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Paenibacillus polymyxa as a water additive improved immune response of *Cyprinus carpio* and disease resistance against *Aeromonas hydrophila*

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ABSTRACT

The present study was undertaken to investigate the impact of *Paenibacillus polymyxa* as water additive probiotic bacterium in common carp, *Cyprinus carpio* based on water quality, survival, innate immune responses and disease resistance. The completely randomized experiment design was conducted for eight weeks and treatments consisted of three levels of *P. polymyxa* added in water at concentration of 10^3 cfu mL^{-1} (PP1), 10^4 cfu mL^{-1} (PP2) and 10^5 cfu mL^{-1} (PP3) and one control (Con, without any probiotic). No significant differences ($p > 0.05$) in water quality parameters, such as temperature, pH, dissolved oxygen, ammonical nitrogen and nitrite nitrogen were observed throughout the experimental period among treatments. The influences of *P. polymyxa* at different concentrations significantly improved survival ($p < 0.05$). Study of different innate immunological parameters viz. lysozyme activity, respiratory burst assay, myeloperoxidase content, catalase and superoxidase dismutase activities showed significant ($p < 0.05$) improved immune responses in fish exposed to *P. polymyxa* as water additive at 10^3 (PP1) and 10^4 (PP2) cfu mL^{-1}. The supplementation of probiotic in challenge test significantly ($p < 0.05$) enhanced the resistance of fish against *A. hydrophila* infection. In view of recent reports of antibiotic failure from many countries to stop spread of fish diseases, renewed interest in a more complete understanding of the fish immune response to infectious diseases will be critical in developing new eco-friendly control strategies for future. Therefore, the application of probiotic *P. polymyxa* as water additive could be applied in aquaculture to improve immune responses and disease resistance of *C. carpio*.

1. Introduction

Aquaculture is an increasingly important and inexpensive source of animal protein. During the last three decades, aquaculture has become the fastest-growing food-producing sector and is contributing significantly to national economic development, global food supply and food security (FAO, 2010). According to a recent data published by the Food and Agriculture Organization, Fisheries and Aquaculture Department, the world aquaculture production of food fish reached 62.7 million tonnes in 2011, up by 6.2% from 59 million tonnes in 2010 and contributing about 40.1% to the world total fish production (FAO, 2011). Indian aquaculture production mainly consists (~87%) of three native major carps and three exotic carps. Among exotic carps, *Cyprinus carpio*, commonly known as common carp, is an important candidate species with global production of approximately 3.7 million tonnes during 2010–11 (FAO, 2011). With the ever increasing demand for this species, there has been a shift in aquaculture practices, moving from extensive systems towards the semi-intensive and intensive systems.

Infectious diseases are considered to be of paramount importance to the development and sustainability of commercial aquaculture, in terms of direct losses of biomass and productivity as well as indirectly as trade restrictions and poor water quality (Verschuere et al., 2000; Sharifuzzaman et al., 2014). The pathogens, however, get congenial environment for multiplication causing disease manifestations, when the fishes constantly suffer from stress due to adverse conditions in the pond ecosystem like higher temperature, higher stocking densities, less oxygen

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Table 1
Composition and proximate analysis of basal pelleted diet for *C. carpio*.

Ingredients	Composition (%)	Proximate analysis	Composition (% dry matter)
Soybean meal	25	Dry matter	89.6
Fish meal	15	Crude protein	30.1
Mustard oil cake	30	Lipid	5.08
Rice bran	20	Ash	13.4
Corn starch	5	Organic matter[a]	76.2
Sunflower oil	3.5	Total carbohydrate[b]	41.0
Vitamin & mineral mixture	1.5	Gross energy (kcal 100 g^{-1})	386.7

[a] Organic matter = dry matter – total ash.
[b] Total carbohydrates = organic matter – (crude protein + total lipid).

and heavy organic load etc. Hence, semi-intensive and intensive systems are very much prone to disease outbreak. Bacterial infections are one of the important causes of disease problems in Indian aquaculture (Sahoo et al., 2011). *Aeromonas hydrophila* is the most common pathogen, and it can easily spread through accidental abrasions and causes haemorrhagic septicaemia, ulcers, exophthalmia, abdominal distension (Austin and Austin, 2012).

