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Evaluation of In Vivo Probiotic Efficiency of *Bacillus amyloliquefaciens* in *Labeo rohita* Challenged by Pathogenic Strain of *Aeromonas hydrophila* MTCC 1739

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Abstract Diseases in aquatic organisms, including fish, are a major concern in aquaculture production. In this present investigation, we have evaluated the beneficial effects of dietary *Bacillus amyloliquefaciens* CCF7 (GenBank Acc. No. KP256501) supplementation in rohu (*Labeo rohita*) challenged by a pathogenic strain of *Aeromonas hydrophila* MTCC 1739. Four experimental diets were formulated: control diet (no probiotics) and three experimental diets (different concentrations of probiotic candidate *B. amyloliquefaciens* CCF7 at 10^5 (T1), 10^7 (T2), 10^9 (T3) CFU/g). Further, we have divided the feeding trial into pre-challenge (70 days) and post-challenge (28 days) periods and various immune parameters (serum protein, globulin, albumin, lysozyme, and IgM), and stress parameters (malondialdehyde, catalase, and superoxide dismutase) were examined during both the periods. Throughout the entire experiment, control group was fed with probiotic free basal diet, while the treatment groups received probiotic supplemented diets (PSD). After challenge test, serum aspartate transaminase (AST), serum alanine transaminase (ALT) activity, and liver malondialdehyde level have increased significantly in control groups; however, level of these parameters were considerably lower in fish fed with PSD. In contrast, liver catalase and superoxide dismutase activities and serum globulin concentration was significantly higher in the group fed with T3 diet followed by T2. Furthermore, an elevated level of serum IgM and higher activity of serum lysozyme was also recorded in PSD fed groups, especially for T3 group which confirmed the probiotic efficiency of the bacterium *B. amyloliquefaciens* CCF7. We strongly believe that *B. amyloliquefaciens* CCF7 will be a good probiotic candidate in aquaculture industries.

Keywords Rohu · Probiotic · Haematological parameters · Oxidative stress profile · Lysozyme · Serum IgM

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Introduction

The ever rising demand for animal proteins has resulted in rapid increase in global aquaculture production in the last few years. However, diseases are considered major constraints in the successful development and sustainability of aquaculture industries, which has been seen to have a worsening effect on the economy worldwide [1]. Fish are susceptible to a wide range of viral, bacterial, parasitic, and fungal diseases. Among different types of infectious agents, bacterial pathogens are often responsible for poor growth and high mortality of a wide range of farmed fish [2, 3]. Traditionally, various types of antibiotics (viz. oxytetracycline, chloramphenicol, and amoxicillin) and chemotherapeutic drugs (potential sulphonamides, furazolidone, nitrofurans, malachite green) have been applied in aquaculture in order to decrease the pathogenic load and improve the health status [4, 5]. As these substances inflict a deteriorating condition upon the environment of the pond (soil quality, water quality, natural biodiversity), likewise they also cause a steady development of resistance in pathogens, which adversely affect the health status of aquatic organisms including fish [6, 7].

Different strains of *Aeromonas hydrophila* are recognized as the most widespread fish pathogens; they function as the most frequent infectant in a variety of freshwater fish species and also occasionally affect the marine fish [7]. This pathogenic bacterium mostly causes ulcerations of skin, gills, opercula, tail and fin rot, dropsy, and haemorrhagic septicaemia in fish [8, 9]. Antimicrobials have had a little effect in controlling *Aeromonas* infection in aquaculture [10]. Probiotics are live microorganisms which when supplemented through food, beneficially affect the host in different ways, such as, production of inhibitory compounds, blocking of the pathogen adhesion sites, immune-modulation, improved gut microbial balance, and disease resistance in the host [7]. However, in India, despite severe economic losses due to *A. hydrophila* infection in Indian major carps, only a few attempts have been made to find an effective probiotic for improving the overall health conditions and strengthening the immunity in carps [11, 12]. Therefore, in the present investigation, an attempt has been made to evaluate the probiotic efficacy of a *Bacillus amyloliquefaciens* CCF7 in *Labeo rohita* challenged with pathogenic strain of *A. hydrophila*. To establish the potential of *B. amyloliquefaciens* CCF7 as probiotic candidate, a number of parameters such as hepatic stress profile, serum biochemical parameters, and immunity in terms of antibody production and lysozyme activity have been measured in *L. rohita*.

