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RESEARCH ARTICLE

EFFECT OF *LYNGBYA HIERONYMUSII* ON IMMUNITY AND SURVIVAL OF *AEROMONAS HYDROPHILIA* INFECTED *PLATYCEPHALUS GIBBOSUS*

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ABSTRACT

The aim of this study was to evaluate dietary dosages of *Lyngbya hieronymusii* on the immune response and disease resistance against infections due to the opportunistic pathogen *Aeromonas hydrophilia*, in fish fingerlings of *Platycephalus gibbosus*. A cyanobacterium, *L. hieronymusii* was incorporated into the diets of *P. gibbosus* fingerlings and different biochemical, haematological and immunological parameters were evaluated. Superoxide anion production, lysozyme, serum bactericidal, serum protein and albumin were improved in cyanobacteria treated groups compared with the control group. Survival was increased in the cyanobacteria treatment group upto 89% survivability in the 500 mg cyanobacteria/kg and 1 g cyanobacteria/kg, and 75% survivability in the 2g cyanobacteria/kg, respectively. These results indicate that *L. hieronymusii* stimulates the immunity and makes *P. gibbosus* more resistant to infection by *A. hydrophilia*.

INTRODUCTION

The use of immunostimulants in aquaculture is becoming popular for enhancing the activity of non-specific defense mechanisms and increasing disease resistance in mangrove fish. The use of antibiotics and other chemotherapeutics has several drawbacks such as risk of generating resistant pathogens, problems of drug residues accumulating in treated fish and detrimental effect on the environment. Commercial vaccines are expensive for fish producers and may not be available for all species and against emerging diseases. Therefore, uses of immunostimulants seem to be an alternative way of reducing disease risk in fish culture (Dalmo and Seljelid, 19951; Raa, 1996). Cyanobacteria are a very old group of organisms and represent relics of the oldest photoautotrophic vegetation that occur in freshwater, marine and terrestrial habitats (Mundt and Teuscher, 1988). Cyanobacteria have drawn much attention as prospective and rich sources of biologically active constituents and have been identified as one of the most promising groups of organisms to be able of producing bioactive compounds (Fish and Codd, 1994; Schlegel *et al*., 1997). Many bioactive and pharmacologically active substances have been isolated from algae. For instance, extracts of algae were reported to exhibit antibacterial activity opined that the fatty acids (PUFA) in litter fall of mangroves might have positive role on the growth of fishes and shrimps. Screening of cyanobacteria for antibiotics and other pharmacologically active compounds, has received ever-increasing interest as a potential source for new drugs (Ostensvik *et al*., 1998). Cyanobacteria from local habitats seem to be a source of potential new active substances that could contribute to reduction of the number of bacteria, fungi, viruses and other microorganisms (Mundt *et al*., 2001). Cyanobacteria have not yet been studied for immunostimulants in aquaculture activity and little work has been done to screen cyanobacteria isolated from mangrove with regard to their production of bioactive compounds. The present study was aimed at determining the immunostimulating effect of cyanobacteria in mangrove carp, *Platycephalus gibbosus* which is an important species in mangrove water aquaculture, using application methodology relevant to practical farm conditions. The study was undertaken to evaluate the effects on biochemical and haematological parameters of the serum/blood of *P. gibbosus* for the first time.

MATERIALS AND METHODS

Experimental fish and husbandry

Fingerlings of fish, *Platycephalus gibbosus* (average wet weight 16±1 g), collected from the Mangrove water, Vedaranyam, Nagappatinam district, Tamil Nadu. Fish were stocked in a 50 litre tank and kept for quarantine and health check. After quarantine, fish were acclimated for 30 days in 20 litre chlorine-free mangrove water and fed with commercial diet. Water exchange (50%) was done daily and water quality was monitored throughout the experiment at three days intervals. Temperature was 28 ± 2^0C, pH, 7.8 ± 2, salinity 28 ppt, dissolved oxygen concentration 6.0 ± 0.4 mg/l, ammonia-nitrogen concentration 9.5 ± 0.08 mg/l and nitrite-nitrogen 5.2 ± 0.02 mg/l. Fish were fed their respective diet at the rate of 4% of body weight per day throughout the experiment.

Cyanobacteria

Five hundred milligrams of powdered cyanobacterium (*Lyngbya hieronymusii*) was collected from the mangrove environs and purified at laboratory in ASN III medium (Desikachary, 1959) and oven-dried at 50^0C, powdered by mortar and pestle and sieved. For each experiment, the required percentage (0.1, 0.5 & 1.0% dry weight

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basis) was included in the feed. These represent diets group II, III and IV, respectively. Diet group I (no cyanobacteria) served as control.

