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Evaluation of immunostimulatory effect of dietary garlic (*Allium sativum*) in fingerlings of Amur carp, *Cyprinus carpio haematopterus*

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Abstract
The present study was designed to evaluate the effect of garlic (*Allium sativum)* powder on immunological parameters in fingerlings of amur carp (*Cyprinus carpio haematopterus*). Fingerlings with an average weight of 16.11± 0.86g were distributed randomly into four treatment groups in triplicates. Experimental diets were were prepared by mixing rice bran, deoiled mustard cake, deoiled soybean cake and vitamin-mineral mixture (28.0% protein and 7.5% lipid on dry matter basis). The dried garlic powder was incorporated at three concentrations (0.5%, 1.0% and 1.5%) in feed for conducting the experiment. Garlic powder was not added to the control diet. The experimental diets were fed to fish @5% of their body weight daily over the period of 245 days followed by challenge with *Aeromonas hydrophila*. Biochemical and immunological examination results indicated that the total serum protein, albumin, globulin, A/G ratio, aspartate aminotransferase (AST**)**, serum alanine aminotransferase (ALT**)**, lysozyme activity and nitro blue tetrazolium (NBT) assay varied significantly ($P<0.05$) between the pre challenge and post challenge groups. From the obtained results it can be concluded that garlic powder in the feed of *Cyprinus carpio haematopterus* can be safely incorporated upto 1.5% for enhanced immunity against aeromonad pathogens

Keywords: Dietary garlic, common carp, immunostimulant, total serum protein and *Aeromonas hydrophila*

Introduction
As the world's human population continues to expand beyond 6 billion, its reliance on farmed fish production as an important source of protein will also increase (Naylor and Burker, 2005) [19]. Cultural intensification is an easy way of increasing fish production, but there may be chance of infection due to various factors which cause stress especially by decreased dissolved oxygen concentration and infectious disease outbreak. The unintentional consumption of antibiotics causes the chemical residues to accumulate in the fish tissues which may be hazardous to the human health. Furthermore, the fish pathogens may develop antibiotic resistance which will result in the suppression of innate immunity of fish. The antibiotic resistance to pathogens is a serious problem around the globe. In this connection, many governments have tightened national regulations on the use of antibiotics in general and aquaculture sector in particular (APHA, 2005) [5].
Many investigators have studied the immune response of fish against bacterial pathogens with the introduction of vaccines to treat the bacterial diseases like furunculosis where it has shown some promise regarding protection against the disease (Mukharjee, 2003) [17]. However, single vaccine has been found to be effective against only one type of pathogen (Hari Krishan, 2011) [14]. Moreover, the commercial vaccines are too expensive rendering them economically less feasible. Keeping all these factors in view, natural products/plant extracts or probiotics can be an effective way for protection of fish against diseases. The use of probiotic Biosyn has shown promising results in terms of immunity enhancement in Indian major carp *Labeo rohita* (Nazir *et al*., 2018) [21]. Arya *et al*., 2018 [8] reported improved haematological and biochemical parameters in fingerlings of *Labeo rohita* fed with nutraceutical, Stimulin. Herbal medicinal plants are used in fish cultures worldwide as a medium of protection against diseases. Attempts to use the natural materials such as medicinal plants could be widely accepted as feed additives to enhance efficiency of feed utilization and animal productive performance (Levic *et al*., 2008) [16]. Upreti and Chauhan, 2018) [25] have suggested that the leaf powder of giloy may be incorporated in fish feed upto 1% for enhancing growth and survival of post larvae of carp fishes up to fry stage.

Traditional herbal medicines seem to have the potential immunostimulation (Harris *et al.,* 2001) [15] Gayatri *et al.,* (2014) [13] reported that *Ocimum basilicum* leaf extract exhibited significant enhancement in total erythrocyte count, total leukocyte count, total serum protein and serum globulin in the blood of fish and also acted as effective defensive mechanism for controlling many diseases.