Table 1 Ingredient composition and proximate composition (on % dry matter basis) of the semi-purified basal diet

Ingredients	Content (g/kg)
Casein (fat free)	350
Gelatin	100
Dextrin	300
Cellulose flour	110
Carboxy-methyl cellulose	20
Vegetable oil	45
Cod liver oil	45
Vitamin-mineral mixture[a]	30
Proximate composition	Value (%)[b]
Dry matter	98.02 ± 3.22
Crude protein	40.56 ± 1.46
Crude lipid	8.1 ± 0.35
Crude fibre	9.45 ± 0.23
Ash	11.23 ± 0.56
Nitrogen-free extract[c]	28.4 ± 1.15

[a] Compositions of vitamin-mineral mixture: every 250 g of mineral-vitamin mixture provided vitamin A, 500,000 IU; vitamin D3, 100,000 IU; vitamin B2, 0.2 g; vitamin E, 75 units; vitamin K, 0.1 g; calcium pantothenate, 0.25 g; nicotinamide, 0.1 g; vitamin B12, 0.6 mg; choline chloride, 15 g; calcium, 75 g; manganese, 2.75 g; iodine, 0.1 g; iron, 0.75 g; zinc, 1.5 g; copper, 0.2 g and cobalt, 0.045 g

[b] Data are presented as mean values ± SD ($n = 3$)

[c] Nitrogen-free extract (%) = 100 − (moisture + crude protein + crude lipid + crude fibre + ash)

Materials and Methods

Probiotic Strain and Diet Preparation

The probiotic candidate *B. amyloliquefaciens* CCF7 (GenBank Acc. No. KP256501) used in the present study was isolated from the proximal intestine of the Indian major carp, *Catla catla* and identified by partial 16S rDNA sequence analysis [13]. The cellular concentration of the strain to be added into the test diets was determined by correlating OD_{600} values and colony-forming unit (CFU) counts on tryptone soya agar plate in triplicate [3].

In order to check the probiotic efficiency, three probiotic mixed diets (T1, T2, and T3) and one control diet (Table 1) were prepared following the method of Nandi et al. [14]. To sum up the points, three graded concentrations (1×10^5, 1×10^7, and 1×10^9 CFU/g feed) of probiotic strain *B. amyloliquefaciens* CCF7 were mixed with T1, T2, and T3 diet, respectively. The viability of the probiotic strain in diets was checked at an interval of 0, 10, 20, and 30 days of storage by spread plating on TSA. On the basis of the viability result, diets were prepared at every 10 days during feeding experiment.

Experimental Layout and Challenge Test

Healthy advanced fingerlings of rohu fish (*L. rohita*) with average weight of 20.23 g were collected from a commercial fish farm near Santiniketan, West Bengal, India (23° 41′ 30″ N latitude and 87° 41′ 20″ E longitude) and acclimated to laboratory conditions for 15 days and fed with the control diet. As per the guideline described by Smith et al. [15], all the fish were healthy with no sign of disease. Two hundred and eighty-eight uniform-sized healthy fish were randomly distributed into four groups (control, T1, T2, and T3) with each of six replicates. All the experiments were conducted in 100 L flow-through circular fibre tubs with continuous aeration. Fish were fed twice daily at 10:00 and 18:00 h for 100 days (pre-challenge feeding for 70 days and post-challenge feeding 30 days), at a feeding rate of 3% body weight per day. Fish were weighed every fortnight, and the quantity of the feed given was adjusted accordingly. Challenge test was done after 70 days of probiotic feeding. In order to conduct the challenge test, 100 μl of fresh cultured (mid log phase culture) pathogenic strain of *A. hydrophila* MTCC 1739 was intraperitoneally (i.p) injected to healthy *L. rohita* at a dose of 10^7 CFU/fish (LC_{50}). The bacterial strain was re-isolated from the dead

specimen and conformed by following the method of Nandi et al. [14].

Sampling

The first sampling was done at the end of 70 days pre-challenge period. Prior to sampling, fish were anaesthetised with MS-222. Blood and liver tissues were collected (n = 6) from each group (control, T1, T2, and T3) and stored properly. The collected blood was used for serum separation using centrifugation (2000×g at 4 °C) and stored at −20 °C until use. Hepatic tissue collected from the anaesthetised fish was rinsed with chilled PBS (50 mM, pH 7.4) and stored at −80 °C for further use. Post-challenge samplings (both blood and liver tissue) were done at 7 days interval up to 28 days.

