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R E S E A R C H   P A P E R

Effects of Dietary Alginic Acid on Growth and Haemato-İmmunological Responses of *Cirrhinus mrigala* (Hamilton, 1822) Fingerlings

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Abstract

A 60 days feeding trial was conducted to study the effects of dietary alginic acid (AA) on growth and haemato-immunological responses in Cirrhinus mrigala fingerlings. One hundred eighty fingerlings were randomly distributed into five treatments in triplicates. Five diets were prepared with graded level of alginic acid and were designated as: control (Basal feed + 0% AA), T1 (Basal feed + 0.2% AA), T2 (Basal feed + 0.4% AA), T3 (Basal feed + 0.6% AA) and T4 (Basal feed + 0.8% AA). Higher weight gain % and specific growth rate (SGR) were observed in T2 group. Feed conversion ratio (FCR) value was found significantly lower in T2 group. Total leucocytes and erythrocytes counts were significantly ($P<0.05$) increased in T2 group. Highest ($P<0.05$) haemoglobin content was observed in T2. Higher ($P<0.05$) serum protein, albumin and globulin were manifested in T2. Albumin/globulin ratio was lowest in T2 group. Higher respiratory burst activity (NBT) was observed in T3. Highest activity of catalase and super oxide dismutase (SOD) were manifested in T2. Lowest lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activities reported in T2. Overall findings suggested that alginic acid at 0.4% dietary level augments growth and haemato-immunological responses in C. mrigala fingerlings.

Introduction

Aquaculture is recognised as the fasted growing agricultural allied sector in the world. It has emerged as one of the most promising and fastest growing industries, which provides high quality animal protein, raises nutritional levels and generates income and employment around the globe (FAO, 2010). Indian aquaculture production mainly consists (~70%) of three major carps (*Labeo rohita, Catla catla,* and *Cirrhinus mrigala*) (FAO, 2007). *Cirrhinus mrigala*, is one of the preferred Indian major carp (IMC) species, constituting about 20-25% of the total IMCs production in India. The intensification of aquaculture

has emerged into various kinds of stress, disease and consequently results in inferior quality of unhealthy fish production. Out of the various pathogens encountered in aquaculture, the bacterial infection is one of the major culprits of disease outbreak leading to huge economic losses to poor farmers (Sahoo *et al.*, 2011). The bacteria causes haemorrhagic septicaemia, which is characterized by the presence of ulcers, abscesses, exophthalmia, abdominal distension, small superficial lesions, local haemorrhages, particularly in the gills and opercula (Kaleeswaran, Ilavenil, & Ravikumar, 2011). To overcome the disease outbreak, aqua-farmers are compelled to use chemicals and antibiotics as quick control measures. However, this is

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not an ideal and sustainable solution since it has a risk of generating resistant pathogen, bioaccumulation and residual effect to the environment (Farag, Dawz, Hewedi, & El-Barotyl, 1989). Besides this, although vaccine is considered more effective to treat fish diseases, but its specificity against particular pathogens and the price are common constraints faced by aquaculturist (Ardo *et al.*, 2008). Therefore, one of the ideal methods for controlling the disease is by strengthening the non-specific immune system of fishes.

Immunostimulants are biological extracts and synesthetic chemicals which stimulate the non-specific defence system in fish by promoting phagocytic cell function, increasing their bactericidal activity and/or non-specific cytotoxic cells and antibody production (Sakai, 1999). Different kinds of substances such as plant extracts, natural compounds, nutritional supplements, microbial products etc., are used as immunostimulants but very few of them are suitable. It has been shown that some substances obtained from sea weeds act as immunostimulants and growth promoter. For example intraperitoneal injection of κ-carrageenan and sodium alginate have been demonstrated to have resistance against pathogens and increase the innate immune response in Common carp, *Cyprinus carpio* (Fujiki & Yano, 1997). Alginic acid, derived from several genera of brown algae like Macrocystis, Laminaria, Lessonia, Ascophyllum, Alaria, Sargassum and Fucus is reported to act as immunostimulants in several studied (Yeh *et al.*, 2008; Ahmadifar, Takami, & Sudagar, 2009). The orange-spotted grouper, *Epinephalus coioides* injected intraperitoneally with κ-carrageenan and sodium alginate had elevated non-specific immune parameter responses and resistance against *V. alginolyticus* (Cheng, Tu., Chen, Nan, & Chen, 2007). In fish, alginic acid enhanced the activity of head kidney phagocytes and their migration in the site of alginate injection by increasing their production of chemotactic factors and their sensitivity (Fujiki & Yano, 1997). The striped snakehead (*Channa striata*) injected intraperitoneally with 0.5 mg alginic acid, suspended in PBS resulted in immunostimulation (Miles *et al.*, 2001). Further, the sodium alginate at supplementation level of 1.0 to 2.0 g/kg in diet promoted growth, enhanced immunity and resistance of *Epinephalus coioides* against *Streptococcus species* and an iridovirus (Yeh *et al.*, 2008). The alginic acid (ergosan) at 4% dietary level enhance the growth and improve the immune system in the great sturgeon *Huso huso* (Ahmadifar *et al.*, 2009). Sodium alginate extracted from the brown algae, *Undaria pinnatifida* and *Macrocystis pyritera*, was found to enhance the non-specific defence system of common carp (*C. carpio*) and its resistance against *Edwardsiella tarda* (Fujiki, Matsuyama, & Yano, 1994).

