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Effects of polyherbal formulation 'ImmuPlus' on immunity and disease resistance of Indian major carp, *Labeo rohita* at different stages of growth

Jaya Kumari, P K Sahoo* & S S Giri

Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar 751 002, India

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A series of experiments were performed to determine the impact of polyherbal immunomodulatory formulation 'ImmuPlus (AquaImmu) on growth, immunity and disease resistance of rohu (*Labeo rohita*), one of the Indian major carp at different stages of growth. Rohu larvae were fed on plankton, ImmuPlus-mixed compound feed, and plankton plus ImmuPlus-mixed compound feed (ImmuPlus added at three dose levels of 0.25, 0.50, and 0.75 g/kg feed) from 4th day of hatching to 14th day. ImmuPlus-mixed diets enhanced growth of larvae, survival and disease resistance against *Aeromonas hydrophila* challenge, compared to only plankton-fed group. In two other experiments, advanced rohu larvae and fingerlings were fed with ImmuPlus-mixed compound feed (at 0, 0.5, 1.0 and 2.0 g/kg) for 15, 30 and 45 days. At the end of 45 days for advanced larvae and 30 days for fingerlings, the fish fed with ImmuPlus at 1.0 g/kg level showed significantly higher growth and disease resistance against *A. hydrophila* challenge. In a separate experiment, juveniles of rohu were fed with 1 g/kg of ImmuPlus incorporated feed for 15 and 30 days. At the end of the trial, the ImmuPlus fed fish showed enhanced non-specific immunity (as measured through nitroblue tetrazolium reduction assay, serum lysozyme activity, serum haemolysin titre and resistance against *A. hydrophila* challenge in non-vaccinated fish as well as specific immunity levels (as measured through bacterial agglutination titre against *A. hydrophila* in vaccinated fish). Incorporation of ImmuPlus at 1 g/kg level in the diet of rohu may be beneficial for enhancing disease resistance.

Keywords: *Aeromonas hydrophila*, Growth, Immunity, Immunostimulation, ImmuPlus (AquaImmu), *Labeo rohita*,

In India, with the emergence of large-scale commercial carp culture, diseases of varied aetiology are being increasingly recognised as a major hurdle to successful and sustainable farming[1]. The use of chemotherapeutics for controlling diseases has been criticized for their negative impacts, including accumulation of tissue residues, environmental pollution, development of drug resistance in pathogens and immunosuppression[2-4]. There is a need to look for ecofriendly disease preventive measures to promote sustainable culture of Indian major carp. In order to reduce the risk of disease at different stages of growth, the level of resistance to infection in the cultured organisms should be increased by the use of better feed, vaccines, immunostimulants or by selective breeding for disease resistance[5]. Immunostimulants increase resistance to disease by enhancing the non-specific immune system and their use has been given considerable attention in aquaculture. Immunostimulants, used in vaccines to amplify the specific immune response or administered as feed additives to modulate non-specific immunity, have been demonstrated to play role in protection against diseases in fish[6-12] and enhancing growth[13]. The modulation of immune response by using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigations[14].

India has a rich resource of traditional herbal medicines to treat human and animal diseases. These have no or little side effects during treatment. Commonly used herbal extracts are from *Ocimum sanctum* (Tulsi), *Withania somnifera* (Ashwagandha), *Tinospora cordifolia* (Guduchi) and *Emblica officinalis* (Amlaki) for the treatment of immunosuppressive conditions for humans and animals[15]. However, they have not been tried systematically to control fish diseases. Recently, it has been demonstrated that feeding of ImmuPlus at 1 g/kg to *Macrobrachium rosenbergii* for 3 weeks significantly raised the immunity level[16]. ImmuPlus (AquaImmu), a polyherbal formulation (Indian Herbs Supply and Research Company Limited, Saharanpur, India) contains the extracts of selected natural Indian medicinal plants *viz.*, *O. sanctum* (Tulsi), *W. somnifera* (Ashwagandha), *T. cordifolia* (Guduchi)

*Correspondent author
Telephone: +91-674-2465421, Fax: +91-674-2465407
E.mail: pksahoo1@hotmail.com

and *E. officinalis* (Amlaki*)* as major constituents in their optimum concentrations.