Until now, prevention or controlling aquatic disease has mainly depended on antibiotics and disinfectants. However, the massive use of these chemicals has led to antibiotic resistance in some instances (Verschuere et al., 2000). Also the rapid expansion of intensive aquaculture industry, are often companied by rotted uneaten feed, sedimentation of feces and organic residue. The water quality rapidly deteriorates as a result. In particular, nitrogenous compounds such as ammonia and nitrite quickly built up, which are both harmful to fish even at low concentrations (Mohapatra et al., 2012; Xie et al., 2013). Water exchange can be applied to maintain good water quality, however frequent exchange is not only laborious and costly, but also may incur disease causing agents and pollute nearby water bodies (Mohapatra et al., 2012). Therefore, there is an urgent demand for cost-effective and environment-friendly approaches for remediation of aquaculture water.

Probiotics is the application of microbial supplements to benefit their host (Fuller, 1989). Although the use of probiotics in aquaculture seems to be relatively recent, the interest in such environment friendly practice is increasing rapidly (Gupta et al., 2016; Gupta and Gupta, 2016). Moriarty (1998) proposed to extend the definition of probiotics in aquaculture to microbial 'water additives'. Probiotics exert beneficial effects on the host by (i) enhancing growth performance through establishment of healthy gut microenvironment (Merrifield et al., 2011; Gupta et al., 2014; Gupta and Gupta, 2016); (ii) providing nutrients to digestion through production of exogenous digestive enzymes and vitamins (Tinh et al., 2008); (iii) inhibiting pathogenic microorganisms through competition (Verschuere et al., 2000; Irianto and Austin, 2002); (iv) enhancing immune response through elevating specific and innate immunity (Irianto and Austin, 2002; Gupta et al., 2014, 2016; Gupta and Gupta, 2016); and (v) improving water quality through water nitrogen remediation (Verschuere et al., 2000; Zhou et al., 2009a).

Remediation of aquaculture water using microorganisms like *Bacillus* species is a burgeoning trend for the sustainable development of aquaculture industries (Verschuere et al., 2000). *Bacillus* species are widely used for water remediation because they are stable for long period due to spore formation, easily prepared by fermentation and possess antagonistic effects on pathogens (Xie et al., 2013; Zhou et al., 2009b; Hong et al., 2005). To date, screening strains with good remediation characteristic in conjunction with their influence on survival, immune response and disease resistance still remains a fundamental step towards developing commercial microbial agents. The principal objective of this study was to obtain information concerning the non-specific innate immune responses and disease resistance of common carp (*Cyprinus carpio*), in response to probiotic *P. polymyxa*, supplemented in water.

2. Materials and methods

2.1. Bacterial strain

The microorganism employed was laboratory maintained *Paenibacillus polymyxa* (MTCC 122) obtained from the Indian Institute of Microbial Technology, Chandigarh, India. *P. polymyxa* is an endospore-forming, non-pathogenic, Gram-positive, anaerobic bacterium is often used as probiotic under biosafety level. To confirm the purity of strain, colonies were identified on the basis of their morphological, Gram's staining and biochemical characteristic using bacterial identification kits (HiMedia, India). The probiotic characteristics of strain such as safety, antagonistic activity against pathogenic bacteria and colonization in the intestine, were reported in elsewhere (Gupta et al., 2014). Cell density was calculated from optical density (OD) at 600 nm and correlated with colony forming unit (cfu) counts using serial dilution and spread plating on normal nutrient agar (HiMedia, India). The quantified bacteria were maintained at 4 °C in a suspended form with PBS (pH 7.5). Cells were re-suspended in the same buffer before use.

2.2. Experimental design

Four trials were carried out with completely randomized design on common carp in twelve aerated fiberglass tanks of 500-L capacity at a density of 20 fish per tank for eight weeks. Common carp, *Cyprinus carpio*, showing no signs of disease, with no previous history of parasitic infections, and having a mean body weight of 32.17 g were obtained from fish farm of College of Fisheries, GADVASU, Ludhiana, India and maintained in aerated freshwater tanks for two weeks. All treatments were used in triplicate. The treatments consisted of three *P. polymyxa* concentrations at 10^3 cfu mL^{-1} (PP1), 10^4 cfu mL^{-1} (PP2) and 10^5 cfu mL^{-1} (PP3) and one control (Con, without any probiotic) and were inoculated thrice a week. The selected strain was grown in nutrient broth (HiMedia, India) in a shaking incubator (Caltan NSW BOD Incubator-Shaker, Narang Scientific Works Pvt. Ltd., India) at 32 °C for 24–48 h. After incubation, the cells were harvested by centrifugation (2000*g*) to obtain microbial pellet. The pellet was washed three times with phosphate buffered saline (pH 7.2), and re-suspended in the same buffer before use. The same amount of solution without any probiotic was applied in the control treatment. The tanks were supplied with running freshwater which had been filtered through the cotton filter.