Relative Percent Survival

Relative percent survival is an important data in challenge test experiment, which was done following the method of Amend [16]. The post-challenge mortalities were recorded in both probiotic fed and control fish groups.

Relative percent survival (RPS) = 1 − (percent of probiotic fed mortality/percent of control mortality) × 100%.

Assessment of Oxidative Stress Parameters

The stress parameters like malondialdehyde (MDA) production, superoxide dismutase (SOD), and catalase (CAT) activities of hepatic tissues were determined. The preparation of liver tissue for different assays has been described elsewhere [14]. MDA, SOD, and CAT activities were measured following the method of Draper and Hadley [17], Beauchamp and Fridovich [18], and Aebi [19], respectively. MDA is expressed as nmol/mg protein (quantified using an extinction coefficient of 1.56×10^5/M/cm) and SOD as unit/mg protein where 1 unit of SOD activity is the amount of enzyme necessary to cause 50% inhibition of photo-reduction reaction. Whereas, CAT activity is expressed as unit/mg serum protein, where 1 unit of CAT activity is the amount of enzyme required to decrease in absorbance of 0.01/min at 240 nm.

Determination of Serum AST and ALT Activities

Commercial diagnostic kits (SPAN Diagonistics, Surat, India) were used to determine the serum aspartate transaminase (AST) and alanine transaminase (ALT) activities.

Determination of Serum Protein, Albumin, and Globulin

Serum total protein and albumin levels were estimated following biuret method and bromocresol green dye binding method, respectively, using commercial diagnostic kits (SPAN Diagonistics, Surat, India). Serum globulin content was calculated by subtracting albumin from the total protein.

Assay for Serum Lysozyme Activity

Lysozyme assay was done following the method of Parry et al. [20] with few modifications. Briefly, 50 μl of serum was carefully mixed with 1 ml of a suspension of *Micrococcus luteus* (MTCC 106) in phosphate buffer (50 mM, pH 5.4), and the reduction in absorbance was measured at 450 nm from 0 to 15 min at room temperature. The lysozyme activity was defined as the sample amount causing a decrease in absorbance of 0.001/min. Hen egg white lysozyme (Sigma, USA) was taken as standard.

Serum IgM Level

Serum IgM plays an important role in immunity. The concentration of serum IgM in both pre- and post-challenge fish was measured by ELISA using a commercial fish IgM kit provided by Cusabio, Wuhan, Hubei, China.

Statistical Analysis

In order to understand the significant difference at $P < 0.05$ level, data were analysed through one-way ANOVA, followed by Duncan's multiple range tests [21]. All the data are taken as mean of standard error of six replicates.

Results and Discussion

Challenge Study and Disease Symptoms

The survival rate of the fish fed with probiotic supplemented diet was higher than the fish provided with controlled diet during the post-challenge sampling. The RPS of the fish was recorded to be the highest in the group T3 (72.72%), followed by T2 (57.56%) and T1 group (36.36%). Thus, the result gives a clear indication of the beneficial effect of the probiotic strain. In a review, Banerjee and Ray [7] have also stated that dietary supplementation of probiotics enhances disease resistance in fish directly (modulating immune system) or/and indirectly (through modulation of gut endosymbionts). In the present study, the disease symptoms of *Aeromonas* infection (haemorrhages on the ventral side of the body with slightly protruding reddish vent, opaque eyes, and distended abdomen) were very prominent in control fish compared to the probiotic fed fish groups (T1, T2, and T3). Thus, there is no doubt about the probiotic potential of this bacterium; however, we have not determined the exact working mechanism.

