity on day 60 (except D) and on day 90 (except B) of experimental feeding respectively as compared to control (Fig. 1).

Similarly ALT activity was significantly ($p≤0.05$) different in all the treated groups of fish before (on day 60 and 90) and after bacterial challenge (on 10th day post-challenge) as compared to control. But no significant difference of ALT activity was found in groups C and D on 30th day of experimental challenge (Fig 2).

Significant ($p≤ 0.05$) difference in ALP activity was found in group D on day 30 as well as in groups B, C

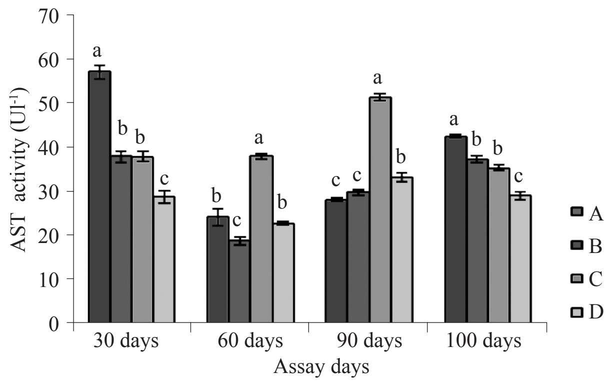

Fig. 1. Effect of *Chlorella* on AST activity of *L. rohita* on different assay days during the experimental feeding

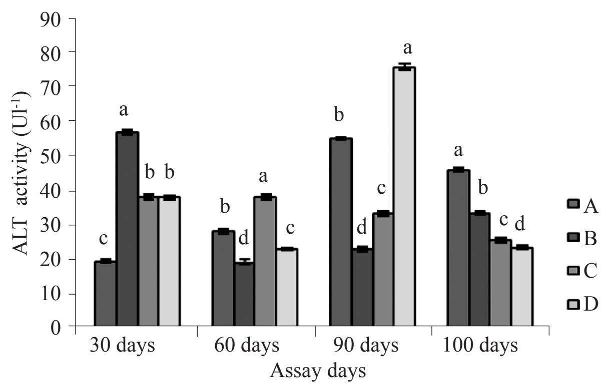

Fig. 2. Effect of *Chlorella* on ALT activity of *L. rohita* on different assay days during the experimental feeding

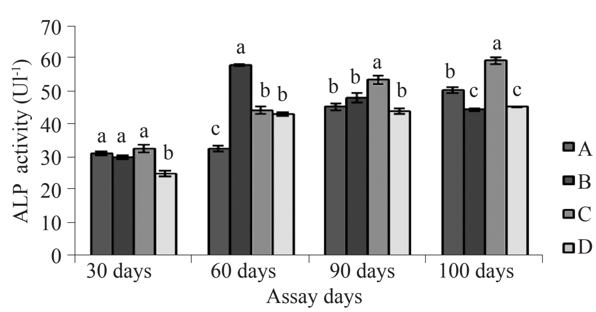

Fig. 3. Effect of *Chlorella* on ALP activity of *L. rohita* on different assay days during the experimental feeding

and D on day 60 of experimental feeding as compared to their respective control. Similarly ALP activity was significantly ($p \leq 0.05$) different in all the treated groups on 10th day bacterial post-challenge as compared to control (Fig. 3). But no significant ($p>0.05$) difference of ALP level was found on day 90 (except in group C) of experimental feeding

ALT and AST are liver specific enzymes and they are more sensitive measures of hepatotoxicity and can be assessed in a shorter time (Balint *et al.*, 1997). However, Oluah (1999), noted that changes in the values of ALT and AST indicate tissue damage in liver, kidney, muscle and gills. ALT and AST are indicators of liver function (Ozaki, 1978) and damage (Oda, 1990). Dietary stimulation enhances the resistance against infection, thereby increasing the serum/blood immune parameters and liver enzyme activities (Pradhan, 2012). The ALT activity was found to increase in all groups of fish fed *Chlorella* supplemented diets in comparison to control group, over 30 days feeding and decreased in most of the groups at day 60 and 90 of experimental feeding and in pathogen challenged groups except in group C and group D over 60 days and 90 days feeding, indicating that the liver function was not impaired due to *Chlorella* feeding.