2.3. Feed and feeding

A basal laboratory prepared pelleted diet comprising soybean meal, fish meal, mustard oil cake, rice bran, corn starch, sunflower

Table 2
Effect of probiotic *P. polymyxa* as water additive on water quality in *C. carpio* tanks.

Treatment	Period (week)	Con	PP1	PP2	PP3
Temperature (°C)	0	29.4 ± 0.1[a]	29.4 ± 0.1[a]	29.5 ± 0.1[a]	29.4 ± 0.1[a]
	4	32.9 ± 0.5[a]	33.1 ± 0.7[a]	32.9 ± 0.4[a]	33.0 ± 0.5[a]
	8	33.7 ± 1.7[a]	33.6 ± 1.2[a]	33.6 ± 1.5[a]	33.8 ± 1.8[a]
pH	0	7.25 ± 0.03[a]	7.27 ± 0.03[a]	7.26 ± 0.03[a]	7.27 ± 0.03[a]
	4	7.93 ± 0.05[a]	7.94 ± 0.04[a]	7.88 ± 0.03[a]	7.82 ± 0.02[a]
	8	8.84 ± 0.12[a]	8.70 ± 0.09[a]	8.68 ± 0.07[a]	8.71 ± 0.09[a]
Dissolved oxygen ($mg\,L^{-1}$)	0	7.18 ± 0.12[a]	7.31 ± 0.05[a]	7.23 ± 0.25[a]	7.16 ± 0.14[a]
	4	5.11 ± 0.14[a]	5.34 ± 0.07[a]	5.29 ± 0.13[a]	5.31 ± 0.06[a]
	8	4.53 ± 0.06[a]	4.97 ± 0.33[a]	4.81 ± 0.46[a]	4.73 ± 0.31[a]
NH_4 −N ($mg\,L^{-1}$)	0	0.0033 ± 0.003[a]	0.0066 ± 0.0003[a]	0.0066 ± 0.0003[a]	0.0033 ± 0.003[a]
	4	0.052 ± 0.002[a]	0.036 ± 0.002[a]	0.039 ± 0.001[a]	0.048 ± 0.001[a]
	8	0.363 ± 0.009[a]	0.283 ± 0.021[a]	0.306 ± 0.005[a]	0.328 ± 0.012[a]
NO_2 −N ($mg\,L^{-1}$)	0	0.0024 ± 0.0003[a]	0.0031 ± 0.0001[a]	0.0029 ± 0.0002[a]	0.0021 ± 0.0001[a]
	4	0.0163 ± 0.0012[a]	0.0084 ± 0.0004[a]	0.0097 ± 0.0002[a]	0.0134 ± 0.0004[a]
	8	0.0637 ± 0.0045[a]	0.0453 ± 0.0037[a]	0.0556 ± 0.0013[a]	0.0587 ± 0.0009[a]

Values are mean of triplicate groups and presented as mean ± SE. Values with different superscripts in the same row are significantly different ($p < 0.05$). Con (control): fish group of treatment without probiotic inoculation. PP1, PP2 and PP3: fish groups of treatments received probiotic inoculation @10^3, 10^4 and 10^5 cfu mL^{-1}, respectively. NH_4−N: Ammonical-Nitrogen, NO_2−N: Nitrite-Nitrogen.

oil and vitamin and mineral mixture was prepared (Table 1). Proximate analysis of the basal feed is presented in Table 1, and was analyzed according to AOAC (1997). To ensure high quality of the feed, fresh diets were prepared on fortnightly basis. Fish were fed at 5% of initial body weight per day for eight weeks. Feeding was done twice a day at 09:30 and 16:30 h.

