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Lactobacillus acidophilus Supplementation Improves the Innate Immune Response and Disease Resistance of Striped Catfish (*Pangasianodon hypophthalmus* Sauvage, 1878) Juveniles against *Aeromonas hydrophila*

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Abstract

A bacterium *Lactobacillus acidophilus* (LAB) was evaluated for its probiotic potential on the health status of striped catfish (*Pangasianodon hypophthalmus,* Sauvage, 1878) juveniles. Triplicate groups of striped catfish (21.69 ± 0.18 g) were fed twice daily at 2.5 % of body weight, with 0 (control), 10^3, 10^5, 10^7 and 10^9 CFU/g LAB diets for 12 weeks. The fish were challenged intraperitoneally with 1×10^6 CFU mL^{-1} of *Aeromonas hydrophila* after 12 weeks of feeding trial and the mortalities were recorded over 21 days. The haematological, immunological parameters and histopathological changes of liver were assessed both in pre- and post-challenged fish groups. Results revealed that feeding the fish with supplemented diets significantly increased ($p < 0.05$) the survival after challenged with *A. hydrophila*. Compared to the control diet, a significant improvement in packed cell volume, red blood cell, white blood cell and lymphocyte counts were observed in LAB supplemented diets in the pre-challenged fish groups. After 3-week post challenged, the hematological parameters, immunoglobulin and survival were significantly high in fish fed with LAB supplemented diets at 10^5 CFU/g and above, while only the lysozyme activity was significantly high after 2-week post-infection. Histopathological result revealed that severe necrosis was found in the liver of striped catfish fed with the control diet after challenged with *A. hydrophila* which was not seen in the case of LAB supplemented diets. Thus, the results suggest that administration of LAB at 10^5 CFU/g of diet improves the innate immune response of striped catfish juveniles.

Keywords: *Hematological* parameters, Innate immune response, Histopathology, *Lactobacillus acidophilus, Pangasianodon hypophthalmus, Aeromonas hydrophila*

Introduction

Manipulation of intestinal microflora through the dietary supplementation of probiotics also known as 'bio-friendly agents' is a novel approach from both the nutritional and immunological stand point. Generally, probiotics are live micro-organisms, when administered in adequate amounts, confer health benefits to the host [1]. These microorganisms are able to colonize and multiply in the intestine of the host and therefore show numerous beneficial effects by modulating various biological systems in the host [2]. Hence, probiotics are broadly used in poultry and swine rearing but it remains to be used extensively in aquaculture [3]. To date lactic acid bacteria (LAB) is one of the most significant probiotic groups commonly used in aquaculture because they are a part of the natural intestinal microflora of a healthy fish [4-7]. *Lactobacillus acidophilus* is one of the more leading probiotic species of *Lactobacillus,* in aquaculture notably as a growth promoter in Nile tilapia [3]; in African catfish [8]; in grass carp [6]; in rainbow trout [7]; in snakehead [9,10], however few studies have been directed at the immunological enhancement of defense mechanisms of fish by the probiotic bacteria [7,10,11].

To date, no literature is available of using *L. acidophilus* for striped catfish, *P. hypophthalmus*. Therefore, the aim of the present study is to evaluate the effectiveness of *L. acidophilus* as a potential probiotic for striped catfish (*P. hypophthalmus) juveniles*, based on the haematologic and immune parameters as well as their ability to resist *A. hydrophila* infection.

Materials and methods

Culture of *L. acidophilus*

One g of commercial *L. acidophilus* (International Food Grade, Laboratory of USA) was grown in 50 mL of De Man, Rogosa and Sharp broth [6,12] (MRS broth 2 % w/v) for 12 h at 37 °C in a shaking incubator as a seed culture, and then 1 mL of overnight cultured bacterial suspension was transferred to 99 mL of MRS broth for mass culture and incubated for 36 h [13] in a constant shaking incubator (INFORS HT electron, 180 rpm) at 37 °C. After incubation, the bacterial cells were harvested by centrifugation at 3,000 g for 10 min, washed twice with phosphate buffered saline (PBS, pH 7.4), and re-suspended in the same buffer [14]. Cell density was calculated from OD_{600} correlated with colony forming unit (CFU) counts using serial dilution and spread plating on MRS agar.

Table 1 Ingredients used and proximate composition of control and varying concentrations of *Lactobacillus acidophilus* supplemented diets (g kg^{-1}).

Ingredients	Treatments[1]				
	Control	**10^3 CFU/g**	**10^5 CFU/g**	**10^7 CFU/g**	**10^9 CFU/g**
Fish meal[2]	305.8	305.8	305.8	305.8	305.8
Casein	129.0	129.0	129.0	129.0	129.0
Corn starch	376.3	376.3	376.3	376.3	376.3
Fish oil	35.0	35.0	35.0	35.0	35.0
Soybean oil	58.9	58.9	58.9	58.9	58.9
Binder[3] (CMC)	40	40	40	40	40
Vitamin mix[4]	30	30	30	30	30
Mineral mix[5]	20	20	20	20	20
L. acidophilus (CFU/g of diet)	0.0	10^3	10^5	10^7	10^9
Cr_2O_3	5.0	5.0	5.0	5.0	5.0
Proximate composition, g kg^{-1}					
Moisture	79.2	83.4	81.9	82.7	78.4
Crude protein	353.3	363.5	350.8	359.6	356.8
Crude lipid	122.2	126.1	130.4	130.8	124.1
Fibre	33.2	30.2	32.0	33.5	32.6
Ash	83.7	88.8	80.9	77.0	81.4
NFE[6]	407.6	391.4	405.9	399.1	405.1
GE (MJ Kg^{-})[7]	19.39	19.30	19.37	19.28	19.25