Hepatic Oxidative Stress Parameters

The oxidative stress parameters like MDA, CAT, and SOD are important indicators of the health status of an animal. The hepatic stress profile of *L. rohita*, in both control group and probiotic fed groups (T1, T2, and T3) was presented in Fig. 1. During pre-challenge period, the liver MDA level (nmol/mg protein) was recorded to be 22.17 ± 1.33, 16.5 ± 0.62, 18.05 ± 1.18, and 15.39 ± 0.73 in control fish, T1, T2, and T3 group, respectively (Fig. 1a). The MDA concentration increased rapidly after challenging the fish with *A. hydrophila*, and significantly ($p < 0.05$) the highest value was recorded in control group at 14 days (59.28 ± 2.12 nmol/mg protein), which is an indication of stress. However, the MDA production in liver was not so elevated in the probiotic fed groups. Interestingly, T2 group showed no significant difference ($P < 0.05$) between the MDA level at pre-challenge and 28 days post-challenge period. Banerjee et al. [22] suggested that, lipid peroxidation is a complex self-propagating process that produces MDA, which is recognized to be a stress indicator of the cell and tissue. The MDA concentration in tissue is directly related to the damaging processes caused by toxic-free radicals like, O_2^- and OH^-, which are very unstable and cause death of the cell [23]. The result of MDA formation was supported by protective antioxidant enzymes (SOD and CAT) production in liver of the different groups (Fig. 1b, c). Both SOD and CAT activities of fish fed with probiotic diets containing 10^5, 10^7, and 10^9 CFU/g of *B. amyloliquefaciens* were considerably ($p < 0.05$) higher from that of the control group after 70 days of feeding period. After challenging with *A. hydrophila*, significantly ($P < 0.05$) increased SOD activity was detected in T3 and T2 groups at 14 days (T3 = 80.86 ± 3.26 U/mg protein, T2 = 69.72 ± 3.17 U/mg

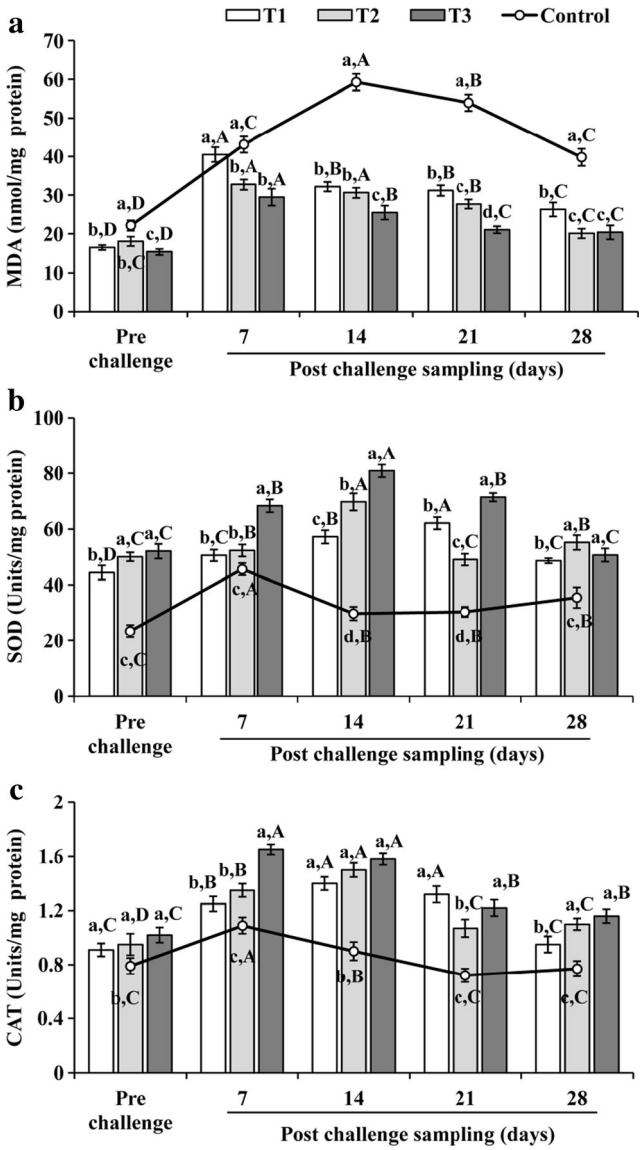

Fig. 1 Effect of dietary supplementation of *Bacillus amyloliquefaciens* CCF7 on MDA level (**a**), SOD activity (**b**), and CAT activity (**c**) of liver in *L. rohita* before and after challenging with *A. hydrophila*. Data are presented as mean values ± SE ($n = 6$). *Different lowercase letters* show significant differences between groups at the same time ($p < 0.05$), and *different uppercase letters* show significant differences between time for the same group ($p < 0.05$)

