Immune protection role and disease prevention in common carp, *Cyprinus carpio* (Actinopterygii, Cypriniformes, Cyprinidae) against a heterotrophic Gram-negative bacteria, *Aeromonas hydrophila* due to spirulina, *Arthrospira platensis* supplement

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Abstract. An eight-week feeding trial was conducted to evaluate the effect of the fish meal, soybean meal and squid meal were replaced by spirulina on growth, survival, immune protection and disease prevention in *Cyprinus carpio* against *Aeromonas hydrophila* infection. Six different experimental diets were prepared which includes a control diet (D1) and 5 different composition (5, 10, 15, 20 and 25%) of spirulina *Arthrospira platensis* diets (D2 to D6) where spirulina replaced the fish meal, soya meal and squid meal. The cumulative mortality was calculated as 27, 19, 14 and 11% in the fish groups received 10, 15, 20 and 25% spirulina respectively; and significantly ($p < 0.05$) high mortality (92%) was noticed against the pathogen in the fish fed control diet. The immunological assays namely, phagocytic, respiratory burst and complement activities were significantly ($p < 0.05$) increased in the individual fed 15, 20 and 25% spirulina diets between 2 and 4 weeks of post-challenge. The lysozyme activity was enhanced significantly ($p < 0.05$) in fish fed all the spirulina diet compositions throughout the experiment. Overall, the findings suggested that the inclusion of spirulina at the dosage between 15 and 25% in the diet enhanced the growth, survival and defense response in *C. carpio* significantly ($p < 0.05$), which ultimately lead to disease resistance and defense protection against *A. hydrophila* infection.
Key Words: *Aeromonas hydrophila, Cyprinus carpio,* spirulina, defense role, disease resistance.

Introduction. Aquaculture is the farming of aquatic organisms in ponds, inland waters and coastal regions, which involve nursery and rearing process to improve the production (Youn et al 2014). The total demand for fish and fishery products all over the world is projected to estimate about 50 million tons (MT) to 300 MT by 2020, and it is predicted that out of this estimate, 73% will come from aquaculture, which accounting nearly 39% of global fish production (Ashley 2007). The overall production of carp (Cyprinids) was about 25.4 million tons which is similar to 72.1% of total freshwater production throughout the world (FAO 2017).

Common carp, *Cyprinus carpio* is one of the economically potential candidate and cultured freshwater candidate worldwide; more than 90% of this production comes from culture source of Asia especially India. This is mainly due to its interbreeding capability in captivity, faster growth rate, high environmental tolerance, shorter cultivation periods, high survival of fish seed and high stocking density (Eddy & Underhill 1974). *C. carpio* is secured the third place as the important aquaculture candidate among the Cyprinidae family (Saikia & Das 2009). *C. carpio* is observed to grow faster than the other Indian major carps such as rohu, *Labeo rohita* and mrigal, *Cirrhinus mrigala*; but its growth is almost equal to that of catla, *Catla catla* (Pierce et al 1993). Larval maintenance especially larval nutrition is crucial in high nursery rearing. However, bacterial fish pathogens, cost of production, non-availability and the ability to afford the adequate fish feed has effectively affected the aquaculture sector (Csizmadia et al 1995).

Aeromonas hydrophila is one of the most spread bacterial fish pathogens in freshwater aquaculture system throughout the world (Larsen & Jensen 1977). In several fish including carps both in the farm and wild *A. hydrophila* is regarded as an opportunistic pathogen; the disease is frequently associated with hemorrhagic septicemia (Angka et al 1995). In worldwide, a wide variety of freshwater fish reported to have a number of diseases such as septicemia, abnormal distension, dropsy, ulcers, tail rot, fin rot, *etc*. due to *A. hydrophila* (Jagruthi et al 2014). However, the use of antibiotics as a preventive measure has been questioned because they can alter the gut microbiota and induce resistant bacterial populations, with unpredictable long-term effects on public health (Kaskhedikar & Chhabra 2010). Recently numerous researchers reported that a variety of natural and herbal immunostimulants have beneficial effects not only as an alternative tool in fish disease management but also to promote growth and trigger non-specific and specific immune systems in fin as well as shellfishes (Harikrishnan & Balasundaram 2005; Harikrishnan et al 2011). In this consideration, the single-cell protein spirulina *Arthrospira platensis* holds immunostimulant and antioxidant activity (Sannasimuthu et al 2018, 2019). Preclinical analysis by Chamorro et al (1996) suggested that *A. platensis* has various number of therapeutic properties including antiviral, antimutagenic, hypocholesterolemia and defense role.