2.4. Analysis and measurements

2.4.1. Physico-chemical parameters of the water

The basic physico-chemical parameters of the water were measured every week. Water sample collection and on-site measurements were carried out in daylight, between 10:00 a.m. and 12:00 p.m. Water temperature was measured by using digital thermometer. The values of pH were measured by using Hach multimeter (HQ40d, Hach Company, Colorado, USA). The concentration of dissolved oxygen of water was measured by Winkler (1888) method and further cross-checked by Hach multimeter oxygen sensitive electrode/probe (Hach HQ40d, USA). The ammonical-nitrogen (NH_4-N) and nitrite-nitrogen (NO_2-N) concentrations of water in the tanks were measured according to the procedures of APHA (1989).

2.4.2. Survival

Survival and relative percent of survival of fish were recorded at the end of the experiment. Relative percent of survival was calculated according the formula proposed by Amend (1981).

2.4.3. Immunological aspects

Innate immune responses were measured separately for probiotic treated and control fish. Sampling was scheduled at the end of 4 and 8 weeks of experimental trial. Four fish were randomly removed from each tank after batch weighing and thus, a total of 12 fish were collected per treatment for immunological assays. Blood samples were collected from the caudal vein using 1-mL syringe after anaesthetizing the fish with clove oil (at 350 μl/L from stock solution of 100 mg of clove oil in 1.0 mL of 95% ethanol). The blood samples were transferred into sterilized Eppendorf tubes. Following centrifugation (2000*g*, 10 min, 4 °C), serum was collected and stored at −20 °C until further use.

Serum lysozyme activity was measured using the turbidimetric assay following Sankaran and Gurnani (1972) with partial modification. Lysozyme activity was expressed as units/mL, where one unit was defined as the reduction in absorbance of 0.001/min. Lyophilized hen egg white lysozyme (HiMedia, India) was used to develop a standard curve. The respiratory burst activity of phagocytes was carried out following the protocol of Anderson and Siwicki (1995) using the nitroblue tetrazolium (NBT, HiMedia, India). The OD of the supernatant was measured at 540 nm in the microplate reader (Model: Infinite M200 PRO, Tecan, Switzerland). *N,N*-dimethyl formamide (Qualigens, Fisher Scientifics, India) was used as blank. Total myeloperoxidase content present in serum was measured according to Quade and Roth (1997) with slight modification. Colour development was measured at 450 nm in a microplate reader using Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} as blank.

Serum superoxide dismutase activity was determined with an enzymatic assay method as described by Sun et al. (2010). One unit of serum superoxide dismutase activity was defines as the amount of enzyme necessary to produce a 50% inhibition of the NBT reduction rate measured at 550 nm. Catalase activity was estimated following the method described by Takahara et al. (1960). One unit of catalase activity was defined as the amount of the enzyme required to decompose one μmole of H_2O_2 per minute at pH 7.0 (25 °C).

2.5. Experimental infection

At the end of experimental trial, 15 fishes from each treatment were randomly captured and subjected to bacterial challenge. *Aeromonas hydrophila* (MTCC 1739) was used as pathogenic agent. The bacterium was grown in nutrient broth and incubated at 37 °C for 24 h. Bacterial growth was measured at an OD of 600 nm followed by plate counting in nutrient agar. The isolated bacteria were identified as a result of an examination of cells morphology, Gram's staining and biochemical method using standard bacterial identification kits (HiMedia, India). The fish were injected intraperitoneally with *A. hydrophila* suspension containing 1×10^5 cfu mL^{-1}. After injection, the fish were distributed into 12 aquariums of 60 l capacity and their mortality was monitored and recorded for 15 days. For negative control, a group of 15 fish were injected with PBS. The cause of death and pathological changes were verified by re-isolation of bacteria from dead and infected samples and identification was achieved using standard bacterial identification kits.

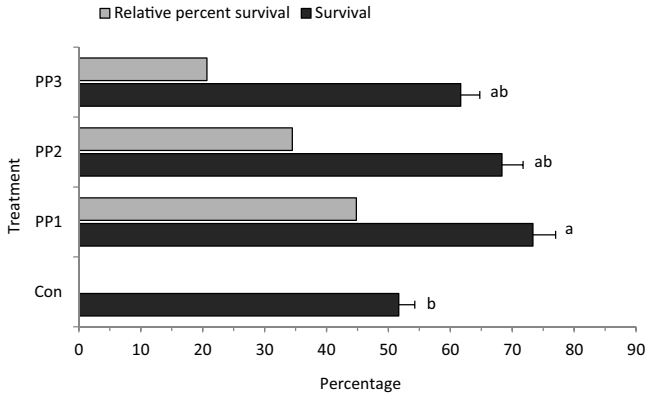

Fig. 1. Survival and relative percent survival of common carp. Con (control): fish group of treatment without probiotic inoculation. PP1, PP2 and PP3: fish groups of treatments received *P. polymyxa* as water additive at 10^3, 10^4 and 10^5 cfu mL^{-1}, respectively. Data of survival is presented as mean ± SE. Different superscripts indicate statistically significant differences ($p < 0.05$) between treatments.