To the best of our knowledge, literature pertaining to the dietary incorporation of alginic acid on growth performance and heamato-immunological response in *C. mrigala* is unavailable. Owing to the above facts the present work was conducted to evaluate the efficacy of dietary alginic acid on growth and haemato-immunoligical response of *Cirrhinus mrigala* fingerlings.

Materials and Methods

Experimental Site and Experimental Animals

The experimental set up was maintained in the wet laboratory of the Central Institute of Fisheries Education (CIFE), Mumbai, India, and the laboratory analyses were carried out at CIFE, Mumbai. Fingerlings of *C. mrigala* (average weight 5.42±0.02 g) were procured from the Aaray Fish Farm, Goregaon, Mumbai, Maharashtra, India, during the month of september, 2013. Fish were transported, stocked in cement tank (1000 L capacity) and left undisturbed during the whole night. The next day, fish were given a salt treatment (5%) to ameliorate the handling stress if any. The stock was acclimatized under aeration, provided through compressed air for 15 days. During acclimation, fish were fed with control diet having (35% CP and 7% Lipid) at 3% of the body wt. Round the clock aeration was provided. The physico-chemical parameters of the water were within the normal range of carp rearing (dissolved oxygen: 6.56–7.1 mgl^{-1}; pH:7.25–7.8; temperature: 26.6–28.2°C; alkalinity 46-58 mgl^{-1} and hardness 48-64 mgl^{-1}) throughout the experimental period.

Experimental Design, Feed and Feeding

One hundred eighty fingerlings of *C. mrigala* were randomly distributed in five treatment groups in triplicates following a completely randomized design (CRD). The experimental rearing system consisted of 15 uniform size rectangular fibre reinforced plastic tanks (150 L capacity) containing 12 fish per tank. The total volume of the water in each tank was maintained at 120 L throughout the experimental period.

Five isonitrogenous and isocalorific experimental diets were prepared. The protein percentage was maintained at the rate of 35% in all the treatments while lipid percentage in all the treatments was kept around 7%. The Fingerlings of *C. Mrigala* required 34-35 % protein and around 7-9 % lipid which comes under the normal requirement of the IMCs including *C. mrigala* fingerlings. The alginic acid was supplied from Fisher Scientific, Across, Mumbai, India. The fish were divided into five different treatment groups as control (Basal feed + 0% AA), T1 (Basal feed + 0.2% AA), T2 (Basal feed + 0.4% AA), T3 (Basal feed + 0.6% AA) and T4 (Basal feed + 0.8% AA) (Table 1). Continuous aeration was provided to all the tanks throughout the experimental duration. Fish meal and soybean, were

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Table 1. Composition of experimental diets (g %)

Ingredients	Experimental diet (%) inclusion				
	Control	T1	T2	T3	T4
Fish meal[a]	29	29	29	29	29
Soybean[a]	14	14	14	14	14
Groundnutoil cake[a]	8	8	8	8	8
Corn flour[a]	11.81	11.81	11.81	11.81	11.81
Wheat flour[a]	14	13.8	13.6	13.4	13.2
Rice powder[a]	16	16	16	16	16
Sunflower oil[a]	3	3	3	3	3
Cod liver oil[a]	2	2	2	2	2
Vitamin-mineral Premix[b]	1	1	1	1	1
Vitamin-C[c]	0.59	0.59	0.59	0.59	0.59
BHT[d]	0.6	0.6	0.6	0.6	0.6
Alginic acid[e]		0.2	0.4	0.6	0.8
Proximate composition of diets (%)					
Crude Protein	35.01	35.06	35.1	35.0	35.14
Ether extract	7.08	7.13	7.06	7.02	7.01
Crude fiber	6.92	6.12	6.45	5.89	6.23
Digestible energy (Kcal/100g)	354.52	354.64	355.32	355.45	356.46
Dry matter	94.61	94.01	94.12	93.12	93.14