[1] Diets with different levels of *Lactobacillus acidophilus* Food Grade, Int. Lab, USA.
[2] Danish fishmeal: Crude protein: 720; Crude lipid: 50.
[3] CMC, Carboxy methyl cellulose.
[4] Vitamin mix kg^{-1} (Rovithai Ltd 700/437 Chonburi THAILAND): Vitamin A 50 MIU, Vitamin D3 10 MIU, Vitamin E 130 g, Vitamin K3 10 g, Vitamin B1 10 g, Vitamin B2 25 g, Vitamin B6 16 g, Vitamin B12 100 mg, Niacin 200 g, Pantothenic Acid 56 g, Folic Acid 8 g, Biotin 500 mg, Antioxidant 0.200 g and Anticake 20 g.
[5] Mineral mix kg^{-1}: Calcium phosphate (monobasic) 397.5 g; Calcium lactate 327 g; Ferrous sulphate 25 g; Magnesium sulphate 137 g; Potassium chloride, 50 g; Sodium chloride, 60 g; Potassium iodide, 150 mg; Copper sulphate 780 mg; Manganese oxide 800 mg; Cobalt carbonate 100 mg; Zinc oxide 1.5 g and Sodium selenite 20 mg.
[6] NFE, Nitrogen free extract, calculated as 1,000 − (protein + lipid + fibre + ash).
[7] GE, gross energy measured in a bomb calorimeter.

Preparation of experimental diets

Five isonitrogenous (360 g kg^{-1}) and isoenergetic diets (19 MJ kg^{-1}) were formulated and utilized for this study by inclusion of the probiotic *L. acidophilus* at various levels 10^3, 10^5, 10^7 and 10^9 CFU/g (**Table 1**). The diet that was not supplemented with *L. acidophilus* served as the control. Experimental diets were prepared by mixing feed ingredients thoroughly in a 5-kg food mixer (Tyrone, Model TR 202, UK) for 30 min [15]. Oils were added and were subsequently mixed for an additional 10 min [15]. To achieve accurate final concentrations of the diet, the *L. acidophilus* suspension prepared as described above was added to the mixed ingredients gradually using the food mixer followed by sufficient distilled water to make dough. The resultant dough was extruded into 3 mm diameter pellets using a pelleting machine (Model MH 237, Miao Hsien Ltd, Taichung, Taiwan), which was then air dried under sterile conditions for 24 h. The dried pellets were then broken into smaller pieces and kept in a plastic bag and stored at –20 °C, and the daily portions were then kept at 4 °C [16]. The viability of *L. acidophilus* in the test diets at 0, 30, 60 and 90 days post-storage at −20 °C was also determined by spread plating on MRS agar.

Experimental fish and culture conditions

The feeding trial was conducted at the Aquatic Research Complex in Universiti Sains Malaysia, Penang, Malaysia. Striped catfish juveniles of an average weight of 13 ± 1 g were bought from a commercial fish farm in Perak, Malaysia and transported in oxygenated plastic bags filled with freshwater. Prior to the commencement of the study, the experimental fish were acclimatized to laboratory conditions for one month so they may adjust to the laboratory systems and were fed with a commercial feed (Cargill Feed Sdn. Bhd, Malaysia). Thereafter, fish with an average body weight of 21.78 ± 1.74 g were randomly distributed into 15 500L-tanks, each with stocking densities of 25 fish/tank. The fish were fed an experimental diet at 2.5 % body weight daily based on a preliminary study carried out previously that determined the optimal feeding rate under these culture conditions [17]. The daily ration was presented in equal portions at 09:00 and 17:00 for a period of 12 weeks. Flow through water systems in each tank was set at the rate of 4.50 L h^{-1} throughout the experiment. Continuous aeration was provided and tanks were cleaned fortnightly.

Challenge test

At the end of the feeding trial, a bacterial challenge test was performed on each experimental group with *A. hydrophila* to evaluate the effectiveness of *L. acidophilus* against bacterial infection. To perform a pathogen challenge test, 150 fish from the feeding experiment were randomly selected and distributed among fifteen (60×30×30 cm^3) glass tanks in the closed recirculating system. Each treatment was maintained on triplicate. Each group of fish was fed the respective experimental diets that it had been administered during the feeding trial. The fish were challenged by intraperitoneal injection of 0.2 mL of 1×10^6 CFU/mL *A. hydrophila* concentration adjusted with sterile phosphate buffered saline. The mortality was recorded daily for 3 weeks following injection.

Sampling procedure

To determine the haematological, immunological parameters and histopathology of striped catfish, the experimental fish were starved for 24 h. Then 3 fish per replicate tank or aquarium (9 fish per treatment) were randomly selected from unchallenged and challenged fish and immediately stabilized using Aquadine (Fish Stabilizer; International Fish S.O.S Association) to reduce the stress during handling. The blood was collected where one part was transferred to a heparinized tube to determine the haematological parameters and the second part was transferred to a non-heparinized microtainer blood collection tube. The latter portion was left to clot at 4 °C for 2 h and the serum collected by centrifuging at 3,000 *g* for 15 min [18]. The separated serum was then stored at −20 °C until further analysis.