2.6. Statistical analysis

Data were analysed by the one-way analysis of variance (ANOVA) and Tukey's range test. Probability levels of 0.05 were used to determine the significance in all treatments. All statistical analysis of data was performed using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Water quality improvement

The effect of probiotic, *P. polymyxa*, on water quality in fish tanks are presented in Table 2. The pH values in treated tanks ranged from 8.68 to 8.71, while that of control tanks was 8.84 during 8th week of the experimental trial. However, there was no significant difference among treatments at the same period (initial, 4th and 8th week) of trial. The values of dissolved oxygen, ammonia and nitrite ranged from 4.73 to 4.97 mg l^{-1}, 0.283–0.328 mg l^{-1}, and 0.045–0.058 mg l^{-1}, respectively in the experimental tanks inoculated with probiotic, whereas, that of control was 4.53 mg l^{-1}, 0.363 mg l^{-1}, and 0.063 mg l^{-1}, respectively during 8th week of the experiment. No significant differences were observed ($p > 0.05$) throughout the experimental period at the same duration although the higher concentration was determined in the control compared with probiotic inoculated treatments (Table 2). Furthermore, there were no significant differences among the three probiotic treatments ($p > 0.05$).

3.2. Survival influence

After 8 weeks of culture, treatment received *P. polymyxa* at 10^3 cfu mL^{-1} in PP1 showed significant ($p < 0.05$) increase in survival rate over the Con (Fig. 1). However, non-significant differences were observed between PP2, PP3 (water inoculated with *P. polymyxa* at 10^4 and 10^5 cfu mL^{-1}, respectively) and Con. Fish group in treatment PP1 exhibited the highest relative percent of survival followed by PP2 and PP3 (Fig. 1).

3.3. Innate immune responses

After 4 weeks of *P. polymyxa* inoculation in water, a marginal increase without significant difference ($p > 0.05$) in lysozyme; RBA and MPO; SOD and catalase activities were observed compared to the Con (Tables 3 and 4).

3.3.1. Lysozyme activity

After 8 weeks of inoculation, the serum lysozyme activity of fish in PP1 treatment (10^3 cfu mL^{-1}) was significantly higher than PP2 and PP3 (Table 3). Significant lower activity was observed in Con fish over that of probiotic inoculated groups.

3.3.2. Respiratory burst activity

Statistical analysis showed that higher significant ($p < 0.05$) respiratory burst activity of common carp blood after 8 weeks of inoculation with different probiotic concentrations when compared to the Con (Table 3). Treatment inoculated with *P. polymyxa* @10^3 cfu mL^{-1} (PP1) and @10^4 cfu mL^{-1} (PP2) exhibited the highest RBA activity. However, the RBA activity was not statistically significant ($p > 0.05$) between PP3 and Con groups (Table 3).

3.3.3. Myeloperoxidase activity

The myeloperoxidase activities (MPO) of fish are presented in Table 3. After 8 weeks, water inoculated with *P. polymyxa* in treatment PP1 exhibited the highest significant MPO activity. A significant decline in MPO activity was observed at the higher probiotic inoculation level (PP2 followed by PP3). Although, numerically higher MPO activity was detected in fish inoculated with probiotic @10^4 and 10^5 cfu mL^{-1} (PP2 and PP3, respectively), the differences were not significant compare to Con group (Table 3).

3.3.4. Superoxide dismutase activity

Superoxide dismutase activities (SOD) are presented in Table 4. After 8 weeks of trial, fish groups treated with probiotic in treatment PP1 and PP2 showed significant higher level of SOD. A decline in SOD activity was observed at the higher probiotic inoculation level (10^5 cfu mL^{-1}). The SOD activity was not statistically significant ($p > 0.05$) between PP2, PP3 and Con groups (Table 4).