[a]Procured from local market Andheri, Versova Mumbai, India
[b]Composition of vitamin mineral mix (PREEMIX PLUS) (quantity/2.5kg) Vitamin A, 55,00,000 IU; Vitamin D3, 11,00,000 IU; Vitamin B2, 2,000 mg; Vitamin E, 750 mg; Vitamin K, 1,000 mg; Vitamin B6, 1,000 mg; Vitamin B12, 6mcg; Calcium Pantothenate, 2,500 mg; Nicotinamide, 10 g; Choline Chloride,150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 L- lysine, 10 g; DL- Methionine, 10 g; Selenium, 50 ppm; Satwari, 2500 mg;
[c]Stay C (Hoffman La Roche, Nutley, NJ, USA) 15% ascorbic acid activity.
[d]Himedia laboratories, Mumbai, India .
[e]Alginic acid was supplied by Fisher scientific, Across, Mumbai branch of India

used as protein sources, sunflower oil was used as lipid source, maize, rice powder and wheat flour were used as carbohydrate sources. All the ingredients were thoroughly mixed with water to make dough form. The dough was steam cooked for 10 min in a pressure cooker. Vitamin-mineral premix and alginic acid were mixed after cooling. Finally, the dough was pressed through a hand pelletizer to get uniform size pellets and sun dried for 4 h. The pellets were then kept in a hot air oven (50-60°C) overnight for complete drying, packed in polythene bags, and stored at 4°C throughout the experimental period. Feed was given to approximate satiation level for 60 days twice daily at 3% of the body weight and adjusted accordingly based on the biomass gain during the trial. Daily ration was divided into two parts: about 2/3rd of total ration was given at 09:00 h and the rest 1/3rd at 18:00 h. The uneaten feed and faecal matters were removed by siphoning out about 50% of the tank water on alternate days.

Growth Study

For the growth performance study, fish were weighed at the start and every 15 days interval till the termination of the experiment on the 60th day. The growth performances of *C. mrigala* fingerlings were evaluated in terms of weight gain (%), specific growth rate (SGR), feed conversion ratio (FCR) by using following equation:

$$\text{Weight gain (\%)} = (\text{final weight} - \text{initial weight}) / \text{initial weight} \times 100;$$

$$\text{SGR} = 100 \times (\log_e \text{average final weight} - \log_e \text{average initial weight})/\text{number of culture days};$$

$$\text{FCR} = \text{Total feed given (dry weight) (g)} / \text{weight gain (wet weight) (g)}$$

Feed Intake

Feed intake was determined in terms of percentage body weight. The feed was given at 2-3% of body weight and total feed intake was determined for each treatment for 60 days experiment.

Blood Collection

At the end of the feeding trial, three fish from each group were anaesthetised with clove oil (50μlL^{-1}) and blood was collected from the caudal vein using a

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syringe, which was previously rinsed with 2.7% ethylene diamine tetra acetic acid (EDTA) solution. The blood was then transferred immediately to a test tube containing a pinch of EDTA powder (as an anticoagulant) and shaken gently. The blood was used for determination of haemoglobin content, total erythrocyte and leucocyte count and for NBT assay. For serum, another three fish from each group were anaesthetised and blood was collected without anticoagulant and allowed to clot for 2 h, centrifuged (3000×g for 5 min) and kept at -80^{0}C until use.

Total Leucocyte and Erythrocytes Count

Total leukocytes and erythrocytes were counted in a haemocytometer using leukocyte and erythrocyte diluting fluid (Qualligens Fine chemicals, Mumbai, India) respectively. Twenty microliters of blood was mixed with 3980 μL of corresponding diluting fluid in a clean test tube and shaken well to suspend the cells uniformly in the solution. Then the cells were counted using a Neaubauer's counting chamber. Cell numbers were calculated according to the following formula:

No. of cells (cu. mm^{-1}) = (no. of cells counted × dilution)/ (area counted × depth of fluid).

Haemoglobin (Hb) Content

The haemoglobin content of blood was analysed following the cyanmethaemoglobin method using Drabkins fluid (Qualigens Diagnostics, Mumbai, India). The absorbance was measured using a spectrophotometer (MERCK, Nicolet, evolution 100, Darmstadt, Germany) at 540 nm and the final concentration was calculated by comparing with a cyanmethaemoglobin standards (Qualigens diagnostics, Mumbai). The haemoglobin concentration was then calculated by using the following formula:

Haemoglobin (g/dl) = 60 [OD (T)/OD (S)] × 251/1000. Where OD (T) is absorbance of test and OD(S) is absorbance of standard.

Total Serum Protein, Albumin, Globulin and Albumin/Globulin Ratio

Serum protein was estimated by Biuret and the bromocresol green (BCG) dye binding method (Secombes & Fletcher, 1992) using total protein and albumin kit (Qualigens diagnostics, Mumbai). Albumin was estimated by the BCG binding method (Stasiack & Bauman, 1996). The absorbance of standards and tests were measured against the blank in a spectrophotometer at 630 nm. Globulin was calculated by subtracting albumin values from total serum protein. The albumin/globulin (A/G) ratio was calculated by dividing albumin values by globulin values.