Freshwater spirulina belongs to blue-green algae or cyanobacteria receiving closer attention as a possible protein source for fish diets, notably in tropical developing countries including India, because of its rich protein content, essential fatty acids, vitamins, minerals and beta-carotene (Nandeesha et al 1998). So, it can partially replace fishmeal in fish feeds and can be manipulated to produce essential amino acids, natural β-carotene, vitamins and antioxidant substances for better quality flesh. It is a well-known fact that spirulina can be used as feed supplement for humans as well as animals especially fishes. For instance, it has been reported that spirulina enhanced growth in the sturgeon, *Acipenser baeri* (Palmegiano et al 2005). Previous researchers proved that spirulina is capable to break down the indigestible feed components, and it improves the intestinal flora in fish (Ramakrishnan et al 2008); in addition, it enhances the production of enzymes that transport fats for growth in fish. Moreover, β-carotene in spirulina firmly maintains the mucous membrane and thereby prevents the entry of toxic elements into the body and chlorophyll acts as a cleansing and detoxifying factor against toxic substances (James 2010). Hence, the present study was undertaken to investigate the immune protection role and disease resistance in *C. carpio* against *A. hydrophila* due to *A. platensis* supplementation.

Material and Method. The common carp *C. carpio* (21.5±1.7 g) were obtained from the commercial fish farm Eluru, West Godavari District, Andhra Pradesh. The fish were transported to the aquarium at SRM Institute of Science and Technology in oxygenated plastic bags and maintained in 300 L fiber tanks for 2 weeks prior starting the experiment (December 2018).

Feed formulation and feeding. Experimental diets were prepared with the ingredients as shown in Table 1. The ingredients were ground properly and mixed well in a blender and the pellet (1 mm) was prepared. The pellets were shade dried at room temperature for three days. The fishes were fed twice a day at 09:00 h and 16.00 h. Feeding rate started with 7% of the total biomass and this percentage was maintained according to the size of the total biomass until the experiment is terminated. Twenty-five individuals were maintained in each treatment and the fishes were weighed randomly every week. Three replicates were maintained for each treatment.

Bacterial culture. *A. hydrophila* was procured from Microbial Type Culture Collections (MTCC), Chandigarh, India. It was grown with slight agitation in shaker incubator at 37°C in a 250 mL conical flask containing tryptic soy broth (TSB; Sigma-Aldrich) to log phase.

Using centrifugation at 3500 × g for 20 min at 4°C, the culture was harvested. Bacterial pellets were washed twice with sterile phosphate buffered saline (PBS) at a concentration of 0.15 M (pH 7.2). The bacterial suspension was divided into aliquots and stored at -80°C until further use.

Table 1

The formulation of experimental diet for *C. carpio*

Ingredients (%)	*D1 (Control)*	*D2*	*D3*	*D4*	*D5*	*D6*
Spirulina	0	5	10	15	20	25
Fish meal	15	13	11	9	7	5
Soya bean meal	32.5	30	27.5	25	22.5	20
Squid meal	3	2.5	2	1.5	1	0.5
Shrimp head meal	5	5	5	5	5	5
Corn gluten	6	6	6	6	6	6
Broken rice	6.2	6.2	6.2	6.2	6.2	6.2
Fish oil	1.3	1.3	1.3	1.3	1.3	1.3
Soya lecithin	1.2	1.2	1.2	1.2	1.2	1.2
Maida	22	22	22	22	22	22
Vitamin mix[1]	1.3	1.3	1.3	1.3	1.3	1.3
Mineral mix[2]	1.3	1.3	1.3	1.3	1.3	1.3
$NaCl_2$	1.2	1.2	1.2	1.2	1.2	1.2
Lime stone powder	2	2	2	2	2	2
Fish soluble paste	2	2	2	2	2	2