Experimental design and feeding diet

Platycephalus gibbosus fingerlings was selected for the study and divided into 4 groups. Each group of 10 fingerlings was again divided into two equal duplicate subgroups. Group I was fed with basal diet and acted as the control. The remaining groups were fed with 500 mg cyanobacteria/kg of feed (Group II), 1 g cyanobacteria/kg (Group III) and 2 g cyanobacteria/kg of feed (Group IV) for 40 days. Blood and serum samples were collected from fish in each subgroup and examined for the following parameters, total protein, albumin, globulin, albumin globulin ratio, blood glucose, haemoglobin, serum bactericidal activity, serum lysozyme activity and superoxide anion production, WBC and RBC.

Collection of blood

Feed was withheld from fish for 24 h before blood samples were collected. From randomly picked fish at 10-day intervals, after anaesthetizing with 0.2 ppm MS-222, blood was collected from the caudal vein with a 1 ml plastic syringe ringed with heparin and stored at 4^0C and used the same day. Blood samples were also collected without heparin, allowed to clot, centrifuged at 1500-rpm and sera collected and refrigerated. From each subgroup six and four fish were sampled for serum and blood, respectively and returned to their respective system. Sera and blood were pooled into six groups, depending upon volume, for estimation of immunological and biochemical parameters.

Superoxide anion production

Determination of immunological parameters Superoxide anion production the superoxide anion production of blood phagocytes challenged with Bacteria was measured with some modifications (Chung and Secombes, 1988). Flat bottom 96-well microtitre plates were coated with 100µl buffer containing poly-L-lysine solution (0.2% Sigma). Blood (100 µl) was added in five wells and incubated at 30^0C for 2 hour, then washed with Hanks balanced salt solution (HBSS). Then 100µl of NBT (1g/ml HBSS) was added containing 100 µl *A. hydrophilia* cells. After incubation for 30 min at 30^0C, the medium was removed and the reaction stopped by adding methanol. The formazone in each well was dissolved with 120 µl of 2 M KOH and 140 µl of DMSO and measured using a multiscan spectrophotometer (Biorad) at 630 nm, with 405 nm as reference.

Lysozyme activity

The turbidimetric assay for lysozyme was carried out according to Parry *et al,* (1965). Briefly, serum (100 µl) was added to 2 ml of a suspension of *A. hydrophilia* in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25^0C and absorbance was measured at 530 nm after 0.5 and 4.5 min on a spectrophotometer. A unit of lysozyme activity was defined as the sample amount causing a decrease in absorbance of 0.001/min.

Bactericidal activity

Serum bactericidal activity was done following the procedure of Kajita *et al,* (1990). An equal volume (100 µl) of serum and bacterial suspension was mixed and incubated for 1 hour at 25^0C. Blank control was also prepared by replacing serum with sterile PBS. The mixture was then diluted with sterile PBS at a ratio 1:10. The serum bacterial mixture (100 µl) was pour plated in nutrient agar and plates were incubated for 24 hour at 30^0C. The number of viable bacteria was determined by counting the colonies grown in nutrient agar plates.

Determination of blood haematological parameters

Blood haemoglobin content was determined following the cyanomethemoglobin method. Total erythrocyte count was performed following the method of Hendricks (1952) using a haemocynometer where a total leucocyte count was determined following the method of Shaw (1930).

Determination of serum/blood biochemical parameters

Serum samples were analyzed for total protein, albumin, globulin content (subtracting albumin from total protein) and albumin: globulin ratio (Lowry *et al*., 1951). Blood glucose content was estimated following the procedure of Schmidt (1974).

Challenge of fish

After 60 days of feeding, 4 fish from each subgroup were challenged intraperitoneally with a lethal dose of *A. hydrophilia* and observed for a 10days period for mortality. Biochemical, immunological and enzymatic parameters were assayed in post-challenged groups as per the methods described earlier. Data were analyzed using one-way analysis of variance (ANOVA) and significant differences among treatment means were compared using Duncan's multiple range test. Significance was tested at 5% level.