3.3.5. Catalase activity

Fish catalase activity was not influenced by the probiotic inoculation of *P. polymyxa* as water additive (Table 4). However, numerically higher catalase levels were found in fish received three concentrations of probiotic.

3.4. Challenge test

During the *A. hydrophila* challenge test, the first dramatic mortality was observed on the fourth day after injection where the fish received probiotic *P. polymyxa* at higher level (10^5 cfu mL^{-1}) and Con showed lower disease resistance compared to other fish groups (Fig. 2). At the end of 15 days challenge test, the control group had the lowest survival rate (23.81%) and significantly differed from those of fish received probiotic at 10^3 cfu mL^{-1} (PP1, 66.65%), 10^4 cfu mL^{-1} (PP2, 52.31%) and 10^5 cfu mL^{-1} (PP3, 47.58%). Also, fish acquired probiotic in treatment PP2 exhibited numerically higher disease resistance than the PP3 group but the difference was not significant (Fig. 2). Negative control group showed 100% survival rate. Typical symptoms of infection such as exophthalmia and skin inflammation, necrosis and haemorrhagic septicaemia on the body surface and dead egg mass, kidney disintegration and liquefaction of internal organs were observed in moribund or dead fish of control group.

4. Discussion

The significance of using probiotics in fish aquaculture were recently reviewed by Qi et al. (2009), Nayak (2010) and Gupta and Gupta (2016). It is very relevant to provide fish with a healthy environment and probiotics has great deal of potential (Zhou et al., 2009a). Wang et al. (2005) investigated the effect of commercial probiotics on water quality in shrimp, *P. vannamei*, ponds and the

Table 3
Innate immune response of *C. carpio* treated with or without probiotic, *P. polymyxa* as water additive for eight weeks.

Treatment	Inoculation level of *P. polymyxa* (cfu mL^{-1})	Immune response					
		Lysozyme activity (Unit mL^{-1})		RBA (OD 540 nm)		MPO (OD 450 nm)	
		4th week	8th week	4th week	8th week	4th week	8th week
Con	0	107.40 ± 1.44^{a}	129.70 ± 9.02^{c}	0.360 ± 0.04^{a}	0.530 ± 0.05^{b}	0.074 ± 0.008^{a}	0.097 ± 0.005^{b}
PP1	1×10^3	117.30 ± 1.78^{a}	217.20 ± 1.85^{a}	0.510 ± 0.06^{a}	0.870 ± 0.05^{a}	0.087 ± 0.010^{a}	0.148 ± 0.011^{a}
PP2	1×10^4	112.50 ± 8.34^{a}	176.70 ± 9.10^{b}	0.480 ± 0.02^{a}	0.710 ± 0.02^{ab}	0.096 ± 0.006^{a}	0.125 ± 0.007^{ab}
PP3	1×10^5	115.40 ± 2.85^{a}	135.60 ± 4.72^{c}	0.430 ± 0.03^{a}	0.570 ± 0.05^{b}	0.081 ± 0.003^{a}	0.103 ± 0.009^{b}

Values are mean of triplicate groups and presented as mean ± SE. Values with different superscripts in the same row are significantly different ($p < 0.05$). Con (control): fish group of treatment without probiotic inoculation. PP1–PP3: fish groups of treatments received probiotic inoculation.

Table 4
Superoxide dismutase and catalase activities of *C. carpio* treated with or without probiotic, *P. polymyxa* as water additive for eight weeks.

Treatment	Inoculation level of *P. polymyxa* (cfu mL^{-1})	Immune response			
		SOD (Unit mL^{-1})		Catalase (Unit mL^{-1})	
		4th week	8th week	4th week	8th week
Con	0	22.70 ± 1.48^{a}	30.40 ± 1.12^{b}	38.540 ± 3.06^{a}	42.950 ± 2.01^{a}
PP1	1×10^3	34.90 ± 2.82^{a}	61.20 ± 7.81^{a}	48.270 ± 1.84^{a}	55.680 ± 4.30^{a}
PP2	1×10^4	36.10 ± 3.42^{a}	47.30 ± 1.52^{ab}	43.400 ± 1.60^{a}	53.370 ± 4.16^{a}
PP3	1×10^5	29.50 ± 3.77^{a}	37.10 ± 1.92^{b}	45.300 ± 1.67^{a}	46.420 ± 1.95^{a}

Values are mean of triplicate groups and presented as mean ± SE. Values with different superscripts in the same row are significantly different ($p < 0.05$). Con (control): fish group of treatment without probiotic inoculation. PP1–PP3: fish groups of treatments received probiotic inoculation.