[1] The vitamin mixture used in the diet with following concentration per kilogram of diet: vitamin A - 5000 IU, vitamin D - 400 IU, vitamin E - 20 mg, thiamin mononitrate (B1) - 4 mg, riboflavin - (B2) 6 mg, nicotinamide - 50 mg; pyridoxine hydrochloride - 3 mg, calcium pantothenate - 10 mg, cyanocobalamine (B12) - 2 mg, ascorbic acid (vitamin C) - 100 mg, biotin - 0.1 mg; [2] The trace mineral mix used in the diet with the following concentrations (ppm): copper - 10, iron - 100, manganese - 50, zinc - 50, cobalt - 0.05, iodine - 0.1.

Experimental system and design. During the experimental period, water samples were collected at every 2 weeks interval for the analysis of pH, temperature, dissolved oxygen, free carbon dioxide and total alkalinity. Using a digital pH meter (Seven2Go, METTLER TOLEDO, India), the pH level was measured. Dissolved oxygen, carbon dioxide and total alkalinity were evaluated using standard procedures as provided in APHA (2005). Also, a daily cleaning by siphon method was applied to remove the feces and uneaten feed from the system. After acclimation, the fish were divided into six groups of 25 each in triplicate and fed with 0 (control without spirulina), 5, 10, 15, 20 and 25% spirulina diets at the rate of 7% of body weight twice a day. Feeding with the respective diets continued till the end of experiment. On 28^{th} day (4^{th} week) of feeding, all fish were injected intraperitoneally (i.p.) with 100 mL PBS containing *A. hydrophila* at 3.1×10^{-7} cfu mL^{-1}. On weeks 1, 2, and 4 post-infection, the blood samples were collected from fish for hematological and immunological assays after anaesthetizing them with MS-222 ($NaHCO_3$ and tricaine methanesulphonate; Sigma-Aldrich) 1:4000 in dechlorinated water for 2 min. Five fish were randomly selected for sampling from each treatment. All the fish in each treatment were used to quantify the cumulative mortality and relative percent survival (RPS) over a period of 4 weeks (post-infection period).

Growth parameters. To determine the growth performance such as the percentage weight gain, specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER), we followed the methodology of Choudhury et al (2005) and calculated it as follows:

Weight gain (%) = final weight - initial weight / initial weight × 100

SGR = Log. of final weight – Log. of initial weight / no. of days

FCR = feed given (as dry weight basis) / body weight × total weight gain (wet weight basis)

PER = net weight gain (wet weight basis) / amount of protein intake (g)

Blood serum and macrophages collection for immunological analysis. The fish were sacrificed with an overdose of anesthetic and exsanguinated by caudal vein puncture using 1 mL capacity vacuette containing a Z Serum Sep Clot Activator (Greiner

Bio-one). The blood was allowed to clot for 2 h at 4°C and the serum was separated by centrifugation at 3500 g for 25 min at 4°C, and retained at -80°C for subsequent analysis. The head kidney macrophages were isolated for the evaluation of immunological parameters according to the method of Secombes (1990).

***Immunological assays**.* The phagocytic activity of macrophages was evaluated following the method described by Sakai et al (1995). The respiratory burst activity was determined as explained by Anderson & Siwicki (1995). The complement activity was investigated as explained by Yano (1992) using rabbit red blood cells (Oxoid). The lysozyme activity was determined by turbidimetric assay according to the method of Sankaran & Gurnani (1972).

***Statistics**.* The data were presented in average of three replicates ± standard deviation. The significant effect of spirulina diets on immunological parameters such as phagocytic activity, respiratory activity, complement activity and lysozyme activity was analyzed using one-way ANOVA followed by Tukey's multiple range test.