Haematological analysis

Erythrocyte sedimentation rate (ESR), packed cell volume (PCV), Red blood cell count (RBC), white blood cell count (WBC), haemoglobin (Hb) content and differential leukocyte count (monocyte, lymphocyte and granulocyte) were determined using microhematocrit, haemocytometer and cyanomethemoglobin methods, respectively as described previously [8,19]. The mean corpuscular haemoglobin concentration (MCHC), the mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) were calculated as follows [19]:

Mean corpuscular haemoglobin concentration (MCHC) gdL^{-1} = [haemoglobin g %/PCV %×100]

Mean corpuscular haemoglobin (MCH) pg $cell^{-1}$ = [haemoglobin g %/RBC (millions mm^{-3})×10]

Mean corpuscular volume (MCV) μm^3 = [PCV %/RBC (millions mm^{-3})×10]

Immunological indices

Total immunoglobulin content (Ig)

The total protein and immunoglobulin content of the serum sample was determined using the methods as previously described by [19-22]. The total immunoglobulin value was expressed as (mg mL^{-1}), calculated according to the following formula:

Total Ig (mg mL^{-1}) = Total protein in serum sample − Total protein treated with PEG

Lysozyme activity

Lysozyme activity in the serum sample was determined according to the method of [19,23], based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus (Sigma)*. The result was expressed in amounts of lysozyme (µg) per mL of sample calibrated to a standard curve.

Histological study of the liver

Light microscopy of the liver samples in the pre- and post-challenged fish groups were performed according to the method as described by [17,24].

Statistical analysis

The results were analyzed statistically using one-way analysis of variance (ANOVA). Duncan's multiple range test [25] was used in order to evaluate mean differences between the 5 different treatments with a significance level of $p < 0.05$. All statistical analysis was carried out using Statistical Package for Social Science (SPSS) software, version 20 for Windows. The data were presented as mean ± standard deviation. A dependent or paired-samples T test was also performed in order to determine the mean differences within the same group.

Results

Viability of *L. acidophilus* in experimental diets

The viability of LAB in the experimental diets at 0 (initial), 30, 60 and 90 days after storage at −20 °C is shown in **Table 2**. As storage time increased, the viability of LAB in the diets decreased. With the exception of the 10^5 CFU/g LAB diet, the viability of LAB in the supplemented diets significantly reduced only after 30 days of storage. Percent viability of LAB in all the test diets at the end of the 90 days of storage period ranged between 90.43 ± 2.35 to 96.98 ± 1.37.

Table 2 Viability of *Lactobacillus acidophilus* in experimental diets (log CFU/g) at a temperature of −20 °C for 90 days of storage (mean ± SD; n = 3)

	Days				% Viability after 90 days storage
Treatments	**Initial**	**30 days**	**60 days**	**90 days**	
10^3 CFU/g	3.37 ± 0.03^c	3.31 ± 0.03^{bc}	3.21 ± 0.10^b	3.05 ± 0.06^a	90.43 ± 2.35
10^5 CFU/g	5.06 ± 0.05^d	5.02 ± 0.03^c	4.97 ± 0.03^b	4.89 ± 0.05^a	95.08 ± 0.77
10^7 CFU/g	7.11 ± 0.02^c	7.08 ± 0.02^{bc}	6.99 ± 0.06^{ab}	6.90 ± 0.10^a	96.64 ± 1.01
10^9 CFU/g	9.10 ± 0.06^c	9.06 ± 0.04^{bc}	9.00 ± 0.03^b	8.88 ± 0.12^a	96.98 ± 1.37

Data with different superscripts in the same row indicate significant differences ($p < 0.05$).

Haematological parameters

Pre-challenged fish

The haematological parameters in the pre-challenged fish groups are presented in **Table 3**. Generally, with the exception of Hb, MCHC, MCH, MCV and monocyte counts, all other parameters responded to LAB supplementation compared to the control fish. ESR and granulocyte values decreased while PCV, RBC, WBC and lymphocyte levels increased significantly. Significant changes compared to the control emerged at10^5 CFU/g supplementation level for most of the parameters and highest significant values were obtained at 10^9 and 10^7 CFU/g for RBC and lymphocyte values, respectively. At the end of the feeding trial, fish fed with LAB supplemented diets showed a decreasing trend in ESR values with fish fed the 10^9 CFU/g diet exhibiting significantly lower ($p < 0.05$) value compared to those fed the control diet. Increasing levels of LAB did not improve PCV and RBC values, but these values were significantly improved ($p < 0.05$) when compared with the control fish group. Consumption of LAB diets by the striped catfish did not show any positive effect ($p > 0.05$) in the Hb content and RBC indices such as MCHC, MCH and MCV compared with the control fed group. However, LAB supplementation showed positive influence evident by the significantly higher ($p < 0.05$) WBC compared to the control diet, but no significant differences were observed among the LAB supplemented diets. Generally, pre-challenged fish groups fed with LAB supplemented diets showed significantly increased ($p < 0.05$) lymphocyte count, whereas the granulocyte count were significantly lower ($p < 0.05$) (except on 10^3 CFU/g) compared with the control fed group. On the other hand, feeding fish with LAB did not show any influence ($p > 0.05$) on the monocyte count compared to the control fish group.

Table 3 Haematological parameters of juvenile striped catfish, *Pangasianodon hypophthalmus*, fed diets containing varying levels of *Lactobacillus acidophilus* and control diets after 12 weeks (mean ± SD; n = 9, 3 fish per replicate tank).