It was noticed that AST activity decreased significantly ($p<0.05$) in all the groups fed with *Chlorella* at day 30, 60 and on day 10 post-challenge, but it increased on days 60 and 90 in group C. Sahu *et al.* (2008) reported decreased AST and ALT activities in serum of rohu fed with turmeric incorporated diet for a period of 60 days and opined that oxaloacetate and glutamate were not available to the Kreb's cycle through this route of transmission. AST can be generally found in the liver, cardiac muscle, kidney, brain, pancreas, lungs leucocytes and erythrocytes whereas ALT is present in highest concentration in liver (Kupeli *et al.,* 2006). The effect of *Chlorella* on serum AST and ALT in rats was reported by Lee *et al.* (2008) and they observed no difference in the activities in the control diet groups and *Chlorella* fed groups. In our study dietary *Chlorella* was found to influence AST and ALT activities in serum which was contradictory to the observations made in rat. This could be attributed to the application doses and mode of utilisation by fish, as fish do not have the ability to digest dietary fibre since they lack cellulose digesting enzyme. Dietary fibre intake due to *Chlorella* fed diets has been thought to reduce risk factor for cardiovascular disease, gastrointestinal disease, colon cancer, obesity. and high dietary fibre reduced lipid level in hypercholesterolemic hamster (Chau and Huang, 2005). Dietary incorporation of *Chlorella* might have influenced flesh protein quality and also physiological processes including liver functions. As *Chlorella* is rich in protein (66 g per 100 g of powder), the intake and availability of this algae will help to enhance the flesh quality, without altering the physiological mechanism of fish.

Phosphatase activity is of significance in pathological conditions (Reddy and Rao, 1990). Increased ALP activity ($p<0.05$) was marked in the group of fish fed with *Chlorella* over different days except 30 days of feeding and at 10 day post-challenge in groups B and D. Increase in phosphatase activity indicates higher breakdown of the energy reserves, which is utilised for the growth and survival of fish. ALP is the brush border enzyme, which splits various phosphorous esterases at an alkaline pH and mediates membrane transport (Goldfisher *et al.,* 1964). ALP is also involved in transport of glycogen (Gupta and Rao, 1974), protein synthesis (Pilo *et al.,* 1972) and synthesis of certain enzymes and secretary activity. Thus, any alteration in the activity of ALP may affect an animal in a variety of ways.

Increased ALP activity was reported by Sahu *et al.* (2008) while feeding rohu at various doses of turmeric for a period of 60 days following challenge with *A. hydrophila*. In the present study, similar type of observations were noticed. Bai *et al.* (2001) reported that optimum dietary *Chlorella* supplementation at 5% of diet had positive effect on growth and feed utilisation without any negative effect on blood parameters and body composition of Korean rock fish. Dietary supplementation of 2% *Chlorella* powder in the commercial diets improved growth, feed utilisation, serum cholesterol and whole body fat contents in juveniles Japanese flounder (Kim *et al.,* 2002). After challenging the fish with *A. hydrophila*, there was no mortality of fish up to 12 h. The groups of fish fed with different percentage of *Chlorella* showed higher percentage of survival as compared to the control (Fig. 4). The highest survival (86.5%) was shown in group D fed with 1 g *Chlorella* per kg feed. Das *et al.* (2009) observed that mortality following challenge with *A. hydrophila* decreased in group of fish fed with *Euglena* incorporated diets, where fishfed on 1 g *Euglena* per kg diet showed highest rate of survival as compared to control. It can be inferred from the challenge study that the increased protection against the pathogen could be due to the enhancement in the defense system, as is evidenced with increase in different immune parameters in fish, post pathogen challenge.

Optimal dietary level (0.8-1.2%) of *Chlorella* promotes growth immune response and the activity of digestive enzymes in Gibel carp (Xu *et al*., 2014). Intake of *C. vulgaris* at various dosages, changes the AST, ALT and ALP activity but the variation was not so high that it will not retard the growth of fish, but has many stimulatory effects in blood, serum protein and nonspecific immune systems and survival of fish challenged with *A. hydrophila*. Increase in transaminase activity leading to elevation of ALT and AST activities occur due to liver damage (Rajyasree and Neeraja, 1989), which is also related to kidney and gill damage (Ortuno *et al.,* 1999). Stress induced alteration in ALP and acid phosphatase (ACP) activities in serum/tissues have been reported in carp (Das *et al.,* 2004). Therefore monitoring these enzymatic activities in serum would provide the information related to stress caused by algal feeding. It is evidenced from the study that *Chlorella* feeding could not suppress the growth and survival of fish and do not cause liver disfunctions as evidenced from the enzymatic profiles.

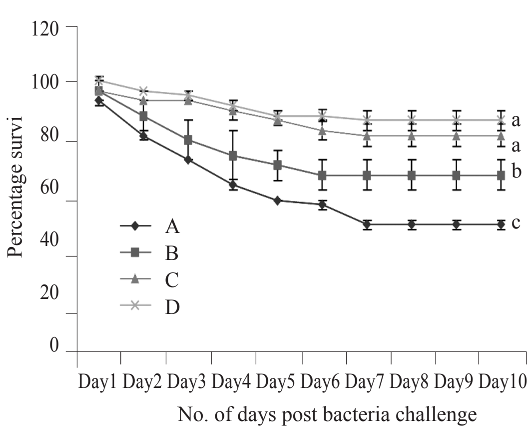

Fig. 4. Effect of *Chlorella* on survivability of *L. rohita* after bacteria challenge

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