Results and Discussion. The fish challenged with *A. hydrophila* and fed 5% spirulina diet for 4 weeks showed the highest cumulative mortality (71%). However, the fish challenged with *A. hydrophila* and increased the spirulina incorporation as 10, 15, 20 and 25% in the diet for 4 weeks reduced the cumulative mortality as 53, 27, 13 and 20%, respectively. The infected fish fed control diet suffered with 92% mortality (Figure 1). The infected common carp *C. carpio* fed with all the doses of spirulina exhibited a significant increase in growth rate and survival. The results strengthen the growing view that some plants possess a key component of immunostimulants and they have potential protection against disease inducing bacterial pathogen, thus effectively stimulating the immune system (Harikrishnan et al 2011).

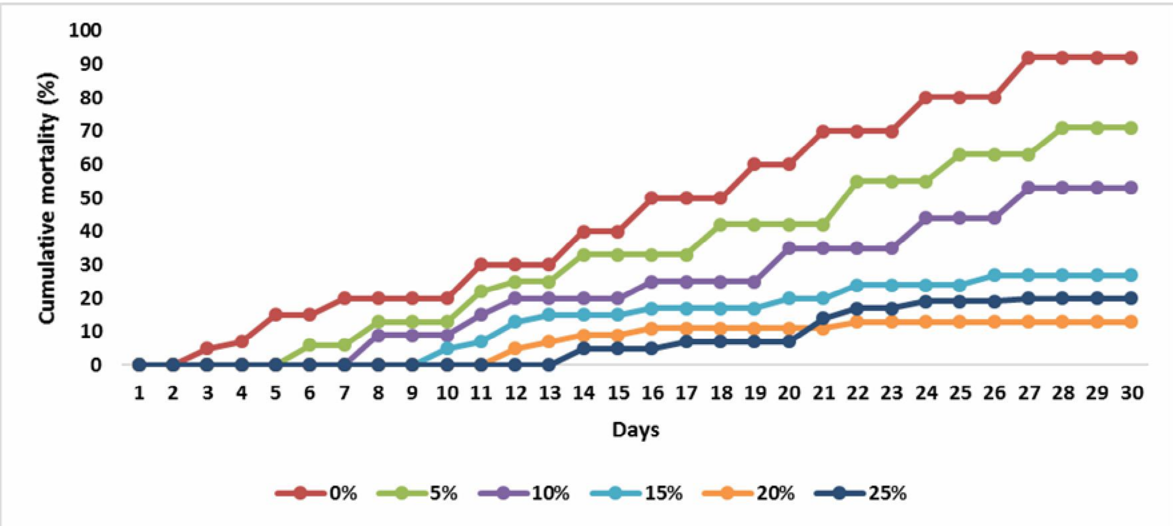

Figure 1. Effect of spirulina diets (0, 5, 10, 15, 20 and 25%) on the cumulative mortality in *C. carpio* against *A. hydrophila* infection for 4 weeks.

***Growth**.* *C. carpio* fed with spirulina (5, 10, 15, 20 and 25%) exhibited a significant growth rate when compared to the control from week 1 to 4 during the post-infected period. However, the specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) did not significantly increase due to the spirulina supplement; although, the fish fed with 15, 20 and 25% spirulina diet, PER significantly ($p < 0.05$) increased from weeks 1 to 4 (Table 2). This study is in accordance with other studies (Anbazahan et al 2014; Jagruthi et al 2014) that pigment diet increased the growth and survival of *C. carpio,* this may due to the amount of chlorophyll and carotenoids present in the spirulina.