Haematological Parameters	**Treatments**				
	Control	**10^3 CFU/g**	**10^5 CFU/g**	**10^7 CFU/g**	**10^9 CFU/g**
ESR (mm h^{-1})	1.14 ± 0.19^b	1.09 ± 0.49^{ab}	0.99 ± 0.14^{ab}	0.90 ± 0.26^{ab}	0.82 ± 0.17^a
PCV (%)	38.00 ± 1.0^a	39.67 ± 2.50^b	40.22 ± 1.30^b	40.56 ± 1.42^b	40.67 ± 1.50^b
RBC ($\times 10^6$ mm^{-3})	3.93 ± 0.22^a	4.20 ± 0.64^{ab}	4.36 ± 0.24^{bc}	4.35 ± 0.24^{bc}	4.62 ± 0.42^c
Hb (gdL^{-1})	16.04 ± 1.05	16.59 ± 1.14	16.72 ± 1.12	16.51 ± 1.51	17.06 ± 0.92
MCHC (gdL^{-1})	42.24 ± 2.93	42.06 ± 4.63	41.61 ± 3.14	40.78 ± 4.23	41.97 ± 2.09
MCH (pg $cell^{-1}$)	41.00 ± 3.88	40.29 ± 6.59	38.40 ± 2.80	38.01 ± 3.73	37.22 ± 3.94
MCV (μm^3)	97.04 ± 5.90	96.31 ± 15.23	92.40 ± 4.79	93.54 ± 6.90	88.55 ± 6.54
WBC ($\times 10^4$ mm^{-3})	4.48 ± 0.49^a	5.57 ± 0.26^b	5.56 ± 0.48^b	5.70 ± 0.67^b	5.65 ± 0.28^b
Lymphocyte (%)	67.17 ± 1.33^a	69.83 ± 0.98^b	70.33 ± 0.52^{bc}	71.50 ± 1.22^c	71.17 ± 1.17^{bc}
Monocyte (%)	4.83 ± 1.83	4.67 ± 1.21	5.33 ± 1.63	5.50 ± 1.52	5.00 ± 1.67
Granulocyte (%)	28.00 ± 2.45^b	25.50 ± 2.07^{ab}	24.33 ± 1.37^a	23.00 ± 2.53^a	23.83 ± 2.14^a

Data with different superscripts in the same row indicate significant differences ($p < 0.05$); ESR, Erythrocyte sedimentation rate; PCV, Packed cell volume; RBC, Red blood cell; WBC, White blood cell; Hb, Haemoglobin; MCHC, Mean corpuscular haemoglobin concentration; MCH, Mean corpuscular haemoglobin; MCV, Mean corpuscular volume.

Post-challenged fish with A. hydrophila

1) Two-week post-challenged

Table 4 presents the haematological indices of striped catfish 2 weeks after infection with *A. hydrophila*. After this post-challenged period, ESR, Hb, MCHC, WBC, Lymphocyte, Monocyte and Granulocyte values remained similar ($p > 0.05$) for all the test diets including the control treatment. However, PCV and RBC recorded significant increases while those of MCH and MCV decreased significantly. Is there any explanation as to why the treatment with 10^9 CFU/g had lower PCV and RBC values?

Table 4 Haematological parameters of striped catfish, *Pangasianodon hypophthalmus*, juveniles fed diets containing varying levels of *Lactobacillus acidophilus* challenged with *Aeromonas hydrophila* after 2-week (mean ± SD; n = 9, 3 fish per replicate tank).

Haematological parameters	**Treatments**				
	Control	**10^3 CFU/g**	**10^5 CFU/g**	**10^7 CFU/g**	**10^9 CFU/g**
ESR (mm h^{-1})	1.43 ± 0.33	1.33 ± 0.48	1.17 ± 0.28	1.0 ± 0.45	1.25 ± 1.35

Haematological parameters	Treatments				
	Control	**10^3 CFU/g**	**10^5 CFU/g**	**10^7 CFU/g**	**10^9 CFU/g**
PCV (%)	36.83 ± 0.41^a	38.17 ± 1.17^{ab}	38.50 ± 0.55^{ab}	39.33 ± 0.82^b	37.33 ± 3.39^{ab}
RBC ($\times10^6$ mm^{-3})	3.35 ± 0.13^a	3.94 ± 0.35^b	4.03 ± 0.43^b	4.22 ± 0.43^b	4.17 ± 0.19^b
Hb (gdL^{-1})	15.01 ± 0.87	15.78 ± 1.52	15.92 ± 0.37	16.05 ± 1.11	16.26 ± 1.30
MCHC (gdL^{-1})	40.75 ± 2.30	41.40 ± 4.56	41.34 ± 0.58	40.81 ± 2.65	43.67 ± 2.89
MCH (pg cell^{-1})	44.88 ± 2.13^b	40.28 ± 5.46^{ab}	39.93 ± 4.77^{ab}	38.28 ± 3.98^a	39.05 ± 3.97^a
MCV (µm^3)	110.25 ± 4.52^b	97.54 ± 10.28^a	96.51 ± 10.56^a	94.05 ± 10.95^a	89.55 ± 8.62^a
WBC ($\times10^4$ mm^{-3})	5.16 ± 0.23	5.73 ± 0.69	5.93 ± 0.69	5.93 ± 0.61	5.79 ± 0.82
Lymphocyte (%)	67.17 ± 2.32	65.50 ± 2.17	66.67 ± 1.97	67.00 ± 1.67	66.83 ± 1.17
Monocyte (%)	4.83 ± 0.98	5.00 ± 0.89	5.00 ± 1.10	5.17 ± 1.17	4.83 ± 1.33
Granulocyte (%)	28.00 ± 2.45	29.50 ± 2.74	28.33 ± 2.25	27.83 ± 1.17	28.33 ± 1.97

Data with different superscripts in the same row indicate significant differences ($p < 0.05$).