RESULTS

The result of different dosages of cyanobacteria on production of superoxide anion is shown in Table 1. Superoxide anion production in the three experimental groups was significantly higher than the control at all the assay periods, except group II in the post-challenge period. Highest superoxide anion production (0.10 O.D.) was found in group IV fish on day 30. Lysozyme activity in the serum of cyanobacteria fed groups was significantly higher at all sampling times including post-challenge, when compared with the control group. A significant difference was also observed between the treated groups at all sampling times. Highest lysozyme activity (145 U/ml) was observed in group III fish on day 30 (Table 1). Serum bactericidal activity in different cyanobacteria fed groups was significantly higher when compared with control at all sampling times, including post-challenge. Highest bactericidal activity (60 cfu/control) was found in group III fish on day 30 (Table 1). Haemoglobin content was significantly higher than control only in group IV on day 10 and groups III and IV on day 20 (Table 1). There was no significant impact of different doses on day 30 and post-challenge. WBC count in different treatments did not show a significant difference on day 10, however, a significantly higher WBC count was found with 500mg cyanobacteria/kg feed and 2g cyanobacteria/kg feed on day 20 as well as post-challenge and on entire groups II, III and IV on day 30 (Table 1). RBC count was significantly higher in all fish fed the different doses of cyanobacteria compared with the control group on all assay days (Table 1).

Serum protein content was significantly different in groups II and IV when compared with group I on day 10 (Table 2). Groups II and III fishes fed for 30 days had a significantly higher serum protein content than group I fish. Post-challenge, a significantly higher serum protein content was found in groups III and IV when compared with control. Serum albumin content in all fish fed the cyanobacteria doses was significantly higher than the control on day 10, whereas only group IV on day 20 and group III on day 30 had significantly higher albumin content than the control fish. Post-challenge, more of the treated fish had elevated serum albumin content compared with control fish. The serum globulin level was significantly higher in group IV on day 20 and group III on day 30 in comparison with the control. However, a significantly higher globulin content was found in groups III and IV when compared with control group I after the post-challenge period. Albumin: globulin ratio was significantly higher in groups II and III fish on day 10; there was no significant

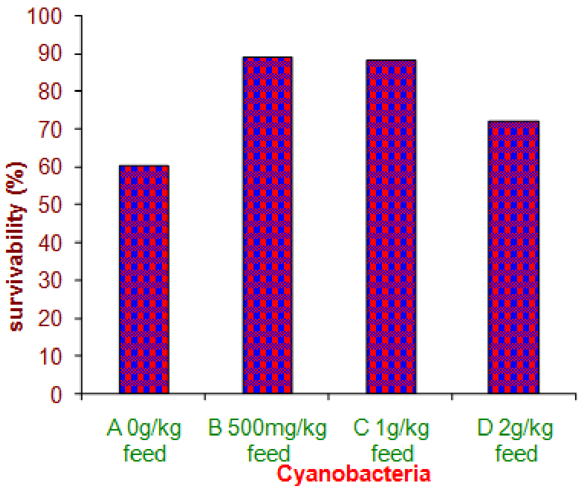

Fig. 1. Effect of *Lyngbya hieronymusii* on survivability of *P. gibbosus* after *Aeromonas hydrophilia* challenge

DISCUSSION

It has been shown that the protective effect of spices may be associated with its antioxidant properties (Pedraza-Chaverri *et al*., 2000; Rahman, 2003). Neutrophil activity can also be an indicator of the non-specific response. Cells become more adherent to tissue cell surfaces by the production of adhesion protein, which facilitates their migration from the capillaries to the site of injury (Kishimoto *et al*., 1989; Magnuson *et al*., 1989). They also exhibit an increased production of oxygen radicals, which are potentially capable of destroying invading pathogens (Hassett and Cohen, 1989). In the present experiment, lysozyme activity in the serum of *Lyngbya hieronymusii* fed groups was significantly higher at all sampling times including post-challenge, when compared with the control group. A significant difference was also observed between the treated groups at all sampling times. Immunostimulants can increase the non-specific immunity by either increasing the number of phagocytes or activating phagocytosis and respiratory burst (Shoemaker *et al*., 1997). Post-challenge with *A. hydrophilia* showed reduced lysozyme activity when compared with the 30-day pre-challenged specimens, but the reduction was not significant.

Table 1. Effect of *L. hieronymusii* powder feeding on immunological and haemtological parameters of *P. gibbosus* followed by challenge of *A. hydrophilia* after 30 days