Respiratory Burst Activity (NBT)

Respiratory burst activity of phagocytes was measured using the nitro-blue tetrazolium (NBT) assay following the method of Secombes & Fletcher (1992) as modified by Stasiack & Bauman (1996). Fifty microliters of blood was placed into the wells of 'U' bottom microtiter plates and incubated at 37^{0}C for 1 h to allow adhesion of cells. The supernatant was then removed and the wells washed three times in phosphate-buffered saline (PBS). After washing, 50 mL of 0.2% NBT was added and incubated for a further 1 h. The cells were then fixed with 100% methanol for 2–3 min and washed three times with 30% methanol. The plates were then air-dried and 60 μL 2N potassium hydroxide and 70μL dimethyl sulphoxide were added to each well. The OD of the solution was then read in an ELISA reader at 540 nm.

Tissue Homogenate Preparation

At the end of the experiment, two fish from each replicate with a total of six fish from each treatment were anaesthetized with CIFECALM (CIFE, Mumbai, India) at 50μl l $^{-1}$. For enzyme assay, tissue samples of liver and muscle were collected and immediately homogenized (5%) in chilled sucrose solution 0.25 M using a mechanical tissue homogenizer. The homogenized samples were centrifuged (5000 × g for 10 min at 4°C) and supernatants were collected and stored at -20°C for subsequent enzyme assays.

SOD (Superoxide Dismutase)

Superoxide dismutase was assayed according to the method described by Misra and Fridovich (1972) based on the oxidation of epinephrine-adrinochrome transition by the enzyme. 50μl of sample was taken in the cuvette and 1.5 ml 0.1M carbonate bicarbonate buffer containing 57mg/dl EDTA (pH 10.2) and 0.5 ml epinephrine (3mM) was added and mixed well. Change in optical density at 480 nm was read immediately for 3 min in UV-VIS spectrophotometer. One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation. SOD expressed as unit activity (amount of protein required to give 50% inhibition of epinephrine auto oxidation).

Catalase

Catalase was assayed according to the method described by Takahara *et al.* (1960). To a reaction mixture of 2.45 ml phosphate buffer (50 mM, pH 7.0), enzyme source was added and the reaction was started by the addition of 1.0 ml of H$_2$O$_2$ solution. The decrease in absorbance was measured at 240 nm at 15 sec

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intervals for 3 min. The enzyme blank was run simultaneously with 1.0 ml distilled water instead of H_2O_2 solution. Enzyme activity was expressed as nano-moles H_2O_2 decomposed/min/mg protein.

Lactate Dehydrogenase (LDH)

The LDH activity was assayed from different tissues of *C. mrigala* by the method of Wroblewski and Ladue (1955). Reaction mixture comprised of 2.7 ml of 0.1 M phosphate buffer (pH 7.5), 0.1 ml of NADH solution (2mg NADH dissolved in 1 ml of phosphate buffer solution), 0.1 ml of tissue homogenate and 0.1 ml of sodium pyruvate. The reaction started after addition of substrate sodium pyruvate. OD was recorded at 340 nm at 15 sec intervals for 3 minutes. LDH activity was expressed as micromoles of NAD released/mg protein at 37 ^{0}C.

Malate Dehydrogenase (MDH)

MDH activity was assayed in different tissues of *C. mrigala* fingerlings by the methods of Ochoa (1955). 3 ml of the reaction mixture comprised of 2.7 ml of 0.1 M phosphate buffer (pH 7.5), 0.1 ml of NADH solution (2mg of NADH dissolved in 1ml of phosphate buffer solution). 0.1 ml of tissue homogenate and 0.1 ml of freshly prepared oxaloacetate solution (2mg Oxaloacetate dissolved in 2ml of chilled distilled water). The reaction started after addition of oxaloacetate solution as substrate. The OD was recorded at 340 nm at 30 seconds interval for 3 minutes. MDH activity was expressed as micro moles of NAD released/mg protein/min at 37 ^{0}C. All the colorimetric assays were carried out using UV-VIS spectrophotometer (Spectroquant Pharma 300 Merck).

Aspartate Amino Transferase (AST)

The AST activity was assayed in different tissue homogenates as described by Wooten (1964). The substrate comprised of 0.2M D, L- aspartic acid and 2mM-ketoglutarate in 0.05 M phosphate buffer (pH 7.4). In the experimental and control tubes, 0.5ml of substrate was added. The reaction was started by adding 0.1ml of tissue homogenate. The assay mixture was incubated at 37 ^{0}C for 60 minutes. The reaction was terminated by adding 0.5ml of 1mM 2, 4 dinitrophenyl hydrazine (DNPH). In the control tubes the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 minutes with occasional shaking. Then 5ml of 0.4ml NaOH solution was added, the contents were thoroughly mixed. After 10 minutes, the OD was recorded at 540 nm against blank.

Alanine Amino Transferase (ALT)

The procedure adopted for ALT activity was same as for AST activity except the substrate comprised of 0.2 M D, L- alanine instead of aspartic acid.

Statistical Analysis

The data were statistically analysed by statistical package SPSS version 16 and data were subjected to one way analysis of variance and Duncan's multiple range tests were used to determine the significant differences if any, between the means. Comparisons were made at the 5% probability level.