2) Three-week post-challenged

Table 5 shows the haematological parameters 3 weeks after infection with *A. hydrophila*. With the exception of RBC, most of the red cell components recorded no significant differences ($p > 0.05$) compared to the controlled fish. However, this period of post-infection revealed marked changes in WBC and its components. With the exception of 10^3 CFU/g of *L. acidophilus*, all supplemented diets showed significantly increased ($p < 0.05$) RBC and WBC count compared to the control fed group. After 3-week infection with *A. hydrophila*, the result in the case of the leukocyte types, particularly lymphocyte and granulocyte count showed significant changes ($p < 0.05$) between the control and LAB supplemented diets. Lymphocyte count in the 3-week post challenged fish group fed with LAB at 10^3 and 10^5 CFU/g showed a significantly lower ($p < 0.05$) level than the control diet, but did not differ significantly from other LAB supplemented diets. However, the significantly higher ($p < 0.05$) granulocyte count was observed in the group fed with LAB up to 10^7 CFU/g compared to those fish fed with the control diet. However, 3-week post-challenged fish groups fed with LAB supplemented diets did not show any significant changes ($p > 0.05$) in the case of ESR, PCV, Hb, RBC indices and monocyte count when compared to the control fed group.

Table 5 Haematological parameters of juvenile striped catfish, *Pangasianodon hypophthalmus*, fed diets containing varying levels of *Lactobacillus acidophilus* and control diets after a 3-week challenged with *Aeromonas hydrophila* (mean ± SD; n = 9, 3 fish per replicate tank).

Haematological parameters	Treatments				
	Control	**10^3 CFU/g**	**10^5 CFU/g**	**10^7 CFU/g**	**10^9 CFU/g**
ESR (mm h^{-1})	1.26 ± 0.50	1.12 ± 0.30	1.11 ± 0.43	1.04 ± 0.64	1.16 ± 1.36
PCV (%)	35.00 ± 2.69	36.67 ± 3.24	37.56 ± 5.36	37.22 ± 4.27	37.11 ± 2.52
RBC ($\times10^6$ mm^{-3})	3.47 ± 0.10^a	3.64 ± 0.38^a	4.03 ± 0.45^b	4.15 ± 0.37^b	4.06 ± 0.30^b
Hb (gdL^{-1})	14.52 ± 1.33	15.37 ± 2.43	15.44 ± 1.34	15.67 ± 2.05	15.71 ± 0.61
MCHC (gdL^{-1})	41.80 ± 5.95	42.01 ± 6.37	41.65 ± 4.82	42.29 ± 4.96	42.54 ± 3.70
MCH (pg cell^{-1})	41.87 ± 4.22	42.65 ± 8.03	38.89 ± 6.23	37.85 ± 5.05	38.86 ± 3.44
MCV (µm^3)	100.80 ± 6.58	101.90 ± 16.61	94.75 ± 18.77	89.99 ± 10.92	91.41 ± 4.17
WBC ($\times10^4$ mm^{-3})	5.46 ± 0.15^a	5.89 ± 0.72^{ab}	6.24 ± 0.37^b	6.78 ± 0.68^c	6.02 ± 0.21^b
Lymphocyte (%)	67.83 ± 1.47^c	65.00 ± 2.28^a	65.33 ± 1.63^{ab}	66.00 ± 1.10^{abc}	67.17 ± 0.75^{bc}
Monocyte (%)	5.00 ± 1.41	5.00 ± 1.26	4.83 ± 0.75	5.00 ± 0.63	4.50 ± 0.84
Granulocyte (%)	27.17 ± 0.75^a	30.00 ± 2.00^b	29.83 ± 1.47^b	29.00 ± 1.26^b	28.33 ± 0.52^{ab}

Data with different superscripts in the same row indicate significant differences ($p < 0.05$).