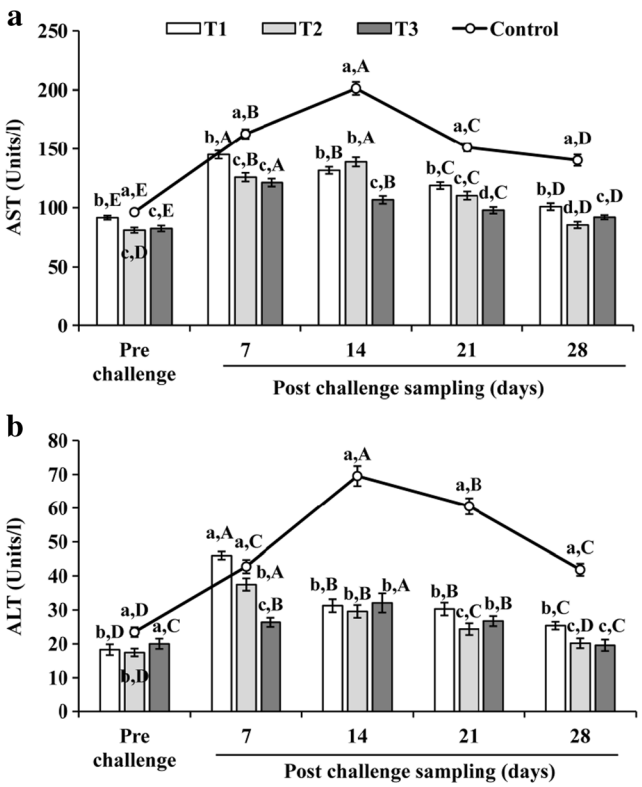

Fig. 2 Effect of dietary supplementation of *Bacillus amyloliquefaciens* CCF7 on serum AST level (**a**) and serum ALT level (**b**) in *L. rohita* before and after challenging with *A. hydrophila*. Data are presented as mean values ± SE ($n = 6$). *Different lowercase letters* show significant differences between groups at the same time ($p < 0.05$), and *different uppercase letters* show significant differences between time for the same group ($p < 0.05$)

protein) and T1 at 21 days (T1 = 62.12 ± 2.18 U/mg protein). On the other hand, the catalase activity in post-challenge probiotic fed fish was significantly ($p < 0.05$) higher in T3 group at 7 days (1.65 ± 0.037 U/mg protein) and 14 days (1.58 ± 0.042 U/mg protein), followed by T2 group at 14 days (1.5 ± 0.052 U/mg protein). To handle the stress situation, cell has its own protective mechanisms like production of SOD and CAT to neutralize the toxic effect of free radicals and to reduce the lipid peroxidation rate [22, 24]. The result of the present investigation has indicated the beneficiary activity of *B. amyloliquefaciens* CCF7 in *L. rohita*, as lower MDA level was detected in probiotic fed group. Furthermore, the activity pattern of SOD and CAT was opposite to MDA level, which has supported the hypothesis and clearly represent the relation between stress and antioxidant enzymes activity.

Serum AST and ALT Activities

AST and ALT are two important enzymes for amino acid metabolism and are considered as biomarkers for liver health. Serum AST and ALT activities (units/l) of the entire test groups (control and probiotic fed groups) during pre-challenge and post-challenge periods were determined and presented in Fig. 2a, b. The level of AST and ALT has elevated drastically ($p < 0.05$) in control group during 7–14 days after challenge test. Highest AST and ALT activities for control fish were recorded to be 201.44 ± 5.37 and 69.53 ± 2.96 units/l respectively, at 14th day post-challenge period. On the other hand, AST and ALT activities were observed not so high in the probiotic-treated fish during challenge test (28 days), as these enzyme levels dropped near the pre-challenge values. Surprisingly, T2 group exhibited no significant ($p < 0.05$) difference between the AST and ALT levels at pre-challenge and 28 days post-challenge period. The profile of serum enzymes (AST and ALT) have been considered as an indicator of stress in fish, and thus, have been commonly used to diagnose various fish diseases. Samanta et al. [25] have evaluated the degree of stress (level of AST and ALT) in *Anabas testudineus* and *Heteropneustes fossilis*, exposed to a toxic herbicide and opined that these parameters might be useful in monitoring herbicidal pollution in aquatic organisms. In general, elevated level of AST and ALT is an indicator of cellular damage or stress-related tissue degeneration [26]. The serum AST and ALT activities were observed to be increased in control group fish compared to probiotic fed fish during 7–14 days post infection period. Hence, it could be inferred that, administration of the probiotic strain *B, amyloliquefaciens* CCF7 had beneficial effects on rohu.