Results

Growth Performances

The growth parameters of the experimental animals of different groups at the end of feeding trials are shown in (Table 2). Supplementation of dietary AA significantly (P<0.05) affected the weight gain%, FCR and SGR of different experimental groups. Highest (P<0.05) weight gain% and SGR was observed in the T2 group fed with 0.4% AA supplementation and were followed by T3 and T1 group. The reverse trend for the FCR was noticed and lowest value was detected in T2 followed by T3 and T1 group (Table 2).

Total Leukocyte Count, Erythrocyte Count and Haemoglobin Content

Dietary supplementation of AA had non-significant (P>0.05) impact on the total leukocyte and erythrocyte count as well as haemoglobin contents (Table 3). T2 group showed higher total leukocyte count and erythrocyte count and was followed by T3 and T4. Higher haemoglobin content was observed in the group fed with 0.4% AA and was comparable to T3 and T4. (Table 3).

Total Serum Protein, Albumin, Globulin and A/G Ratio

A significant difference (P<0.05) in the total serum protein, albumin, globulin and A/G ratios were manifested among the different treatment groups fed with graded level of AA diet (Table 3). Highest total serum protein, albumin and globulin contents were observed in the T2 group compare to all other treatment groups whereas lowest values of A/G ratios were manifested in the same T2 group fed with 0.4% AA incorporated diet level.

Respiratory Burst Activities

The production of superoxide radical examined by NBT reduction was significantly (P<0.05) influenced by

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dietary supplementation of AA (Figure 1). Highest NBT values were observed in the T3 group followed by T2 group. Lowest NBT value was noticed in T4 group fed with 0.8% AA supplementation (Figure 1).

Catalase and SOD Activities

Dietary supplementation of AA led to significant impact on the activity of both antioxidative enzymes. Significantly ($P<0.05$) highest catalase activity in both liver and muscle was noticed in T2 group followed by T3 and T1. Similar trend for muscle SOD level was manifested and highest was observed in T2 group

followed by T3. The hepatic SOD level was higher in T2 group fed with 0.4% AA. (Table 4).

LDH and MDH Activities

Addition of AA in the diet had significant impact ($P<0.05$) on the LDH activities in both liver and muscle. The activity of LDH in both the tissues were higher in comparison to MDH activity. The lowest LDH activity in both liver and muscle reported in T2 group fed with 0.4% AA and was followed by T3 group. Similarly, there was significant ($P<0.05$) effect of AA supplementation on MDH activity in both liver and muscle. T2 group

Table 2. Growth parameters of *Cirrhinus mrigala* fingerlings fed with alginic acid supplemented diets for 60 days

Treatments	Initial weight	Final weight	Weight gain %	Feed Intake (g)	SGR[2]	FCR[1]
C (0%)	4.13±0.01	8.17±0.02[a]	97.83±0.03[a]	8.36±0.02[a]	1.13±0.04[a]	2.15±0.04[d]
T1(0.2%)	4.12±0.06	8.69±0.0[c]	110.92±0.03[c]	8.45±0.02[b]	1.28±0.01[b]	1.84±0.02[c]
T2(0.4%)	4.23±0.01	9.86±0.02[e]	133.08±0.02[e]	8.46±0.02[b]	1.46±0.03[d]	1.46±0.02[a]
T3(0.6)%	4.08±0.02	9.11±0.05[d]	123.28±0.04[d]	8.51±0.01[b]	1.31±0.04[c]	1.61±0.04[b]
T4(0.8%)	4.15±0.02	8.39±0.01[b]	102.16±0.02[b]	8.46±0.02[b]	1.12±0.05[a]	1.89±0.05[c]

[1]FCR: Feed conversion ratio
[2]SGR: Specific growth rate
Values in the same column with different superscript (a, b, c, d, e) differ significantly ($P<0.05$). Data are expressed as mean ± SE, n=6.

Table 3. Haemato-immunological parameters of the *Cirrhinus mrrigala* fingerlings fed with alginic acid supplemented diet for 60 days