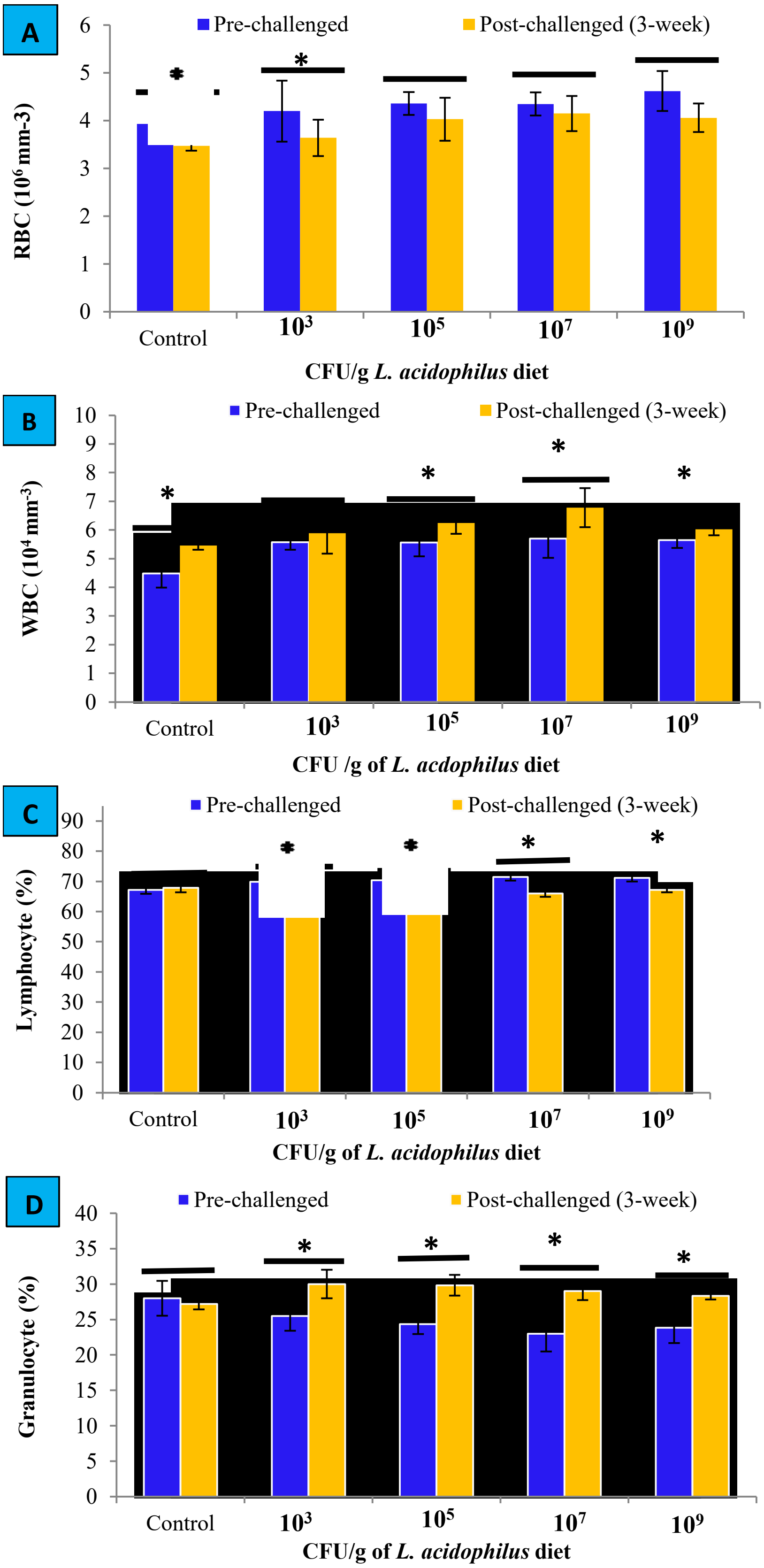

Figure 1 A) RBC, B) WBC, C) Lymphocyte and D) Granulocyte count in pre- and 3-week post-challenged striped catfish, *Pangasianodon hypophthalmus*. The asterisk indicates significantly different ($p < 0.05$) between the pre- and 3-week post-challenged within the same group.

As the 3-week post-challenged fish group fed with LAB supplemented diets significantly influenced some important haematological parameters such as RBC, WBC, lymphocyte and granulocyte count. A comparison was done in the pre-challenged and 3-week post-challenged fish group by using T test (**Figure 1**) in order to determine the effectiveness of the probiotic LAB supplementation in immune response and disease resistance. Haematological parameters including RBC, WBC, lymphocyte and granulocyte count were significantly influenced in the 3-week post-challenged fish group fed with LAB supplemented diet (except on 10^3 CFU/g) compared to control fed fish (**Table 5**). Generally, RBC count showed a decreasing trend in the 3-week post-challenged fish groups compared to the pre-challenged, and it was only significant ($p < 0.05$) in the case of the control and 10^3 CFU/g of LAB supplemented fed groups. However, except on 10^3 CFU/g of LAB, WBC count showed a significantly increasing ($p < 0.05$) trend in the 3-week post-challenged fish groups than the pre-challenged fish group. The lymphocyte counts in the LAB supplemented diets significantly decreased ($p < 0.05$) in the 3-week post-challenged fish group compared to pre-challenged groups, whereas an opposite trend was observed in the case of granulocyte count.

Immune indices

Total immunoglobulin content (Ig)

The total Ig in the blood serum of striped catfish fed with various levels of LAB supplemented diets in both pre- and post-challenged fish groups are presented in **Figure 2**. Pre-challenged fish showed significantly higher ($p < 0.05$) Ig content when the fish were fed a 10^7 CFU/g of LAB supplemented diet compared to the control and remaining treatments. On the other hand, in the 2- and 3-week post-challenged data showed that fish groups fed the control (18.67 ± 2.92; 17.76 ± 0.49) and 10^3 CFU/g (18.63 ± 2.46; 16.79 ± 1.70) LAB supplemented diets had shown Ig content that were significantly lower ($p < 0.05$) than fish fed 10^5 (23.71 ± 2.62; 20.77 ± 2.01), 10^7 (23.67 ± 2.18; 22.88 ± 2.67) and 10^9 CFU/g of diets (24.37 ± 1.74; 20.20 ± 2.92), respectively.