Alambra *et al.,* 2012 [2] reported that dried turmeric powder has the capacity to modulate the antimicrobial peptides, particularly crustin and lysozyme, of the shrimp *M. rosenbergii* when challenged with *V. Alginolyticus*. Dash *et al.,* 2014 [11] observed improved biochemical, haematological and immunological values in *Labeo rohita* infected with *Aeromonas hydrophila* with use of garlic–mineral oil. Among the various plants garlic, *Allium sativum*, is the herb which is most widely quoted in the literature for medicinal properties and is the natural antibiotic known to be effective for treatment of various diseases in humans and animals owing to its antibacterial, antiparasitic, antimicrobial, antioxidant, anticancer and antihypertensive properties. In aquacultural operations, optimized dose of garlic is strongly recommended. Hence the present study was made with the prime objective to evaluate the immunomodulatory potential of garlic powder in fingerlings of Amur carp.

Material and methods

The experimental work was carried out at Wet Lab of the College of Fisheries, G. B. Pant University of Agriculture and Technology, Pantnagar, in tarai region of Uttarakhand. The hatchery reared fingerlings of Amur carp (*Cyprinus carpio haematoptereus*) having average weight of 16.11±0.86 g were stocked in 1 ton capacity tanks. The experimental fishes were subjected to four treatments (T1, T2, T3 and T4) in three replicates (4 x 3 = 12). In treatment T1 (control), the fishes were fed with diet without garlic powder (D1). The fishes stocked in treatments T2, T3 and T4 were fed with diets containing 0.5% (D2), 1.0% (D3) and 1.5% (D3) garlic powder, respectively at the rate of 5% of body weight daily. On the 230th day fishes were challenged with *Aeromonas hydrophila*. The physico chemical water quality parameters were regularly monitored as per standard methods (APHA, 2012) [6].

Calculation of Biochemical parameters

Total serum protein (TSP) (in g/dl) = (Absorbance of test / Absorbance of standard) ×6.5

Albumin (in g/dl) = (Absorbance of test / Absorbance of standard) ×4

Globulin = Total serum protein – Albumin

$$\text{Albumin - globulin ratio} = \frac{\text{Serum albumin (g/dl)}}{\text{Serum globulin (g/dl)}}$$

Serum Aspartate aminotransferase (AST)

Method of International Federation of Clinical Chemistry (IFCC) kinetic was followed to estimate AST activity in serum using Erba Diagnostic Kit (Transasia Bio-medicals Ltd., Solan H. P., India) at 340 nm wave lengths. The result was expressed in IU/L. The general formula for converting absorbance change into International Units (IU) of activity is:

$$(\text{IU/L}) = \frac{\Delta\text{Abs./min.} \times \text{Total reaction volume (µl)} \times 1000}{\text{Sample Volume (µl)} \times \text{Absorptivity} \times \text{Cuvette light path (cm)} = 1\text{ cm}}$$

Where, Absorptivity = Millimolar absorptivity of NADH at 340 nm = 6.22

Activity of AST = ΔAbs./min. x 1768

Serum Alanine aminotransferase (ALT)

ALT activity in serum was estimated by International Federation of Clinical Chemistry (IFCC) kinetic method using Erba Diagnostic Kit (Transasia Bio-medicals Ltd., Solan, H. P., India) at 340 nm wavelength and the result was expressed in IU/L.

The general formula for converting absorbance change into International Units (IU) of activity is:

$$(\text{IU/L}) = \frac{\Delta\text{Abs./min.} \times \text{Total reaction volume (µl)} \times 1000}{\text{Sample Volume (µl)} \times \text{Absorptivity} \times \text{Cuvette light path (cm)} = 1\text{ cm}}$$

Where, Absorptivity = Millimolar absorptivity of NADH at 340 nm = 6.22

Activity of ALT = ΔAbs./min. x 1768 at 37^0 C (IU/L)