Measurement of Serum Proteins

Total serum protein (TSP) is another important indicator of health. Jean-Luc et al. [27] stated that measurement of TSP is easy and cheap, and thus should be used to monitor the health status of fish. In this investigation, the total protein, albumin, and globulin contents in blood serum of rohu were

Table 2 Effect of dietary supplementation of *Bacillus amyloliquefaciens* for 70 days on serum protein of *L. rohita* before and after challenging with *A. hydrophila*

Parameters studied	Sampling schedule (days)		Control	Diets with *Bacillus amyloliquefaciens* CCF7		
				T1	T2	T3
Total protein (g/dl)	0 (BC)		$1.36 \pm 0.051^{c, B}$	$1.58 \pm 0.041^{b, B}$	$1.66 \pm 0.055^{b, B}$	$1.84 \pm 0.056^{a, B}$
	AC	7	$1.5 \pm 0.06^{d, A}$	$1.62 \pm 0.04^{c, B}$	$1.75 \pm 0.046^{b, A}$	$1.88 \pm 0.05^{a, B}$
		14	$1.48 \pm 0.062^{c, A}$	$1.75 \pm 0.063^{b, A}$	$1.83 \pm 0.038^{b, A}$	$1.99 \pm 0.033^{a, A}$
		21	$1.44 \pm 0.043^{c, A}$	$1.77 \pm 0.03^{a, A}$	$1.74 \pm 0.04^{b, B}$	$1.85 \pm 0.05^{a, B}$
		28	$1.41 \pm 0.036^{c, A}$	$1.57 \pm 0.052^{b, B}$	$1.68 \pm 0.06^{a, B}$	$1.73 \pm 0.062^{a, C}$
Albumin (g/dl)	0 (BC)		$0.9 \pm 0.021^{b, A}$	$0.99 \pm 0.022^{a, A}$	$0.97 \pm 0.034^{a, A}$	$1.04 \pm 0.026^{a, A}$
	AC	7	$0.81 \pm 0.041^{b, A}$	$0.88 \pm 0.027^{a, B}$	$0.87 \pm 0.033^{a, B}$	$0.92 \pm 0.041^{a, B}$
		14	$0.78 \pm 0.038^{b, B}$	$0.9 \pm 0.031^{a, B}$	$0.88 \pm 0.036^{a, B}$	$0.89 \pm 0.03^{a, B}$
		21	$0.79 \pm 0.032^{b, B}$	$0.88 \pm 0.03^{a, B}$	$0.85 \pm 0.027^{a, B}$	$0.9 \pm 0.028^{a, B}$
		28	$0.76 \pm 0.044^{b, B}$	$0.89 \pm 0.021^{a, B}$	$0.9 \pm 0.025^{a, B}$	$0.92 \pm 0.032^{a, B}$
Globulin (g/dl)	0 (BC)		$0.46 \pm 0.037^{d, C}$	$0.59 \pm 0.033^{c, D}$	$0.69 \pm 0.04^{b, C}$	$0.8 \pm 0.024^{a, C}$
	AC	7	$0.65 \pm 0.022^{d, B}$	$0.74 \pm 0.03^{c, B}$	$0.88 \pm 0.038^{b, B}$	$0.96 \pm 0.03^{a, B}$
		14	$0.72 \pm 0.031^{d, A}$	$0.85 \pm 0.025^{c, A}$	$0.95 \pm 0.021^{b, A}$	$1.1 \pm 0.045^{a, A}$
		21	$0.67 \pm 0.03^{c, A}$	$0.89 \pm 0.04^{a, A}$	$0.85 \pm 0.032^{b, B}$	$0.95 \pm 0.037^{a, B}$
		28	$0.65 \pm 0.025^{b, A}$	$0.78 \pm 0.012^{b, C}$	$0.78 \pm 0.03^{a, C}$	$0.81 \pm 0.04^{a, C}$