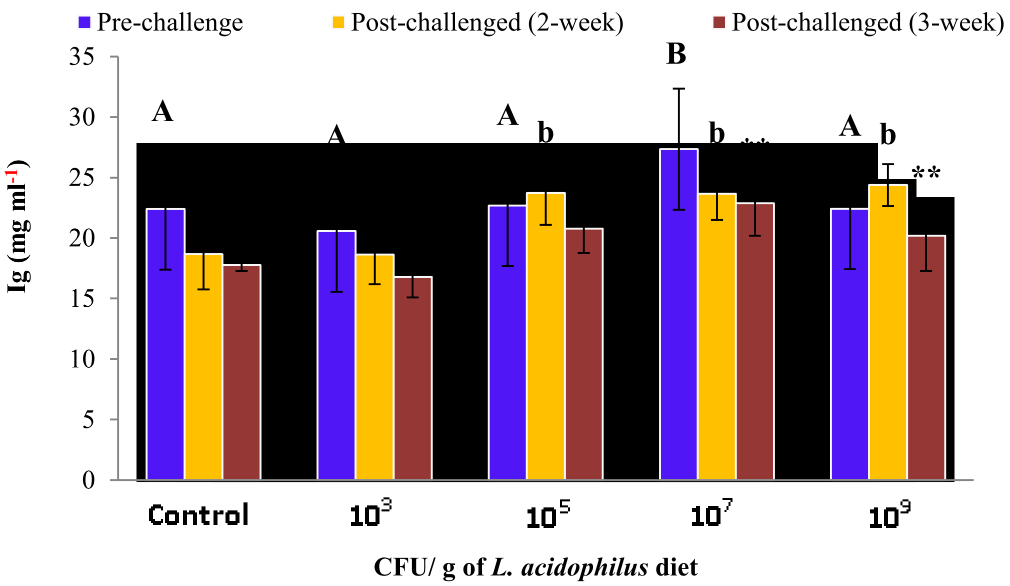

Figure 2 Immunoglobulin content of pre- and post-challenged striped catfish, *Pangasianodon hypophthalmas,* feeding with various concentrations of *Lactobacillus acidophilus* supplemented diets. Bars with different uppercase latters indicate pre-challenged and lowercase indicate post-challenged (2-week) and the asterisk indicates post-challenged (3-week) are significantly different ($p < 0.05$). Data presented as mean ± SD, (n = 9; 3 fish per replicate tank).

Lysozyme activity

Lysozyme activity in pre- as well as post-challenged fish groups with *A. hydrophila* is shown in **Figure 3**. Lysozyme activity did not differ significantly ($p > 0.05$) when the fish were not infected regardless of feeding with LAB diets. Lysozyme activity was peaked at 2 weeks after infection, with significantly highest ($p < 0.05$) at 10^5 CFU/g of diet (40.23 ± 5.69), beyond which it showed a decreasing trend. All the treatment groups showed a drastically decreased in lysozyme activity after 3-week infection and retained the similar values as reported in pre-challenged fish groups.

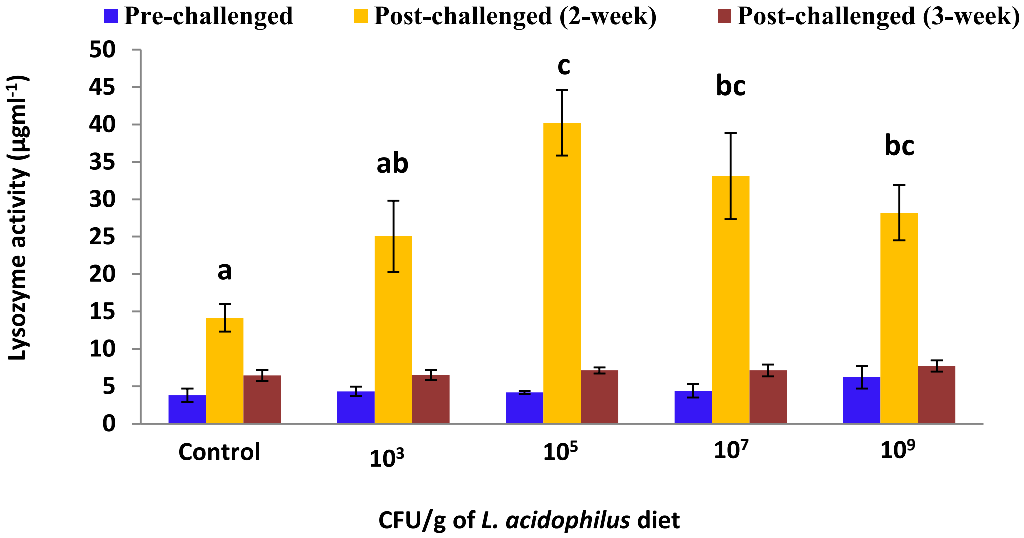

Figure 3 Lysozyme activity of striped catfish, *Pangasianodon hypophthalmas,* fed with *Lactobacillus acidophilus* supplemented diets in pre- and post-challenged with *Aeromonas hydrophila*. Bars with different lowercase letters indicate post-challenged (2-week) significantly different ($p < 0.05$). Data presented as mean ± SD (n = 9; 3fish per replicate tank).

Challenge test

Survival of fish in the post-challenged test after 3-week is shown in **Table 6**. The results of challenge test indicated that, the fish fed with LAB supplemented diets at 10^5 CFU/g and above showed a significantly higher ($p < 0.05$) survival rate than the control fed group. Similar survival rate (93.33 ± 5.77) was observed when fish fed with 10^5 to 10^9 CFU/g of LAB supplemented diets. However, the survival improved with increased LAB upto 10^5 CFU/g, beyond which survival did not change significantly. The morality of the fish was started after 4 days of injection and continued until 10 days.

Table 6 Effect of various concentrations of *Lactobacillus acidophilus* supplementation on the post-challenged survival of juvenile striped catfish, *Pangasianodon hypophthalmus,* with *Aeromonas hydrophila* (mean ± SD; n = 3).

	Treatments				
Parameters	**Control**	**10^3 CFU/g**	**10^5 CFU/g**	**10^7 CFU/g**	**10^9 CFU/g**
No of infected fish	30	30	30	30	30
Route of injection	IP	IP	IP	IP	IP
Dosage of bacteria injected	0.2 mL of 10^6CFU/mL	0.2 mL of 10^6CFU/mL	0.2 mL of 10^6CFU/mL	0.2 mL of 10^6CFU/mL	0.2 mL of 10^6CFU/mL
Survival rate (%)	80.00 ± 0.00^a	86.67 ± 5.77^{ab}	93.33 ± 5.77^b	93.33 ± 5.77^b	93.33 ± 5.77^b

Survival data with different superscripts in the same row indicate significant differences ($p < 0.05$).