Immunological parameter

NBT assay

For NBT assay, 50µl of blood sample was taken and transferred to "U" bottom ELISA plate. The sample was incubated in a dry bath at 37°C for one hr to facilitate cell adherence. The ELISA plate was washed thrice with 100µl of PBS to remove non adherent blood cells. As coloring agent, 100µl of 0.2% NBT (nitroblue tetrazolium assay) was added to plate and again incubated for 1 hr. 100µl of 100% methanol was used to fix the blood cells for 2-3 minutes. Cells were then washed with 100µl of 70% methanol and allowed to dry. 120µl of 2N KOH and 140µl DMSO was added and mixed properly to dissolve formazene blue precipitation. Absorbance was recorded in ELISA plate reader (Tecan) 620nm.

Lysozyme activity

The lysozyme activity was studied by turbitimeter assay (Parry *et al*., 1965) [25]**.** Overnight grown *Micrococcus luteus* (ATCC 49732) in nutrient broth (10^7 - 10^8 CFU/ml) was centrifuged (800rpm, spinwin) to collect the pellet cell. Pelleted bacterial cell was allowed to suspend in 0.05M sodium phosphate buffer (pH 6.2). To 1 ml of bacterial suspension, 50µl of serum was added. After addition of the serum, the absorbance was recorded twice (at time interval of 0.5 min. and 4.5min) in spectrophotometer (Thermo scientific U V 1) at 530 nm. A unit of lysozyme activity was defined as the amount of sample causing the decrease in absorbance of 0.001/min.

Results and discussion

The observations of biochemical and immunological parameters in different treatment groups have been presented in Tables 1 and 2.

Biochemical results

Total serum protein (TSP)

Total serum protein content (TSP**)** for Amur carp in pre challenged fish ranged from 2.68±0.068 g/dl (T_4) to 2.94±0.063 g/dl (T_1). In case of post challenge fish, the range of TSP is in between (2.40±0.007 g/dl (T_1) to 3.03±0.068 g/dl (T_4). In post challenge group, total serum protein content was enhanced with enhancing concentration of garlic powder. The comparison among pre challenge and post challenge data

showed that the value of total protein content was significantly different (p> 0.05) in T_1, T_3 T_2 and T_4 among the pre challenge and post challenge fishes, whereas the value did not differ significantly among the treatments over the study period (Tables 1 and 2). Weaned Rabbits fed on dietary rich *Allium sativum* and ginger (*Zingiber officinale*) feed showed significant increase in TSP (P<0.05) as compared to control (Onu and Aja, 2011) [23].

Albumin

In case of pre challenged groups the albumin content varied from 0.68±0.063 (T_1) to 0.99±0.007g/dl (T_4). Whereas the range of albumin content in case of post challenged group recorded was 0.61±0.01 g/dl (T_1) to 0.98±0.04 g/dl (T_4). The value of albumin content between pre challenged and post challenged groups differ significantly (p>0.05) among the treatments. The albumin content within the same group of fishes in post challenged and pre challenged fishes did not differed significantly (p>0.05) within the duration of experiment (Tables 1 and 2). Similarly observation recorded by Das *et al.,* (2009) [10] showed significant difference in albumin content in fish fed with *Euglena* powder at different assay of treatment groups as compared to the control one, thus supporting the findings of the present investigation

Globulin

In case of pre challenged fishes the globulin content were varied from of 1.09±0.007 g/dl (T_1) to 2.13±0.026 g/dl (T_4) (Table 1). While as the range of globulin content recorded in post challenged fish was 0.95±0.04 g/dl (T_1) to 2.0±0.07 g/dl (T_4). The value of globulin content between pre challenged and post challenged fish did not differ significantly (P> 0.05) among the treatments (Table 2). Similar findings were also recorded by Nahak and Sahu (2014) [18] in *Clarias batrachus* fed with O*cimum basilicum* added diets.