The present study has been undertaken to evaluate the modulatory role of polyherbal formulation 'ImmuPlus' on immunity, disease resistance and growth at different stages in the Indian major carp, *Labeo rohita* through oral feeding. *Aeromonas hydrophila*, one of the important bacterial pathogens of cultured carp, was used as model pathogen for challenge/susceptibility studies because of its ubiquitous nature and involvement in many disease outbreaks.

Materials and Methods

Four experiments were conducted at different stages of growth of rohu *(Labeo rohita)* to evaluate the impact of feeding with ImmuPlus on growth, immunity and disease resistance.

Experiment one

Fish and experimental systems—Three days old apparently healthy rohu larvae (average weight 0.02 mg), used for this experiment were obtained from the Central Institute of Freshwater Aquaculture (CIFA) hatchery. Fish (n=1755) were distributed randomly to 27 numbers of 10 litre capacity glass jars stocked with 65 spawns in each jar and maintained under continuous aeration. Half of the water in the jar was replaced daily throughout the experimental period after siphoning out the let over feed and faecal matter. The water temperature ranged between 26-31°C during the experimental period.

Experimental diet—A standard powdered feed mixture for rohu larvae was used as the basal diet[11]. ImmuPlus was incorporated into the basal diet at the ratio of 0.25, 0.50 and 0.75 g/kg feed. The basal diet without ImmuPlus was used as control. Apart from the artificial diet, plankton was also used as natural source of food.

Experimental design—The experimental design was based on the feeding categories. Three random groupings were made for this experiment. One group of larvae was provided with plankton only as feed source. The second group was provided with measurable amount of plankton (10^5 numbers/jar) as well as artificial basal diet (*ad libitum*) supplemented with graded levels of ImmuPlus (0, 0.25, 0.50, 0.75 g/kg) as moist balls to four different subgroups of rohu larvae, respectively. In the third group, the larvae received only ImmuPlus-supplemented compound feed in the moist ball form with similar graded levels of ImmuPlus without supplementary plankton. All the three groups were fed at *ad libitum* twice daily. The experimental feeding was continued from day 4 to 14 (till the end of larval stage). The experiment was run in triplicate.

Growth—After 10 days of feeding, 15 larvae were removed from the jar of each group/subgroup and their weight and the percent mortality was recorded.

Challenge experiment—To study the susceptibility of the various groups to *A. hydrophila* (pathogenic isolate from carp ulcer lesions maintained at Aquatic Animal Health Division of CIFA), 45 larvae from each glass jar from each subgroup were utilised. Larvae were challenged with lethal dose of *A. hydrophila* (10^8 live cells/ml PBS) by suspension method for 1 hr. The mortality (%) pattern was studied for 10 days. The cause of death was confirmed by isolating the organism from the 10% of moribund or dead larvae.

Experiment two

Fish and experimental system—Advanced rohu larvae (240; average weight 1.92 g) were obtained from CIFA farm (from one pond). Fish were held in 40 litre capacity circular plastic aquaria under continuous aeration. Waste feed and faecal material along with 50% of water were removed daily from the aquaria and exchanged with fresh dechlorinated tap water. Fish were acclimated for 15 days before the experiment.

Experimental design and diet—The trial consisted of four groups, run in triplicate, with 60 fish (20 × 3) in each group. A basal feed mixture was prepared [11]. Four experimental pelleted diets with the above mixture containing graded levels of ImmuPlus at the ratios of 0 (control), 0.5, 1 and 2 g/kg feed were prepared from the basal diet, and served to groups A, B, C and D, respectively. The fish were fed with these experimental diets at a ratio of 5% of their body weight twice daily throughout the experimental period. All the groups were fed the experimental diet for 45 days, twice daily (1000 and 1700 hrs). The body weight of fish was recorded at 15 days intervals. At the end of the trial, fish were subjected to intraperitoneal challenge with lethal dose (2.3×10^7 cells in 50 µl of PBS) of *A. hydrophila* (previously calculated using same larvae) and mortality (%) was recorded up to 10 days. The cause of death was confirmed by isolating the organism from the liver and kidney of 10% of dead fish.

Experiment three

Fish and experimental system—Rohu fingerlings (180; weighing 7.12-7.46 g), were obtained from CIFA pond. Fish were maintained in 1000 litre capacity circular cement tanks (with 200 litre of dechlorinated water) under continuous aeration. Each tank contained 10 fishes and other experimental systems were similar to the previous experiment.