Parameters	Group	Before challenge			After challenge
		10	20	30	40
Superoxide anion production (O.D.)	I	0.03 ± 0.02	0.04 ± 0.02	0.05 ± 0.03	0.04 ± 0.04
	II	0.08 ± 0.02	0.07 ± 0.08	0.07 ± 0.05	0.06 ± 0.06
	III	0.07 ± 0.06	0.09 ± 0.04	0.09 ± 0.04	0.07 ± 0.02
	IV	0.06 ± 0.04	0.08 ± 0.06	0.10 ± 0.02	0.09 ± 0.02
Lysozyme activity (U/ml)	I	100 ± 0.04	102 ± 0.08	125 ± 0.02	100 ± 0.02
	II	120 ± 0.04	124 ± 0.05	130 ± 0.06	120 ± 0.06
	III	140 ± 0.02	135 ± 0.04	145 ± 0.04	132 ± 0.08
	IV	130± 0.06	126 ± 0.06	140 ± 0.08	128 ± 0.04
Bactericidal activity (cfu/control)	I	22 ± 0.04	25 ± 0.06	28 ± 0.02	21 ± 0.06
	II	30 ± 0.02	36 ± 0.02	43 ± 0.06	39 ± 0.02
	III	35 ± 0.06	42 ± 0.06	60 ± 0.08	40 ± 0.05
	IV	38 ± 0.02	50 ± 0.04	47 ± 0.04	50 ± 0.04
Haemoglobin (g%)	I	6.8 ± 0.22		6.8 ± 0.62	9.0 ± 0.04
	II	7.9 ± 0.06		7.6 ± 0.28	10.5 ± 0.2
	III	7.2 ± 0.41		9.2 ± 0.84	7.3 ± 0.64
	IV	8.8 ± 0.66		10.1 ± 0.6	8.0 ± 0.08
WBCcount (1000cells mm^3)	I	13.0 ± 0.2		14.5 ± 0.6	13.0 ± 0.3
	II	14.0 ± 0.6		17.1 ± 0.3	14.5 ± 0.2
	III	14.5 ± 0.4		23.0 ± 0.4	19.3 ± 0.1
	IV	15.0 ± 0.8		22.1 ± 0.2	20.2 ± 0.3
RBCcount (1000000 cells mm^3)	I	0.7 ± 0.22	0.8 ± 0.26	0.92 ± 0.6	8.0 ± 0.14
	II	0.8 ± 0.08	1.3 ± 0.54	1.01 ± 0.8	9.6 ± 0.32
	III	1.0 ± 0.34	1.2 ± 0.20	1.20 ± 0.4	9.5 ± 0.08
	IV	1.2 ± 0.28	1.3 ± 0.46	1.38 ± 0.2	9.9 ± 0.42
					14.0 ± 0.2
					16.0 ± 0.8
					20.8 ± 0.6
					21.2 ± 0.2

elevation in the treated groups thereafter. Blood glucose level at all sampling times was significantly lower in fish fed with different doses of *Lyngbya hieronymusii* compared with the control. After challenging fish with *Aeromonas hydrophilia*, the mortality was recorded for 10 days. There was no mortality of fish up to 12 hours. The group of fish fed with different percentages of *Lyngbya hieronymusii* showed higher survival percentage when compared with control. The highest survival was shown in groups II and III (Fig. 1).

Many things might happen following challenge but it is necessary to speculate that production of superoxide anions by the *Lyngbya hieronymusii* in fish act against *Aeromonas hydrophilia* infection. It has been found that aqueous extract of raw garlic and dried powder scavenges hydroxyl radicals (Yang *et al*., 1993; Kim *et al*., 2001) and superoxide anion (Kim *et al*., 2001). Similar types of activities might have occurred in the present work. Immunostimulants can increase serum lysozyme activity, due to either an increase in the number of phagocytes secreting lysozyme or to an increase in the amount of

Table 2. Effect of *L. hieronymusii* powder feeding on biochemical parameters of *P. gibbosus* followed by challenge of *A. hydrophilia* after 30 days

Parameters	Group	Before challenge			After challenge
		10	20	30	40
Blood glucose (g/dl)	I	155 ± 0.22	149 ± 0.88	141 ± 0.02	122 ± 0.09
	II	138 ± 0.40	132 ± 0.44	125 ± 0.08	126 ± 0.27
	III	134 ± 0.52	122 ± 0.62	112 ± 0.06	119 ± 0.50
	IV	109 ± 0.02	96.8 ± 0.40	94.8 ± 0.02	93.6 ± 0.02
Total protein (g/dl)	I	1.20 ± 0.22	1.70 ± 0.04	2.80 ± 0.06	2.25 ± 0.10
	II	1.80 ± 0.34	1.90 ± 0.38	2.30 ± 0.02	1.91 ± 0.20
	III	1.10 ± 0.14	1.80 ± 0.22	3.90 ± 0.02	3.50 ± 0.28
	IV	1.60 ± 0.12	2.60 ± 0.12	2.70 ± 0.08	2.99 ± 0.10
Albumin (g/dl)	I	0.42 ± 0.02	0.99 ± 0.02	1.35 ± 0.03	0.89 ± 0.05
	II	0.85 ± 0.01	0.98 ± 0.01	1.15 ± 0.04	0.74 ± 0.12
	III	0.65 ± 0.01	1.09 ± 0.01	1.58 ± 0.09	0.95 ± 0.02
	IV	0.69 ± 0.13	1.27 ± 0.01	1.34 ± 0.01	1.01 ± 0.00
Globulin (g/dl)	I	0.77 ± 0.03	0.79 ± 0.09	1.39 ± 0.10	1.19 ± 0.04
	II	0.88 ± 0.11	0.99 ± 0.10	1.35 ± 0.07	0.60 ± 0.23
	III	0.39 ± 0.03	0.94 ± 0.04	2.48 ± 0.06	2.33 ± 0.22
	IV	1.03 ± 0.13	1.54 ± 0.11	1.43 ± 0.05	1.91 ± 0.07
Albumin : globulin	I	0.50 ± 0.01	1.26 ± 0.18	0.86 ± 0.04	0.72 ± 0.03
	II	0.96 ± 0.12	0.95 ± 0.09	0.85 ± 0.03	0.68 ± 0.03
	III	1.58 ± 0.16	1.04 ± 0.05	0.58 ± 0.05	0.41 ± 0.04
	IV	0.69 ± 0.10	0.81 ± 0.04	0.90 ± 0.04	0.52 ± 0.02