Table 2

Weekly growth performance of *C. carpio* fed spirulina diets during the post-infection period of *A. hydrophila* for a total of 4 weeks

Growth parameters	*Doses (%)*	*Week 1*	*Week 2*	*Week 4*
Weight gain	0	28.5±1.3	29.7±1.1	31.2±1.5
	5	29.6±1.2	35.1±1.3	38.3±1.3
	10	31.9±1.6	38.4±1.5	45.8±1.7
	15	40.7±1.5	49.3±1.4	57.6±1.8
	20	37.2±1.4	40.8±1.7	50.4±1.5
	25	35.4±1.9	39.3±1.3	48.9±1.6
SGR	0	1.5±0.2	1.6±0.1	1.7±0.3
	5	1.4±0.1	1.6±0.3	1.8±0.2
	10	1.4±0.2	1.7±0.1	1.8±0.3
	15	1.5±0.3	1.7±0.5	1.9±0.4
	20	1.5±0.1	1.6±0.4	1.8±0.3
	25	1.4±0.2	1.6±0.3	1.7±0.2
FCR	0	1.7±0.5	1.8±0.2	1.8±0.3
	5	1.6±0.2	1.7±0.2	1.9±0.4
	10	1.5±0.4	1.6±0.3	1.8±0.2
	15	1.3±0.3	1.4±0.2	1.5±0.3
	20	1.4±0.5	1.5±0.2	1.7±0.2
	25	1.5±0.2	1.6±0.5	1.7±0.4
PER	0	0.7±0.2	0.9±0.1	0.8±0.2
	5	1.0±0.1	1.1±0.2	1.2±0.1
	10	1.2±0.2	1.2±0.1	1.3±0.2
	15	1.5±0.1	1.7±0.0	1.8±0.0
	20	1.7±0.1	1.8±0.2	1.8±0.1
	25	1.9±0.2	1.9±0.1	2.0±0.1

Phagocytic activity. The phagocytic activity increased significantly ($p < 0.05$) in 20 and 25% spirulina diets on first week itself, but this is not the scenario in other cases. Although, feeding with 15, 20 and 25% spirulina significantly ($p < 0.05$) elicited the phagocytic activity on week 2 and 4 but it was not the scenario in other diets (Figure 2). Cha et al (2008) reported that antimicrobial mechanisms, which include release of lysosomal enzymes, cationic peptides, complement components and production of reactive oxygen species are activated by phagocytic cells upon administration of immunostimulant. This result indicated that spirulina have the capability to enhance those mechanisms.

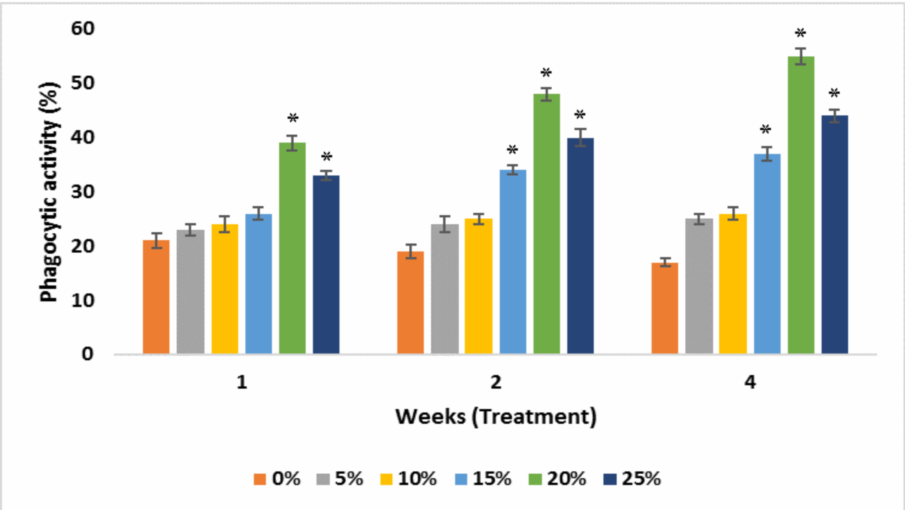

Figure 2. Effect of spirulina diets (0, 5, 10, 15, 20 and 25%) on the phagocytic activity in *C. carpio* against *A. hydrophila* post-infection for 4 weeks. The asterisk (*) indicates the significant difference between control and treatments (weeks 1, 2 and 4) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test.