Experimental design—The fish were randomly divided into three groups. Group A received pelleted control diet[11] throughout the experimental period of 30 days. Groups B and C received ImmuPlus supplemented diet at a rate of 0.5 and 1 g/kg feed, respectively for the same period twice daily at a ratio of 5% of their body weight. The experiment was run in triplicate.

Growth and non-specific immune response—The impact of ImmuPlus on growth was studied by recording the individual weight of 10 fish of one tank at 0, 15, 30 days. At the end of 30 days blood samples were collected from the same fish. The sera obtained were stored at –20 °C until further analysis.

Part of the serum samples of each group were analysed for total protein following the dye binding method of Bradford[17] using bovine serum albumin (BSA) as a standard, albumin by the bromocresol green method[18] and globulin (subtracting albumin from total protein). The remaining serum was used to measure the natural serum haemolysin titre following Sinha and Chakravarty[19].

Challenge experiment—The fish of another tank (n=10) of each group were challenged with 10^6 live cells of 24 hr culture of *A. hydrophila,* ip on 30th day and the per cent mortality was recorded up to 40th day of the experiment. The experiment was run in triplicate.

Experiment four

Fish and experimental system—Rohu juveniles (weighing 30-50 g) obtained from CIFA ponds were maintained in 1000 litre capacity cement tanks. The basic physico-chemical water parameters were measured systematically to maintain at their optimal level throughout all the experiments (dissolved oxygen: 4.5 mg/l, *p*H: 7.3, nitrites: 0.005 mg/l, ammonia: 0.021 mg/l). Daily 10% of the water was changed and waste feed and faecal materials were removed at every 24 hr.

Experimental diet—A pelleted feed was prepared, as described previously, which served as control/basal diet. Fish feed (basal diet incorported with 1 g ImmuPlus/kg feed) was used as experimental diet. Basal diet was fed to fish at a ratio of 3% of their body weight twice daily. The fish were acclimated for 15 days with basal diet.

Experimental design—The fish were randomly distributed in two groups for studying the effect of ImmuPlus on non-specific and specific immunity levels, and disease resistance against *A. hydrophila*. Group A served as control and was provided with basal pelleted diet whereas group B was provided with ImmuPlus-mixed diet (1 g/kg feed) based on the results of experiments 2 and 3 above. Each group was divided into two subgroups. Subgroups A_1 and A_2 received control diet for 15 and 30 days, respectively. Subgroups B_1 and B_2 received ImmuPlus-mixed diet for 15 and 30 days, respectively. All the experiments were run in triplicate.

Non-specific resistance factors—On days 15 and 30, five fish from each group were bled with heparinised syringe to study neutrophil activity and eight fish were bled to collect serum to study serum lysozyme level, haemolysin titre, total protein and albumin-globulin ratio (A:G) to measure the non-specific immunity level. All fish were anaesthetised with 2-phenoxyethanol (Sigma) before any injection or blood collection from the caudal vein.

The oxidative radical production by neutrophils was assessed by the NBT assay following the method of Anderson *et al*[20].

Lysozyme levels in serum were measured using turbidimetric assay[21,22]. Lyophilized hen egg white lysozyme, HEWL (Sigma) was used to develop a standard curve. Serum lysozyme values were expressed as µg/ml equivalent of hen egg white lysozyme activity. Haemolysin titre and total protein levels were measured in the similar manner as described earlier.

Specific immune response—To study the effect of ImmuPlus on specific immunity level, 20 fish of each group were injected intraperitoneally with 0.1 ml formalin-killed *A. hydrophila* bacterin (2.5×10^9 cell/ml PBS mixed with equal amount of Freund's complete adjuvant) per fish. After 15 and 30 days of ImmuPlus feeding, 10 fish from each group were bled and collected sera were used to study bacterial agglutination titre against *A. hydrophila* following the method of Plumb and Areechon[23]. The antibody titre was expressed as the reciprocal of last dilution at which agglutination of bacteria occurred.

Disease resistance—Fish, 20 from each group were challenged, with *A. hydrophila* (10^6 cells/ml, grown in TSB, washed and diluted in PBS) on 16th and 31st day of the ImmuPlus feeding and the percentage mortality was observed for 10 days.

Statistical analysis—The data generated in all the experiments were subjected to one or two-way ANOVA test followed by DMRT[24]. Significance level used was $P < 0.05$.