| Treatments	Total leucocyte count (10^3 cells mm^{-3})	Total erythrocyte count (10^6 cells mm^{-3})	Haemoglobin content (g dL^{-1})	Protein (g dL^{-1})	Albumin (g dL^{-1})	Globulin (g dL^{-1})	A/G ratio
C (0%)	0.88±2.71[a]	0.94±0.07[a]	4.43±0.33[a]	2.21±0.04[a]	1.08 ±0.05[a]	1.12±0.05[a]	0.97±0.05[e]
T1 (0.2%)	1.22±1.43[b]	1.27±0.03[b]	5.41±0.08[b]	2.52±0.03[b]	1.23±0.01[b]	1.28±0.02[b]	0.95±0.05[d]
T2 (0.4%)	1.66±3.06[e]	1.64±0.02[d]	6.14±0.09[c]	3.22±0.03[e]	1.49±0.02[e]	1.72±0.05[e]	0.86±0.05[a]
T3(0.6)%	1.48±1.41[d]	1.44±0.03[c]	5.80±0.05[bc]	2.88±0.01[d]	1.37±0.01[d]	1.53±0.02[d]	0.88±0.05[b]
T4 (0.8%)	1.34±2.60[c]	1.33±0.03[cb]	5.66±0.02[bc]	2.69±0.04[c]	1.30±0.01[c]	1.40±0.01[c]	0.92±0.05[c]
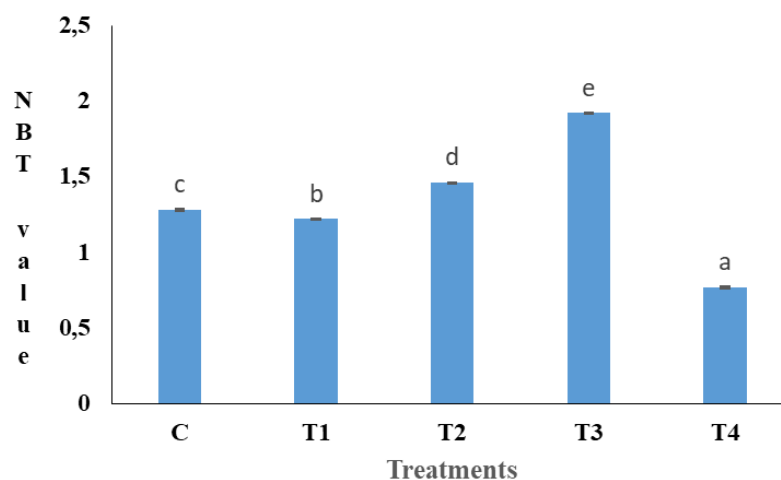
Figure 1. Impact of dietary doses of alginic acid on respiratory burst activity (NBT) of *Cirrhinus mrigala* fingerlings fed for 60 days. Different superscript (a, b, c, d, e) on each bar differ significantly (p<0.05). Values are expressed as mean ± SE, n=6.

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Table 4. Catalase and SOD activity in the liver and muscle of *Cirrhinus mrigala* fingerlings fed with alginic acid supplemented diet for 60 days.

Treatments	Catalase (Liver)	Catalase(Muscle)	*SOD(Liver)	*SOD(Muscle)
C (0%)	0.49±0.01[a]	0.28±0.01[a]	11.27±0.67[a]	12.48±0.72[a]
T1 (0.2%)	0.64±0.01[b]	0.46±0.03[b]	14.21±0.55[b]	16.70±0.62[b]
T2 (0.4%)	0.94±0.02[d]	0.87±0.01[d]	20.35±1.17[c]	22.97±0.73[d]
T3(0.6)%	0.82±0.02[c]	0.70±0.03[c]	19.54±0.80[c]	20.14±0.70[c]
T4 (0.8%)	0.61±0.03[b]	0.38±0.02[b]	15.22±1.26[b]	17.00±1.10[b]

Table 5. AST, ALT, LDH and MDH activity in the liver and muscle of *C. mrigala* fingerlings fed with alginic acid supplemented diet for 60 days

Treatments	AST		ALT		LDH		MDH	
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
C(0%)	48.63±1.40[c]	53.20±2.73[d]	45.49±2.22[d]	51.57±1.81[d]	6.18±0.29[d]	6.49±0.57[c]	0.89±0.04[d]	0.92±0.03[c]
T1(0.2%)	44.52±2.45[c]	47.38±1.46[dc]	35.55±2.13[c]	36.62±2.82[c]	5.45±0.60[dc]	4.56±0.29[b]	0.72±0.02[c]	0.66±0.05[b]
T2(0.4%)	21.99±1.37[a]	24.58±1.65[a]	18.65±1.30[a]	22.02±1.56[a]	2.26±0.21[a]	1.75±0.26[a]	0.24±0.02[a]	0.31±0.01[a]
T3(0.6%)	32.03±1.83[b]	41.23±3.03[bc]	24.82±1.27[b]	26.09±2.00[ab]	3.77±0.16[b]	3.94±0.39[b]	0.32±0.02[a]	0.21±0.01[a]
T4(0.8%)	25.61±1.23[a]	35.11±1.66[b]	33.59±1.66[c]	31.75±2.19[bc]	4.58±0.26[bc]	4.94±0.51[b]	0.55±0.04[b]	0.73±0.04[b]

Enzymes activities are expressed as follows:
AST (Aspartate Aminotransferase): expressed as nano moles of oxaloacetate mg^{-1} protein min^{-1} at 37 °C;
ALT (Alanine Aminotransferase): expressed as nano moles of sodium pyruvate formed mg^{-1} protein min^{-1} at 37 °C; LDH (Lactate dehydrogenase) mg^{-1} protein min^{-1} at 37°C, MDH (malate dehydrogenase): mg[-1] protein min^{-1} at 37 °C,
Values in the same rows with different superscript (a, b, c, d) differ significantly ($P<0.05$).
Data are expressed as mean ± SE, n=6.

showed lowest MDH activity in both liver and muscle (Table 5).