Respiratory burst activity. The respiratory burst activity was determined by the production of reactive oxygen species (ROS) which significantly ($p < 0.05$) increased when feeding 20 and 25% spirulina almost a week. Although, in 15, 20 and 25% spirulina diet groups the increment was observed between 2nd and 4th weeks compared to the control (Figure 3). The ROS produced under normal oxidative damage, can be scavenged by the body's antioxidant system. As Kumaresan et al (2018) reported when the intracellular ROS increased in the body, spirulina can easily scavenge or eliminate the ROS because of its well-known potential antioxidant property.

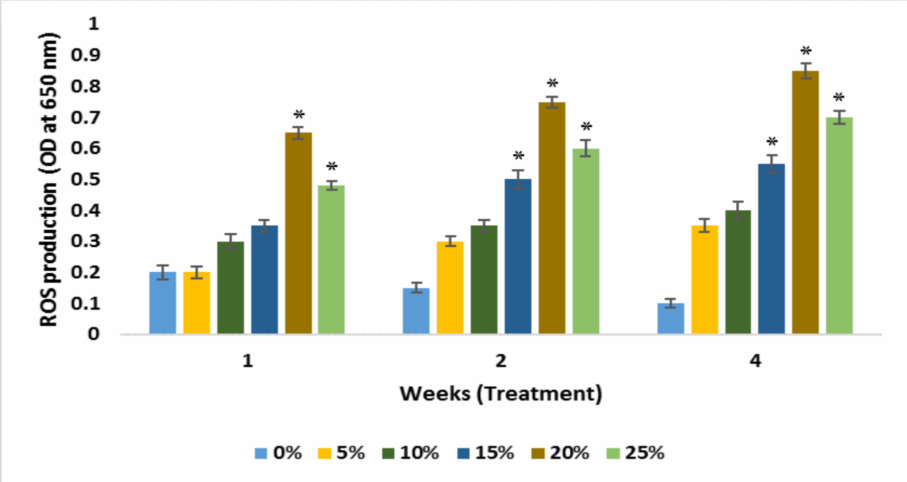

Figure 3. Effect of spirulina diets (5, 10, 15, 20 and 25%) on the respiratory burst activity in *C. carpio* against *A. hydrophila* post-infection for 4 weeks. The asterisk (*) denotes the significant different between control and treatments (weeks 1, 2 and 4) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test.

Complement activity. In innate immune system, complement is a major humoral component which plays an essential role in alerting the host immune system by the presence of virulent pathogens as well as their clearance via opsonization, activation of phagocytosis and inflammation (Boshra et al 2006). In this study, the complement activity was significantly ($p < 0.05$) enhanced with 20 and 25% spirulina diets on week 1, but not with other 5, 10 and 15% spirulina diets. Although, the complement activity was significantly ($p < 0.05$) increased with increment of spirulina (15, 20 and 25%) between second and fourth week compared to the control (Figure4). The results also indicated that the alternative serum complement as a component of the non-specific defense mechanism is more important in fish.