lysozyme synthesized per cell (Engstad *et al*., 1992). Changes in lysozyme activity are greatly influenced by the potency and type of immunostimulants to which fish are exposed. Elevation of lysozyme following immunostimulation has been demonstrated in a number of fish species (Lapatra *et al*., 1998; Paulsen *et al*., 2003). Lysozyme activity was elevated significantly in the groups of fish fed all three levels of *Lyngbya hieronymusii* when compared with the control. Serum bactericidal activity was also enhanced in all treated groups when compared with the control group. Many investigators have reported enhanced bactericidal activity by the phagocytic cells of different fish species treated with immunostimulants (Jorgensen *et al*., 1993). The number of leucocytes is known to increase sharply when infections occur, as one of the first lines of body defense. The increase in total white blood cell counts, and neutrophils, lymphocytes and monocytes counts following 20-day cyanobacteria feeding supports the anti-infection properties of cyanobacyetia (Iranloye, 2002). The erythrocyte count increased with the administration of cyanobacteria, which might indicate an immunostimulant effect and the findings conform to those by Duncan and Klesius (1996) who reported that the number of erythrocytes was significantly greater in channel catfish fed with a diet containing b-glucan. The haemoglobin content in the blood and oxygen consumption increases when fishes are under stress. Under such conditions there will be an increase in release of immature RBCs from the haemopoietic organs, which in turn elevate haemoglobin concentration in blood (Duncan and Klesius, 1996). In our experiment, the change in haemoglobin content was not significant from control, which indicates the fish was not under stress. The serum total protein after long-term feeding with *Lyngbya hieronymusii* increased in comparison to the control diet. Siwicki (1989) observed an increase in total protein content after feeding of b-glucan (0.2%) and chitosan (0.5%) in the diet. Serum albumin and globulin values in fish fed with *Lyngbya hieronymusii* were higher than the control. Increases in serum protein, albumin and globulin levels are thought to be associated with a stronger innate immune response of fish (Wiegertjes *et al.,* 1996).

Dietary garlic decreases blood glucose by increasing the level of serum insulin (Chang and Johnson, 1980; Ahmed and Sharma, 1997). According to Sheela and Augusti (1992), s-allyl cysteine sulfoxide present in garlic is responsible for its hypoglycaemic activity. Results of the present study indicate that continuous feeding of raw *Lyngbya hieronymusii* powder fights against stressors, as was evident from the low glucose value in fishes of groups II, III and IV during the experiment. Reduced mortalities against pathogenic challenges at lower dosages of herbal principals were also reported (Kim *et al*., 2001; Jain and Wu, 2003). Citarasu *et al.* (2002) developed an Artemia-enriched herbal diet for *Penaeus monodon* with a combination of five herbs, which significantly increased the growth and survival during stress conditions. Several herbs were tested for their growth-promoting activities in aquatic animals (Jayaprakas and Eupharsia, 1996; Sivaram *et al*., 2004). It is evident from the present work that *Lyngbya hieronymusii* could enhance fish immunity after incorporation in feed, even at a lower dose, i.e. 500 mg/kg of feed. The present results suggest that inclusion of *Lyngbya hieronymusii* in the diet would improve the non-specific immunity of fish and prevent bacterial infections in culture systems. Field trials incorporating these doses merit investigation. Further purification of the active compounds and their evaluation may substantially improve quality as well as their usage in the culture system.

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