Data are presented as mean values ± SE ($n = 6$)

Different lowercase letters show significant differences between groups at the same time ($p < 0.05$), and different uppercase letters show significant differences between time for the same group ($p < 0.05$)

BC before challenge, *AC* after challenge

Table 3 Effect of dietary supplementation of *Bacillus amyloliquefaciens* for 70 days on serum lysozyme activity (U/mg protein) of *L. rohita* before and after challenging with *A. hydrophila*

Sampling schedule (days)		Control (U/mg protein)	Diets with *Bacillus amyloliquefaciens* CCF7		
			T1 (U/mg protein)	T2 (U/mg protein)	T3 (U/mg protein)
0 (BC)		$390.15 \pm 7.05^{d, C}$	$501.5 \pm 7.94^{c, D}$	$560.41 \pm 9.01^{a, C}$	$522.1 \pm 10.15^{b, C}$
AC	7	$387.09 \pm 9.12^{d, C}$	$608.15 \pm 8.21^{c, B}$	$639.36 \pm 10.79^{b, B}$	$717.91 \pm 10.14^{a, A}$
	14	$488.8 \pm 8.89^{c, A}$	$630.47 \pm 9.43^{b, A}$	$705.3 \pm 11.91^{a, A}$	$713.58 \pm 11.3^{a, A}$
	21	$401.42 \pm 8.47^{c, B}$	$537.29 \pm 8.82^{b, C}$	$567.72 \pm 9.51^{a, C}$	$575.8 \pm 9.65^{a, B}$
	28	$410.3 \pm 8.92^{d, B}$	$486.3 \pm 7.28^{c, D}$	$561.4 \pm 9.65^{a, C}$	$504.21 \pm 8.02^{b, C}$

Data are presented as mean values ± SE ($n = 6$)

Different lowercase letters show significant differences between groups at the same time ($p < 0.05$), and different uppercase letters show significant differences between time for the same group ($p < 0.05$). U = A unit of lysozyme activity was defined as the sample amount causing a decrease in absorbance of 0.001/min

BC before challenge, *AC* after challenge

measured (pre-challenge and post-challenge period) and presented in Table 2. The total protein concentration in all the pre-challenge fish (T1 = 1.58 ± 0.041, T2 = 1.66 ± 0.055, and T3 = 1.84 ± 0.056 g/dl) has increased significantly ($p < 0.05$) in comparison to control fish (1.36 ± 0.051 g/dl). In the post-challenge fish, the total protein level was significantly ($p < 0.05$) higher in T3 (1.99 ± 0.033 g/dl), followed by T2 (1.83 ± 0.038 g/dl). In the case of albumin content, the differences were not significant ($p < 0.05$) between T1, T2, and T3 groups at pre-challenge and post-challenge period. Alternatively, the concentration of serum globulin showed significant ($p < 0.05$) increase in all the test groups fish between 7 and 14 days post-challenge period. Highest globulin content was recorded in T3 group (1.1 ± 0.045 g/dl) at 14th day of challenge period. Fluctuation of the concentration of the serum proteins are vital indicator of stress, and thus have been investigated in several fish species [28–30]. Serum globulins are important fractions of blood proteins that play a critical role in modulating fish immunity. Increase in the amount of total protein and globulin in serum is thought to be a positive sign of health and stronger innate immunity [31, 32]. In this investigation, higher level of globulin was detected in probiotic fed groups, and Rao et al. [33] pointed out that, rise of globulin in serum is directly associated to immunoglobulin production; therefore, probiotic fed fish have better immunity compared to control fish.

Assessment of Lysozymes Activity

Lysozyme is an antimicrobial enzyme and is considered as a part of innate immune system. In this study, we have measured the activity of lysozyme in all experimental groups (Table 3). During pre-challenge period, the serum lysozyme activity was significantly ($p < 0.05$) higher in the probiotic supplemented groups (501.5 ± 7.94, 560.41 ± 9.01, and 522.1 ± 10.15 U/mg protein for T1, T2, and T3, respectively) than that of control group. Following infection with *A. hydrophila*, the lysozyme activity increased in all probiotic fed groups and maximum activity was observed at 7th day in T3 (717.91 ± 10.14 U/mg protein), followed by T2 and T1 (705.3 ± 11.9, 630.47 ± 9.43 U/mg protein) at 14th day post infection period. Lysozyme is known to attack mainly Gram-positive bacteria; however, the antagonistic effect of lysozyme was also reported in some Gram-negative bacteria, and its activity pattern varies from one phylum to another [34, 35]. Researchers have reported the elevated level of serum lysozyme in several fish species fed with probiotic rich diet [14, 36–38]. Recently, Panase et al. [39] have stated the importance of lysozyme in immunity and compared its activity in the brain, serum, liver, and kidney of several fish