AST and ALT Activities

There was significant ($P<0.05$) impact of AA supplementation on AST and ALT activity in both liver and muscle. The lowest AST in both liver and muscle was noticed in T2 group fed with 0.4% AA followed by T4. Similarly, T2 group manifested lowest ALT activity in both liver and muscle. (Table 5).

Discussion

Alginic acid, a sea weed extracts has shown great potential as dietary supplements in the aqua-feed for the better growth, biochemical and immune performance of the fishes. Besides its immune boosting characteristic alginic acid also act as a feed binder which help in reducing the nutrients leaching from feed. This has great importance in the aqua-feed to reduce the feed loss and increase the feed intake of fishes. Supplementation of AA, in present work led to significant increase in the SGR and wt. gain % at 0.4 % incorporation level. Ahmadifar *et al.* (2009) also reported improvement in the growth parameter in great sturgeon, *Huso huso* juvenile at 0.4% dietary alginic acid. In case of crustaceans, incorporation of alginic acid into diets at a concentration of 0.5% (w/w) enhanced the growth of shrimps (*Litopenaeus vennamei*) over the 15 days experimental period (Montero-Rocha, McIntosh, Sanchez-Merino, & Sakai, 1999). Peddie, Davidson, Eccersall, & Wardle (2005)

demonstrated that dietary administration of alginic acid increased growth and survival of juvenile Chinok salmon (*Onchorhynchus tschawytscha*). The possible reasons of growth augmentation might be attributable to improved palatability, digestion and absorption of nutrients in the gut with the dietary supplementation of AA. Thus, the beneficial effects of AA indicate the efficient nutrient utilization in practical diet of *C. mrigala* fingerlings. However, the overdose of AA would not be favourable to growth, which might probably cause imbalance of bacterial population in intestine as reported by Liu *et al.* (2004) or affect the palatability of feed resulting into immune-suppression and negative effects on growth. To support our findings Kumar *et al.* (2015) reported reduction in growth % and SGR in *C. mrigala* fingerlings at higher doase (2%) of dietary anthraquinone extracts. Dietary yeast glucan (Macrogard) and alginic acid (Ergosan) had no effect on sea bass *Dicentrachus labrax* growth performance (Bagni *et al.,* 2005).

Leukocytes are one of the main parts of the cellular immunity system and fluctuation of them is increasingly used as indicators of stress response in fish (Stoskop, 1993). Highest leucocytes counts of T2 group fed with 0.4 % AA supplementation might be due to immune-boosting properties of alginic acid. Erythrocytes are indicator of various sources of stress. RBCs transport Hb that in turn transports oxygen, and the amount of oxygen received by tissues depends on the maturity of RBCs and amount of Hb (Rehulka,1989). Thus highly significant increase of RBC and Hb levels in the T2 group fed with 0.4% AA is a response to tolerate stress and to maintain general health status.

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Intraperitoneal administration of both glucans and alginic acid has been shown to activate leukocyte responses that can represent, in turn, a prelude of multifaceted inflammatory like immune response (Peddie, Zou, & Secombes, 2002). In support to above findings, Cheng *et al.* (2007) indicated that grouper *E. coioides* injected with 20 mg/kg sodium alginate showed a significantly increased non-specific immune response and resistance to *Vibrio alginolyticus.* However, with the increasing concentration of AA in the diet beyond 0.4%, led to reduction in the leucocytes, erythrocytes and Hb content which might be due to immunosuppression properties of AA in *C. mrigala* fingerlings at higher concentration.

There is a close relationship between the level of protein synthesis in liver tissue and plasma protein pools, total protein levels in plasma may be elevated due to the increased levels of protein synthesis in liver tissue**.** Serum albumin not only maintains osmotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues but also acts as plasma carrier protein to transport steroid hormones, haemin, fatty acids and also compounds like drugs **(**Asadi, Mirvaghefei, Nematollahi, Banaee & Ahmadi, 2012). Thus highest level of serum total protein and albumin in the T2 group might have increased non-specific immune system and the increased albumin levels may facilitate the transport of more humoral compounds. Highest total serum protein and globulin content of T2 group was in favour of the findings obtained by Abdel-Tawwab, Mohammad, Ahmad, Seden, & Sakr (2010) they observed an increase in serum protein, albumin and globulin levels in *O. niloticus* fed *C. sinensis* incorporated diet. Lowest albumin-globulin ratio observed in the T2 group indicates the presence of more amount of globulin and indicates higher resistance to pathogen infection (Wiegertjes, Stet, Parmentier, & Muiswinkel, 1996). These results are supported with increased SGR, leucocytes and erythrocyte counts of the T2 group.