Results

Experiment one—The details of the growth obtained in various larval groups, mortality observed during the experiment and in response to *A. hydrophila* challenge are presented in Table 1.

The pattern of mortality observed during the experiment did not follow any of the feeding patterns. However, a distinct difference was observed in larvae fed with only artificial feed, indicating a clear impact of ImmuPlus supplemented diet giving higher survival. There was no difference observed within various levels of ImmuPlus feeding in that group.

The growth rate was the least in only plankton fed group and other ImmuPlus fed groups had higher growth, thus clearly indicating the better efficacy of artificial feeding. Although a clear impact of ImmuPlus feed on growth was not marked, significantly ($P < 0.05$) lower growth was obtained at higher level (0.75 g/kg) of incorporation of ImmuPlus into the diet in the absence of plankton.

ImmuPlus incorporation up to a level of 0.5 g/kg feed seemed to have a beneficial effect on growth. Artificial diet as well as incorporation of ImmuPlus into the feed raised the survival significantly in response to *A. hydrophila* challenge compared to only plankton feeding group. However, in one subgroup of second group receiving plankton and ImmuPlus incorporated diet (0.5 g/kg) revealed sudden higher mortality and the cause could not be explained.

Experiment two—There was no significant ($P < 0.05$) difference in growth obtained in various dose levels of ImmuPlus feeding in comparison to control till one month of feeding. On the 45th day, group C fed with ImmuPlus @ 1 g/kg feed showed significant rise in body weight compared to control and the highest dose ImmuPlus-fed groups. However, at the highest level of Immuplus feeding i.e. at a rate of 2 g/kg feed, the growth rate declined compared to group C (1 g/kg feed) fish (Table 2).

When the fish were subjected to *A. hydrophila* challenge, the control group fish recorded 96.67% mortality. The feeding of ImmuPlus @ 1 and 2 g/kg raised the survival significantly in comparison to other two groups (Table 2). However, there was no significant difference in mortality in these two higher levels of ImmuPlus feeding.

Experiment three—A marked increase in growth was recorded in group C fish fed with ImmuPlus at a rate of 1 g/kg for 30 days in comparison to other groups (Table 3). Feeding of ImmuPlus did not affect non-specific immunity as measured through total protein, albumin, globulin levels and natural haemolysin titre (Table 3). On the other hand, ImmuPlus supplementation reduced the mortality (%) by half compared to the control at both the level of incorporation against *A. hydrophila* infection (Fig. 1).

Experiment four—Feeding of ImmuPlus for 15 or 30 days significantly ($P < 0.05$) raised the neutrophil

Table 1—Growth, survival and mortality (%) due to *Aeromonas hydrophila* challenge in larvae of rohu (*Labeo rohita*) fed with plankton and/or feed supplemented with graded levels of ImmuPlus.

[Values are mean ± SE]

Group	Subgroup/feed (Immuplus level g/kg feed)	Growth (mg)	Mortality (%) during growth	Mortality (%) due to *A. hydrophila* challenge
1 (Plankton only)	Plankton (Pl)	1.98 ± 0.25 ^{a}	4.28 ± 0.48 ^{a}	35.84 ± 9.01 ^{b}
2 (Plankton + feed)	A (Pl + 0 g/kg)	2.68 ± 0.21 ^{bc}	1.63 ± 1.63 ^{a}	3.90 ± 2.22 ^{a}
	B (Pl + 0.25 g/kg)	3.00 ± 0.14 ^{bcd}	5.32 ± 1.59 ^{b}	8.98 ± 4.50 ^{a}
	C (Pl + 0.50 g/kg)	3.64 ± 0.16 ^{e}	5.08 ± 2.84 ^{b}	29.99 ± 7.84 ^{b}
	D (Pl + 0.75 g/kg)	3.31 ± 0.16 ^{de}	0.79 ± 0.79 ^{a}	7.79 ± 0.80 ^{a}
3 (Feed only)	A (0 g/kg)	3.22 ± 0.08 ^{ce}	5.27 ± 1.69 ^{b}	14.10 ± 1.82 ^{a}
	B (0.25 g/kg)	3.33 ± 0.31 ^{de}	0.53 ± 0.53 ^{a}	10.85 ± 6.50 ^{a}
	C (0.50 g/kg)	2.93 ± 0.04 ^{bcd}	1.85 ± 0.44 ^{a}	12.26 ± 3.49 ^{a}
	D (0.75 g/kg)	2.60 ± 0.04 ^{b}	1.29 ± 0.66 ^{a}	11.52 ± 3.92 ^{a}

Means bearing the different superscript(s) column-wise are significantly ($P < 0.05$) different.

and lysozyme activities as well as haemolysin titre compared to that of control (Table 4). The effect of feeding ImmuPlus on serum protein, albumin and globulin levels are presented in Table 5. Feeding of ImmuPlus significantly increased the serum total protein, albumin and globulin concentrations, and lowers the albumin-globulin ratio.