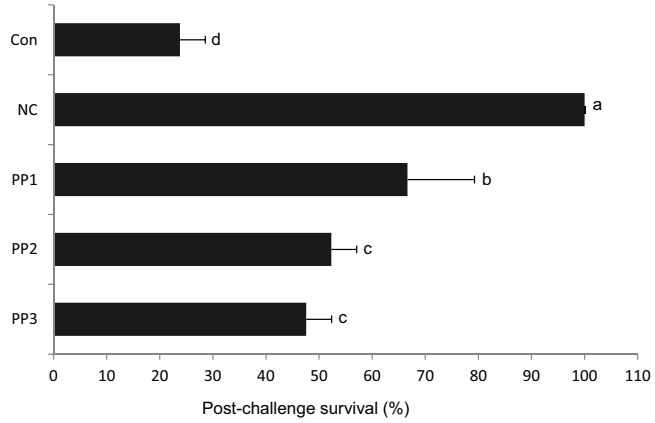

Fig. 2. Effects of probiotic *P. polymyxa* as water additive on the post-challenge survival of *Cyprinus carpio* after infection with *A. hydrophila*. Con (control): fish group of treatment without probiotic inoculation. PP1–PP3: fish groups of treatments received probiotic inoculation @10^3, 10^4 and 10^5 cfu mL^{-1}, respectively. NC = Negative control. Data are presented as mean ± SE. Different superscripts indicate statistically significant differences ($p < 0.05$) between treatments.

results showed that probiotics could significantly reduce the concentrations of nitrogen in pond water. However, Zhou et al. (2009b) observed inconsistence results by using different concentrations of *B. coagulans* as water additive in the culture of shrimp *P. vannamei*. In this study, the use of varied concentrations of *P. polymyxa* in common carp as water additive had shown inconsistent results. There was no obvious effect of *P. polymyxa* on the water quality during the study. This result may be explained by the good water quality, high quality of pelleted diet and other conditions in this study. Moreover, the pH values, and concentrations of ammonia and nitrite determined in this study were stable and within acceptable ranges (Boyd and Tucker, 1998).

In the present study, the inoculation of varied concentrations of *P. polymyxa* in water resulted in significant improvements in survival and relative percent survival of *C. carpio*. The relative percent survival would be a good index for an aquaculture (Ding et al., 2004), which in this study we achieved higher values in PP1 (44.82) treatment (Fig. 1). In fish, *P. polymyxa* used as probiotic, was able to colonize both in the culture water and the fish digestive tract, thereby increasing the fish survival (Rengpipat et al., 1998; Zhou et al., 2009b; Gupta et al., 2014). Similar experiments had previously been documented in preliminary trials on Chinese carp, *Cyprinus carpio* (Ramkrishnan et al., 2008) and giant freshwater prawn, *Macrobrachium rosenbergii* (Gupta and Dhawan, 2011, 2012; Gupta et al., 2016). However, Shariff et al. (2001) found that treatment of *P. monodon* with a commercial *Bacillus* probiotic did not significantly increase survival. It was difficult to directly assess different studies using probiotics, because the efficacy of probiotic application depended on many factors (Gomez-Gil et al., 2000; Gupta et al., 2014) such as species composition, application level, frequency of application and environmental conditions. Moreover, there was no significant difference between probiotic inoculated groups (PP1–PP3). This indicates that the concentration/quantity of probiotics was only one of the factors promoting the survival of common carp.