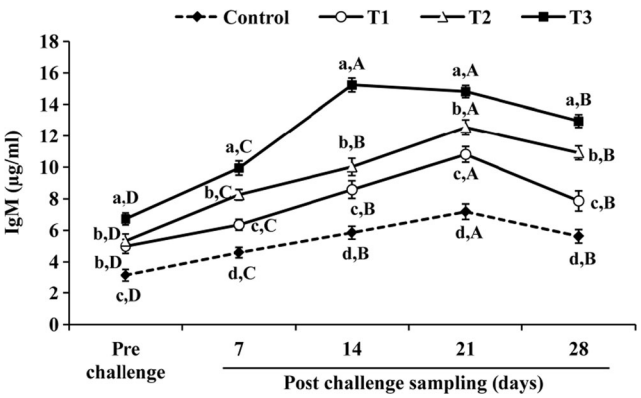

Fig. 3 Effect of dietary supplementation of *Bacillus amyloliquefaciens* CCF7 on serum IgM level in *L. rohita* before and after challenging with *A. hydrophila*. Data are presented as mean values ± SE ($n = 6$). *Different lowercase letters* show significant differences between groups at the same time ($p < 0.05$), and *different uppercase letters* show significant differences between time for the same group ($p < 0.05$)

species. In general, lysozyme degrades the glycosidic bond of the bacterial peptidoglycan layer, and thus plays a defensive role during infection. In our experiment, the higher activity of lysozyme was observed in fish fed with probiotic supplemented diet, which again confirms the beneficial nature of *B. amyloliquefaciens* CCF7.

Determination of Serum IgM Levels

The immune system in fish is not so specific like that of the mammals. Unlike mammalian immune system, fish has only three types of immunoglobulins: IgM, IgD, and IgT [40]. Along with mucosal immunity, adaptive immune system also plays a protective role during infection. Upon binding on mucosal surface, probiotics stimulate the innate and adaptive immune network and enhance the disease resistance capability of the hosts [7]. The serum IgM level in different fish groups during pre- and post-challenge period was presented in Fig. 3. After 70 days of probiotic feeding, a significant ($p < 0.05$) increase in serum IgM titre was observed in treated groups (4.98 ± 0.35, 5.31 ± 0.34, and 6.72 ± 0.45 μg/ml for T1, T2, and T3, respectively) compared to the control group (3.15 ± 0.37 μg/ml). During post-challenge period, a considerable elevation in the level of serum IgM for each group fed CCF7 diet was detected. The highest peak for serum IgM was observed in T3 group at 14th day (15.24 ± 0.44 μg/ml), followed by T2 group at 21st day of post-challenge period (12.55 ± 0.48 μg/ml). Interestingly, the IgM titre declined in all groups at 28th day of infection. The lowering of IgM level at the end of experiment might be due to resonation of fish health from the infection. In fish, the IgM is the predominant and most important immunoglobulin in the circulation [41]. Few reports are available that have confirmed the increasing level of immunoglobulin in fish fed with probiotic candidates [2, 12, 42, 43]. In light of our findings and previous reports, it could be inferred that the stimulation of immunoglobulin levels in rohu might be the result of *B. amyloliquefaciens* CCF7 administration.

Conclusion

In the present investigation, we have evaluated the effects (in term of stress physiology and immunity) of probiotic supplemented feed in *L. rohita* challenged with pathogenic strain of *A. hydrophila*. Controlling fish disease using probiotic candidates has several advantages over chemical pesticides and antibiotics. The examined results show the effectiveness (such as reduction of stress and enhancement of serum protein, lysozyme, and IgM level) of *B. amyloliquefaciens* CCF7 supplemented feed in *L. rohita*. We strongly believe that the use of this bacterial strain might prove to be useful in aquaculture sectors to reduce the disease load and improve the production rate.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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