The effect of ImmuPlus feeding on specific immunity level was observed through measuring antibody titre against *A. hydrophila* vaccination (Table 6). A time-related significant increase in bacterial agglutination titre was noticed in ImmuPlus fed fish compared to control fish. On the contrary, when the non-vaccinated fish were challenged with *A. hydrophila,* only a numerical decrease in percent mortality was noticed in ImmuPlus-fed fish (Table 7).

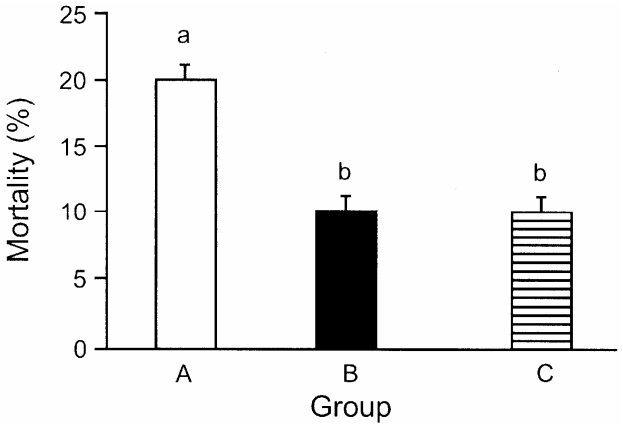

Fig. 1—Mortality (%) in rohu fingerlings fed graded levels of ImmuPlus on and intraperitoneal challenge with *A. hydrophila* (A = control, B = 0.5 g, and C = 1.0 g ImmuPlus/kg feed group). Bars showing different letter are significantly different ($P < 0.05$)

Discussion

This work evaluated the influence of ImmuPlus-supplemented feed on growth, immunity and disease resistance against *A. hydrophila* challenge at different stages of growth of rohu. The use of natural immunostimulants in fish culture for the prevention of diseases is a promising new development[6,8,25,26]. Natural immunostimulants are biocompatible, biodegradable and safe for the environment and human health[27]. Immunostimulants do not eradicate pathogens, but can increase non-specific immunity and may lead to reduction in mortality caused by opportunistic pathogens or stressors.

An oral administration permits mass treatment of fish in a population and does not impose handling related stress, and the present work was planned accordingly. Since time and/or dose-dependent effects of immunostimulants are to be investigated, we tested three dose levels of ImmuPlus at different periods, parameters that are fundamental in any immunomodulatory strategy[8, 26]. Few of the plant extracts/products have been evaluated for their

Table 2—Growth and mortality (%) due to *Aeromonas hydrophila* challenge in a 45 days feeding trial of advance larvae of rohu (*Labeo rohita*) fed with graded levels of ImmuPlus supplemented feed.

[Values are mean ± SE]

Group (ImmuPlus/kg feed)	Initial weight (g)	Body weight (g)			Mortality (%) due to *A. hydrophila* challenge
		15 days	30 days	45 days	
A (0 g)	1.92	2.74 ± 0.19	2.73 ± 0.11	2.94 ± 0.02 ^{a}	96.67 ± 1.93 ^{b}
B (0.50 g)	1.92	2.65 ± 0.21	2.83 ± 0.21	3.09 ±0.24 ^{ab}	98.25 ± 1.75 ^{b}
C (1.00 g)	1.92	2.41 ± 0.11	2.68 ± 0.32	3.12 ± 0.05 ^{b}	87.87 ± 0.21 ^{a}
D (2.00 g)	1.92	2.60 ± 0.26	2.81 ± 0.20	2.69 ± 0.02 ^{a}	84.29 ± 3.30 ^{a}

Means bearing the different superscript column-wise are significantly ($P < 0.05$) different.