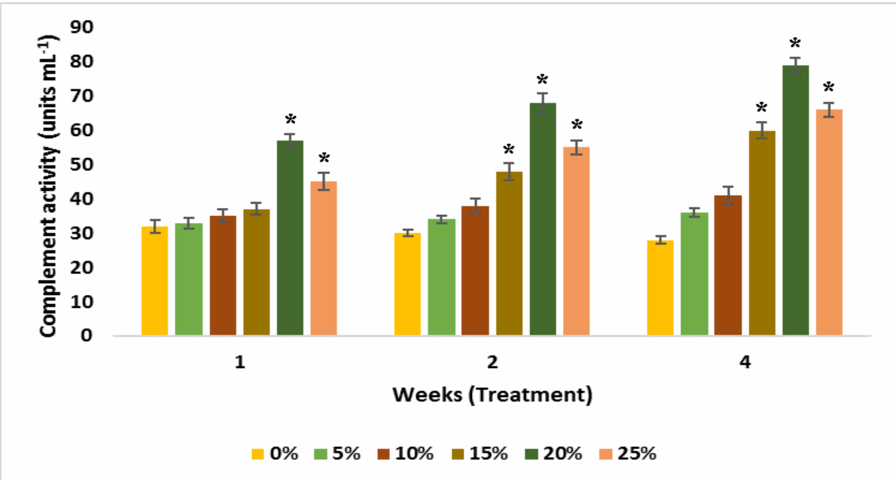

Figure 4. Effect of spirulina diets (5, 10, 15, 20 and 25%) on the complement activity in *C. carpio* against *A. hydrophila* infection for 4 weeks. The asterisk (*) represents the significant different between control and treatments (weeks 1, 2 and 4) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test.

Lysozyme activity. Lysozyme is a cationic enzyme and it is an important component of the innate immune defense and it is widely used as humoral indicator. During pathologic infection or disease, lysozyme can potentially involve to break the peptidoglycan of bacterial cell wall and protect the cells against the pathogenic infection and is known to attack mainly Gram-positive bacteria and few Gram-negative bacteria in conjunction with complement system (Jha et al 2007; Saurabh & Sahoo 2008). In this study, the serum lysozyme activity showed that all the spirulina diets (5, 10, 15, 20 and 25%) was significantly ($p < 0.05$) enhanced compared to the control group (Figure 5). It has been reported that herbals showed increased lysozyme activity in fish against various disease (Harikrishnan & Balasundaram 2005, 2008; Harikrishnan et al 2009). In our finding also, we observed that the serum lysozyme activity is increasing in fish which infers spirulina diets might have contributed in the non-specific defense mechanism against *A. hydrophila.*

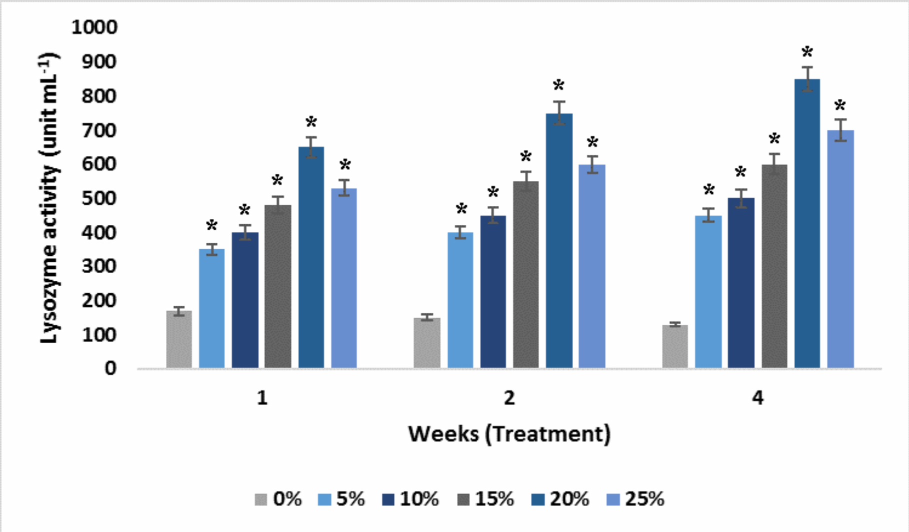

Figure 5. Effect of spirulina diets (5, 10, 15, 20 and 25%) on the lysozyme activity in *C. carpio* against *A. hydrophila* infection for 4 weeks. The asterisk (*) indicates the significant different between control and treatments (weeks 1, 2 and 4) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test.

Conclusions. Spirulina *A. platensis* diet has potential to modulate the immune defense, disease protection, growth and survival in common carp *C. carpio* against *A. hydrophila.* The optimum dose of enriching diets with spirulina is between 15 and 25% which might differ among pathogen levels and its virulence nature. Based on our findings, it is suggested that spirulina diet has potential value for aquaculture in terms of convergent growth, survival, immune response and protection against pathogenic bacterial diseases. However, further study is required to evaluate their mode of mechanism at molecular level.

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