A/G Ratio

In case of pre challenge groups the albumin and globulin ratio was varied in between 0.37±0.007 (T_3) to 0.62±0.007 (T_1) whereas in post challenged fish value was ranged from 0.44±0.007 (T_4) to 0.64± 0.007 (T_1) (Tables 1 and 2). The A/G ratio of T_1 and T_4 varied significantly (p< 0.05) from pre challenge to post challenged fishes. The value A/G ratio significantly differ (p> 0.05) among the treatments in pre challenged group. This flexible values in A/G ratio observed in present investigation is also supported by the findings recorded by Abasali and Mohammad (2010)[1], where experiment carried out in *Cyprinus carpio* fed with plant based immunostimulant diet followed by challenge with *Aeromonas hydrophila* depicted flexible values of A/G ratio in some treatment fish.

Alanine Aminotransferase (ALT)

The Alanine Aminotransferase **(**ALT) content in pre challenged fishes was in the range of 29.80±0.542IU/L (T_3) to 43.43±0.639IU/L (T_1) (Table 1). In case of post challenged fishes, the range of ALT content recorded was 23.43±0.688 IU/L (T_4) to 41.20±0.349 IU/L (T_3). The values of ALT content among pre challenged and post challenged fishes varied significantly (p>0.05) amongst the treatments (Tables 1 and 2). The experiment conducted by Fazlolahzadeh *et al.*, 2011 [12] on rainbow trout, *Oncorynchus mykiss,* fed with garlic rich diet resulted in variable values of ALT which did not vary significantly in treatments group compared to control.

Aspartate Aminotransferase (AST)

The Aspartate Aminotransferase **(**AST) content in pre challenged fishes was in the range of 85.33±0.202 IU/L (T_4) to 112.57±0.693 IU/L (T_1) (Table 1). In case of post challenged fishes, the range of AST content recorded was 77.83±0.984 IU/L (T_4) to 110.73±1.392 IU/L (T_1). The values of AST among pre challenged and post challenged fishes varied significantly (p>0.05) among the treatments. Al-Salahy *et al.,* (2002) [3] found that C*larias lazera* fed with garlic and onion diet has resulted variable values of AST and did not vary significantly in treatments compared to control. Decrease in enzymatic activities (ALT and AST) values in post challenge group fed with 1.5% garlic powder may prove protective mechanism of the garlic powder incorporated diet against *Aeromonas hydrophila* over the period of experiment.

Table 1: Values of different biochemical and immunological parameters in different treatment groups of pre challenged fishes, *Cyprinus carpio haematopterus*

Parameters	T_1	T_2	T_3	T_4	SEM	CD	F-Value
TSP (g/dl)	2.68±0.068b	2.77±0.068b	2.70±0.077b	3.94±0.063a	0.0895	0.291	4290**
Albumin (g/dl)	0.68±0.063b	0.72±0.007a	0.74±0.007a	0.99±0.007a	0.0088	0.028	18.147**
Globulin (g/dl)	1.09±0.007b	1.74±0.258a	1.98±0.007a	2.13±0.026a	0.1675	0.054	9.626**
A/G ratio (g/dl)	0.62±0.007a	0.41±0.007a	0.37±0.009a	0.46±0.007a	0.0095	0.031	26.421*
ALT(IU/L)	43.43±0.61a	37.20±0.69b	34.20±0.31c	29.80±0.54d	0.7327	2.38	61.009*
AST(IU/L)	112.57±0.693a	107.47±0.211b	68.23±0.27c	85.33± 0.20	0.5153	1.679	537.931**
NBT assay (OD)	0.065±0.001d	0.074±0.001b	0.09±0.001b	0.102±0.001a	0.0013	0.004	158.365**
Lysozyme Activity (U/ml)	124.58±0.917d	142.98±0.778c	153.12±0.096b	186.38±0.135a	0.78312	2.554	1.95.983**

* Significant at 5% level, ** Significant at 1% level, ns = non-significant

Table 2: Values of different biochemical and immunological parameters in different treatment groups of *Cyprinus carpio haematopterus* post challenged with *Aeromonas hydrophila*