Table 3—Impact of feed supplemented with various levels of ImmuPlus on growth and non-specific immune (NSI) parameters of rohu fingerlings.

[Value are mean ± SE]

Group	Dose of ImmuPlus (g/kg)	Body weight (g)			Non-specific immune parameters			
		0 days	15 dahys	30 days	Haemolysin titre	Total Protein (g dl^{-1})	Albumin (g dl^{-1})	Globulin (g dl^{-1})
A	0	7.41 ±1.03	8.15 ± 1.89	8.48 ± 1.01	8.00 ±0.67	2.23±0.52	1.02± 0.11	1.22± 0.23
B	0.5	7.46 ±1.58	9.79 ± 1.05	9.98 ± 0.25	7.00 ±0.59	2.12± 0.32	0.91± 0.15	1.21± 0.40
C	1.0	7.12 ±1.92	10.08 ± 1.33	11.31* ± 0.51	7.00 ±0.59	2.25±0.31	1.15± 0.17	1.10± 0.31

Means with superscript (*) are significantly different from their respective control value in a column.

Table 4—Effect of ImmuPlus feeding on NBT activity, serum lysozyme level and haemolysin titre of rohu juveniles.

[Value are mean ± SE]

Group	NBT activity		Lysozyme activity (μg ml^{-1})		Haemolysin titre	
	15 day	30 day	15 day	30 day	15 day	30 day
Control	$37.28^{a} \pm 4.19_{x}$	$29.19^{a} \pm 0.58_{x}$	$4.38^{a} \pm 0.22_{x}$	$4.17^{a} \pm 0.12_{x}$	$367.8^{a} \pm 9.82_{x}$	$512.3^{b} \pm 18.76_{x}$
ImmuPlus fed	$72.67^{a} \pm 1.59_{y}$	$89.44^{b} \pm 1.29_{y}$	$7.60^{a} \pm 0.06_{y}$	$8.17^{a} \pm 0.32_{y}$	$768.3^{a} \pm 9.53_{y}$	$832^{a} \pm 27.14_{y}$

Means bearing different superscript (a, b) within a row are significantly different ($P < 0.05$). Means bearing different subscript (x, y) within a column are significantly different ($P < 0.05$).

Table 5—Effect of ImmuPlus feeding on serum total protein, albumin, globulin levels and albumin-globulin ratio of rohu juveniles

[Values are mean ± SE]

Group	Total protein (g/dl)		Total albumin (g/dl)		Globulin (g/dl)		A-G ratio	
	15 day	30 day	15 day	30 day	15 day	30 day	15 day	30 day
Control	$2.29^{a} \pm 0.11_{x}$	$2.13^{b} \pm 0.01_{x}$	$0.96^{a} \pm 0.01_{x}$	$0.94^{a} \pm 0.01_{x}$	$1.47^{a} \pm 0.07_{x}$	$1.25^{b} \pm 0.02_{x}$	$0.84^{a} \pm 0.04_{x}$	$0.76^{a} \pm 0.02_{x}$
ImmuPlus fed	$2.77^{a} \pm 0.02_{y}$	$2.55^{b} \pm 0.02_{x}$	$1.10^{a} \pm 0.04_{y}$	$1.00^{a} \pm 0.02_{y}$	$1.70^{a} \pm 0.03_{y}$	$1.55^{a} \pm 0.07_{y}$	$0.67^{a} \pm 0.02_{y}$	$0.70^{a} \pm 0.01_{y}$

Means bearing different superscript (a, b) within a row are significantly different ($P < 0.05$). Means bearing different subscript (x, y) within a column are significantly different ($P < 0.05$). A-G ratio: albumin-globulin ratio

immunomodulatory and antioxidant properties in fish[28]. ImmuPlus is a polyherbal formulation, which has been evaluated in this study for its efficacy to stimulate the non-specific and specific immunity in rohu juveniles. The herbs included in this formulation have been shown to stimulate immune system of human beings and animals through enhanced phagocytosis, production of reactive oxygen and nitrogen species[15]. The herbal product containing Amlaki, as one of the ingredient, is rich in vitamin C. The potential immunostimulant and antioxidant properties of vitamin C is well documented in fish[6,8,11,29]. The ascorbic acid present in this product is conjugated to gallic acid and reducing sugars, forming a tannoid complex, which is more stable in nature and enhancing its bioavailability[15]. Similarly, one of the other constituent plant, Guduchi, is well known to augment phagocytic cell functions and enhance protection against infections in animals and human beings[30]. The other plant constituents, Aswagandha and Tulsi are also well known for their immunomodulatory roles[15,31]. All these constituents of ImmuPlus are collectively responsible for raising the immune status of rohu at different growth stages.