In general fish phagocytes after activation 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 as respiratory burst activity (Secombes and Fletcher 1992). These reactive oxygen species are considered to be toxic for fish bacterial pathogens (Hardie, Ellis, & Secombes,1996). Different agents, glucans (Secombes and Fletcher 1992), yeast RNA (Sakai, 1999) and levan (Gupta *et al.*, 2008) are known to stimulate phagocytes for protection against pathogen. Hence it is evident that increase in respiratory burst activity can be correlated with increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes 1993). The respiratory burst activity of phagocytes used to measure by reduction of NBT by intracellular

superoxide radicals produced by leukocytes. Increase in NBT with AA supplementation is evidence of improvement in the non-specific immune system which is supported by increase in leucocytes, erythrocytes counts, total serum protein and globulin contents of T2 and T3 groups. In support of above result, respiratory burst, SOD activity and phagocytic activity of head kidney phagocytes significantly increased in *E. coioides* that had been intraperitoneally injected with sodium alginate at 20 mg/kg (Cheng *et al.,* 2007).

The antioxidant enzymes system composed of SOD and catalase can play a significant role in resisting oxidative damage. SOD is one of the main anti-oxidant defensive enzymes generated in response to oxidative stress that converts the highly toxic superoxide anions into hydrogen peroxides (Fridovich, 1995). SOD catalyzed the dismutation of superoxide anion to molecular oxygen and hydrogen peroxides and catalase enzyme decomposes hydrogen peroxides into oxygen and water which constitute the cellular antioxidant mechanism. In stress condition the activity of SOD and catalse rise to overcome the stress condition. Higher level of catalase and SOD in the T2 group supplemented with 0.4% AA showed the antioxidant properties of alginic acid. Cheng *et al.*(2007) also reported that respiratory burst, SOD activity and phagocytic activity of head kidney phagocytes significantly increased in *E. coioides* that had been intraperitoneally injected with sodium alginate at 20 mg/kg. The fact suggest in present finding is that sodium alginate via dietary administration may increase phagocytic activity of phagocytes of *C. mrigala* resulting in enhanced release of superoxide anions including an increase in SOD activity of phagocytes.

Metabolism is the process in any organisms including fishes for the production of energy to sustain the life. Lactate is used for the glucose production and repletion of glycogen in the liver by a process called cori cycle (Vijayan, Mommsen, Glemet, & Moon, 1996). Glycogen synthesis from liver is mainly by cori cycle. However, cori cycle in most of the fishes are low and fate of lactate is only immediate energy production in the peripheral tissues. In the present study LDH activity decreased in the liver and muscle of group supplemented with AA compare to control which showed the stress ameliorating efficacy of alginic acid. LDH activity increased in confinement stress (Chatterjee *et al.,* 2006). LDH activity significantly increased at higher temperature in *L. rohita* (Das, Ayyappan, & Jena, 2006). However, Lower LDH activity in the AA treated group might be due to the immune protecting and stress mitigating efficacy of alginic acid. The results of present investigation are in agreement with the findings of Ojha *et al.* (2013) and Gupta *et al.* (2008) they observed decreased level of LDH and MDH in liver and muscle of *L. rohita* and *C. carpio* fingerlings fed with dietary supplements of *Mucuna prurient* and microbial levan respectively. Tejpal *et al.* (2009)

reported that dietary supplementation of L – tryptophan mitigates crowding stress (MDH activity) in *C. mrigala*.

Gluconeogenesis process is prominent in fishes during the stress condition. In this process amino acids convert into glucose which indicates the metabolism of alanine or aspartate through gluconeogenesis for glucose production to cope up with stress. Knox and Greengard (1965) and Chatterjee *et al.* (2006) also reported that elevated level of transaminase activity during stress would lead to increased feeding of keto acids (Pyruvates) into TCA cycle, thereby affecting oxidative metabolism. Decrease in the AST and ALT activity in both muscle and liver in the group supplemented with 0.4% AA might be attributed to the stress mitigating properties of alginic acid. Kumar *et al*. (2005) reported decrease in the AST & ALT activity in the muscle and liver of *C. mrigala* fingerlings fed diet supplemented with 1% anthraquainone extract. Gupta *et al*. (2008) indicated decreasing trend in the ALT activity of *C. carpio* in both liver and muscle with gradual increase of dietary levan.

Conclusions

In conclusion, the present study documented that *C. mrigala* fingerlings fed diet containing 0.4% AA led to significant augmentation in the growth performance as well as increase in heamato-immunogical response together with increased respiratory burst activity. Similarly, the activity of antioxidative enzymes (SOD and catalase) were elevated at 0.4% AA incorporation. However, *C. mrigala* fed diets containing higher inclusion levels (0.6% and 0.8%) of AA do not further increase growth, immune and biochemical response suggesting that optimal dose of AA is 0.4% for commercial culture.

Acknowledgements

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