Parameters	T_1	T_2	T_3	T_4	SEM	CD	F-Value
TSP (g/dl)	2.40±0.007c	2.87±0.06a	2.97±0.052a	3.03±0.068a	0.0866	0.28226	10.91**
Albumin (g/dl)	0.61±0.01b	0.80±0.01a	0.85±0.02a	0.98±0.04a	0.0088	0.28740	18.14**
Globulin (g/dl)	0.95±0.04b	1.55±0.02a	1.82±0.03a	2.0±0.07a	0.0899	0.293036	33.44**
A/G ratio (g/dl)	0.64±0.007a	0.52±0.005a	0.47±0.007a	0.44±0.007a	0.9279	0.03024	44.93**
ALT(IU/L)	41.20±0.349a	31.40±0.65b	28.37±0.66c	23.43±0.68d	0.7824	2.5502	91.65**
AST(IU/L)	110.73±1.392a	98.63±1.73b	98.23±1.69b	77.83±0.98c	1.9118	6.231	50.93**
NBT assay (OD)	0.069±0.002b	0.080±0.003a	0.052±0.06b	.0389±0.001c	0.0389	0.127	70.515**
Lysozyme Activity (U/ml)	174.54±13.135c	221.97±0.250b	242±0.233a	252.84±0.115a	8.4819	27.644	16.682**

* Significant at 5% level, ** Significant at 1% level, ns = non-significant

Immunological parameters

NBT Test

Respiratory burst activity can be used as an indicator of infection. NBT plays an important role in assessing the control of pathogen. In case of pre challenged groups the level of superoxide anion production nitro blue tetrazolium (NBT) were ranged from 0.065±0.001 (T_1) to 0.101 ±0.001 (T_4) (Table 1). The tendency was enhancing from T_1 to T_4 in pre challenged fishes. For the post challenge groups, level of superoxide anion production also varied from 0.0691±0.002 (T_1) to 0.1134± 0.001 (T_4) (Table 2). Comparison within the pre challenged and post challenged fish clearly depicted that the level of NBT significantly (P >0.05) enhanced from pre challenged to post challenged groups over a period of time. Superoxide anions are produced by the respiratory burst of phagocytes and it is one of the important factors that restrict the growth of pathogen. The results recorded in the current study are in conformity with the findings of Sahu *et al.*, (2007) [24]. Das *et al.*, (2009)[10] reported significant increment in NBT levels in the groups of fish fed with garlic powder at different assay period of the treatment groups as compared to the control one.

Lysozyme activity

In case of pre challenge fishes the lysozyme activity ranged from 124.58 ± 0.917 U/ml to 186.38 ±0.135 U/ml (Table 1). The lower value was 124.58 ±0.197 U/ml shown by (T_1) and higher 186.38 ±0.135 U/ml by (T_4). In case of post challenge, the value varied from 174.54 ±1.135 to 252.84 ±0.115 U/ml (Table 2). The minimum level was recorded as 174.54±1.135 SE U/ml for (T_1) and maximum level was 252.84±0.115 for (T_4). The lysozyme activity was significantly (P < 0.05) enhanced in all treatment groups among the pre challenged and post challenged groups. Some other authors also recorded appreciable enhancement in this innate factor stemming from dietary supplementation of *Eclipta alba* leaf extract for *Oreochromis mossambicus* (Chyristybapita and Divyagnaneswari, 2007) [9] and garlic powder for *Oncorhynchus mykiss* (Nya and Austin 2011) [22].

The values of water quality parameters were not significantly different to each other at 5% level of significance. Hence, there was no adverse effect of water quality parameters on experimental fish among all the treatments. The results are in accordance with earlier findings of Anita *et al.,* (2016) [4], Arya *et al.,* (2016) [20] and Nazir *et al.,* (2015) [14].

Conclusion

On the basis of the present investigation, it could be summarized that inclusion of dried garlic garlic (*Allium sativum)* powder in carp feed at the rate of 1.5% has potential for enhancing immunity against aeromonad pathogens in fingerlings of amur carp (*Cyprinus carpio haematopterus*) and does not have any adverse effect in the culture system.

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