The results of experiment I show that artificial feed is necessary to enhance growth of larvae at early stage with or without supplementary plankton, thus giving a scope for incorporation of suitable immunostimulant in the diet. Supplementation of ImmuPlus was not able to enhance growth in this 10 days-duration experiment. The short length of exposure period of ImmuPlus may be a reason for this. On the other hand, feeding of ImmuPlus significantly reduced the mortality during growth as well as in the experimental challenge study with *A. hydrophila* and maintained a stable growth and survival. The cause of spontaneous higher mortality in plankton plus ImmuPlus (0.5 g/kg)-based subgroup could not be explained.

In the second experiment, prolonged feeding of ImmuPlus-incorporated diet to advance larvae of rohu significantly reduced the mortality due to *A. hydrophila* challenge at 1 g/kg and above incorporation level. Marked increase in body weight was noticed only after 45 days of feeding in group C fed with ImmuPlus at a ratio of 1 g/kg diet. Above this dose, ImmuPlus-mixed diet at 2 g/kg further reduced the growth which was even lesser than the control weight. The reduction in growth after prolonged exposure with high level of ImmuPlus could be due to as yet undescribed 'regulatory' mechanisms or adaptation processes[32]. Similarly, the growth enhancement and reduction of mortality were significant after day 30 in ImmuPlus-added (1 g/kg) group C rohu fingerlings. In contrast, there was no change in haemolysin titre and protein levels in ImmuPlus-fed fingerlings compared to control. The absence of variations in immune parameters speaks about their sensitiveness in response to

Table 6—Effect of ImmuPlus feeding on specific immunity level of rohu juveniles.

[Values are mean ± SE]

Group	Bacterial agglutination titre	
	15 day	30 day
Control	$35.2^{a} \pm 1.88_{x}$	$39.37^{a} \pm 4.33_{x}$
ImmuPlus-fed	$128.60^{a} \pm 8.09_{y}$	$403.67^{b} \pm 11.34_{y}$

Means bearing different superscript (a, b) within a row are significantly different ($P < 0.05$). Means bearing different subscript (x, y) within a column are significantly different ($P < 0.05$).

Table 7—Effect of ImmuPlus feeding on mortality pattern (%) of rohu juveniles after *A. hydrophila* challenge.

[Values are mean ± SE]

Group	Non-vaccinated challenge (% mortality)	
	15 day	30 day
Control	$41.18^{a} \pm 9.28_{x}$	$60.70^{a} \pm 9.41_{x}$
Immuplus fed	$30.08^{a} \pm 7.99_{x}$	$39.00^{a} \pm 9.75_{x}$

Means bearing different superscript (a, b) within a row are significantly different ($P < 0.05$). Means bearing different subscript (x, y) within a column are significantly different ($P < 0.05$).

immunostimulants, which have also been noticed in earlier studies[8, 9].

From the above base-line data, it could be derived that supplementation of ImmuPlus @ 1 g/kg feed for a period of around one month may be beneficial for raising rohu fingerlings. A similar increase in immune response was obtained in with freshwater prawn, *Macrobrachium rosenbergii*, provided with ImmuPlus at 1 g/kg for a period of 3 weeks[16]. It was further confirmed in the experiment four conducted with rohu advanced fingerlings fed with ImmuPlus-supplemented diet for one month in which a clear picture of immunostimulation was evident from raised specific and non-specific immune parameters *viz.*, neutrophil and lysozyme activities, haemolysin titre and protein levels. Increase in neutrophil and lysozyme activities are good indicators of activations of non-specific defence mechanisms[6, 9 -12]. However, ImmuPlus feeding did not increase protection against *A. hydrophila* challenge compared to control, indicating that specific immunity might be playing major role in protection against *Aeromonas hydrophila* infection.

The present results provides evidence that commercial herbal product ImmuPlus (AquaImmu) added to rohu diet, particularly at 1 g/kg feed for 30 days activates the non-specific immunity, growth as well as specific immune response.

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