Despite a large number of research publications on the use of probiotics to improve overall health of cultured fish, there is little information regarding the effects of probiotics on the immunological aspects of fish when administrated as water additive. Administration of *Bacillus* strains could significantly enhance serum lysozyme activity of rainbow trout, *O. mykiss* (Merrifield et al., 2009) and common carp, *C. carpio* fry (Gupta et al., 2014). In contrast, the serum lysozyme content of tilapia (*O. niloticus*) was not affected by treatment with *B. subtilis* B10 and *B. coagulans* B16 as water additive (Zhou et al., 2009a). In the present study, lysozyme content of the PP3 group, which was given the highest concentration of probiotic (10^5 cfu mL^{-1}), was not significantly greater than that of the other probiotic treatment groups. The differences in the effects of lysozyme activity can be due to the inclusion levels, suggesting probiotic strain has an inherent limit, as well as the fish species under study. Our study showed that fish groups received *P. polymyxa* at 10^3 and 10^4 cfu mL^{-1} (PP1 and PP2, respectively) had significantly higher respiratory bursts than the control group, confirming that non-specific immunity was enhanced in fish received

P. polymyxa. Similar trends in the stimulation of respiratory burst activity after dietary probiotic supplementation involving feeding regimes and feeding durations have been previously reported in various fish (Giri et al., 2012; Sun et al., 2010; Gupta et al., 2014). We also observed reduction in respiratory burst activity at high probiotic level (i.e. 10^5 cfu mL^{-1}) after 8 weeks of inoculation in water. Previous studies have demonstrated that dietary administration of high levels of probiotics for longer periods affects respiratory burst activity in *L. rohita* (Kumar et al., 2008; Giri et al., 2012) and *O. niloticus* (Aly et al., 2008).

In the present study myeloperoxidase content of serum was significantly higher after 8 weeks of *P. polymyxa* supplementation in water. Similar result of elevated myeloperoxidase level in serum was observed for *B. amyloliquifaciens* in carp, *C. catla* (Das et al., 2013); *B. subtilis* in rainbow trout, *O. mykiss* (Newaj-Fyzul et al., 2007) and *P. polymyxa* in common carp, *C. carpio* (Gupta et al., 2014). However, to our knowledge, no reports are available regarding the myeloperoxidase content of common carp received *P. polymyxa* as water additive.

Supplementation of probiotic at 10^3 and 10^4 cfu mL^{-1} as water additive improved the serum SOD activities after 8 weeks of inoculation suggesting a better immune response could be stimulated by *P. polymyxa*. However, inoculation of *P. polymyxa* did not improve significant catalase activity of fish. Zhou et al. (2009a) and Sun et al. (2010) demonstrated that the SOD activities of tilapia (*O. niloticus*) and grouper (*E. coioides*), respectively increased significantly after treated with *Bacillus* spp. However, Son et al. (2009) found that dietary administration of different levels of *L. plantarum* for 4 weeks significantly decreased the SOD activity. The authors hypothesized that the decreased SOD in those fish fed *L. plantarum*-supplemented diets may occur in order to retain the superoxide anion level or to convert it into the singlet oxygen ($^1O_2$) and/or hydroxyl radicals (OH) via a metal-catalyzed interaction to enhance the microbial-killing capacity of phagocytes. Therefore, further study is needed to illustrate the effects of probiotics on the antioxidant enzymes and their related immune function in fish.

Probiotics help in achieving natural resistance and controlling disease-related loss among farmed fish (Gupta et al., 2014, 2016). In this study, inoculation effects of the *P. polymyxa* as water additive were examined in vivo by injection of *A. hydrophilla* to the fish. This bacterium is the causative agent of haemorrhagic septicaemia in a wide range of commercially important fish species including carps (Zhang et al., 2012; Gupta et al., 2014). Occurrence of antibiotic resistant strains of *A. hydrophilla* in fish was reported (Giri et al., 2012). Therefore, prophylactic against this bacterium by the inclusion of immunostimulants in water becomes more practical to implement in a fish farm. In the current study, fish in the control group showed high mortality after a few days of bacterial challenge, while the groups received *P. polymyxa* in water showed significantly higher disease resistance. Such enhancement in fish disease resistance can be partially due to the facilitated non-specific immune responses particularly in PP1 treated fish where significantly higher lysozyme, RBA, MPO activities as well as SOD level were obtained.

As a final note, the present study delineates the efficacy of *P. polymyxa* as a potential water additive in aquaculture. This strain at moderate concentrations was effective in maintaining optimum water quality, survival and induces upregulation of innate immunity. Disease resistance can also be positively affected by *P. polymyxa* with an optimal supplementation of 10^3 cfu mL^{-1} in water. The data obtained here might be of help to work as health indicator in stress, nutrition or infection-related studies and developing new eco-friendly control strategies and intervention programs for the future aquaculture practices. Further studies are needed to verify the application of *P. polymyxa* as water additive in other fish species and specific rearing conditions.

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