Histology of liver

Monitoring of histopathologic changes in the liver of striped catfish in the pre- and post-challenged fish groups are shown in **Figures 4 - 6**. The histopathological observation revealed that no pathological changes were observed in the pre-challenged fish groups based on the intact hepatocytes (H), acini of the exocrine pancreas (AEP) with centro acinar cells (CAC) and portal vein (PV) which indicated that the healthy condition of the fish were maintained throughout the experimental period. Severe cellular damage of the liver was only noted after the fish were challenged with *A. hydrophila* (**Figures 5** and **6**). Massive damage with severe necrotic areas in the liver of the A) control fed group was observed after 2-and 3-week challenged with *A. hydrophila* compared with minor pathological changes including nucleoli displayed to nuclear periphery (PN), normal hepatocyte with central nucleoli (CN), acini of exocrine pancreas (AEP), pyknotic nuclei (PK), swelling of hepatocytes (H) by cytoplasm vacuolization (V), and melanomacrophage centre (MMC) deposited near acini of exocrine pancreas (AEP) and centro acinar cells (CAC) in the B) 10^3 CFU/g diet; lower number of swelling hepatocytes (H) with central nucleoli (CN), nucleoli displayed to nuclear periphery (PN), bile duct (BD), acini of exocrine pancreas (AEP) with centro acinar cells (CAC) and portal vein (PV), and melanomacrophage centre (MMC) in the C) 10^5 CFU/g diet; hepatocytes (H) with cytoplasm vacualization (V), malanomacrophage centre (MMC) deposition near the acini of exocrine pancreas (AEP), ruptured centro acinar cells (CAC) and portal vein (PV), in the D) 10^7 CFU/g diet; hepatocyte with periphery nucleoli (PN), central nucleoli (CN), complete disappearance of nucleoli (DN), hepatocyte (H) with some vacualization (V), acini of exocrine pancreas (AEP) with portal vein (PV) and centro acinar cells (CAC) in the hepatocytes of E) 10^9 CFU/g diet.

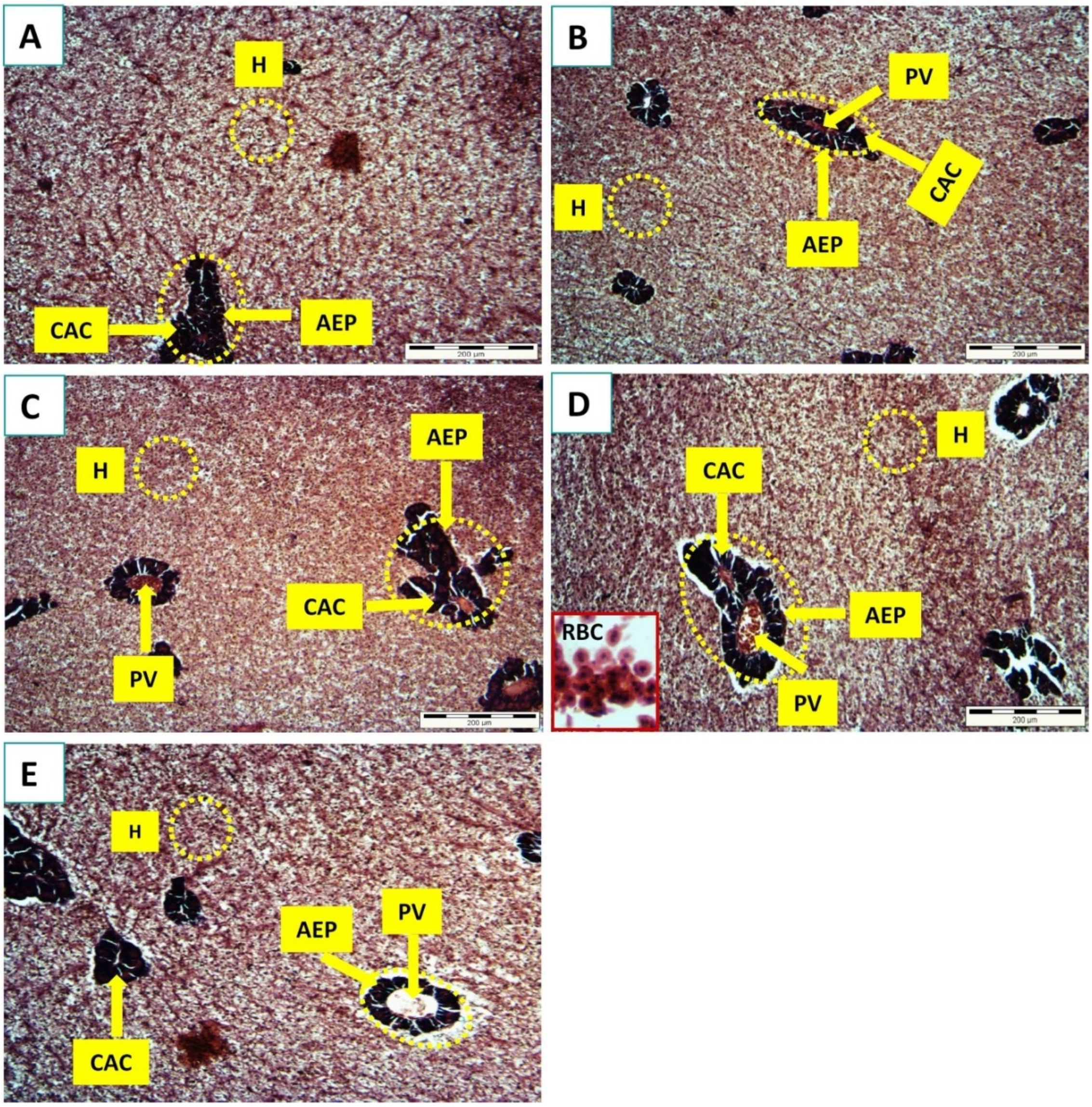

Figure 4 Liver stained with haematoxylin and eosin, bar 200 µm; sample collection at pre-challenged striped catfish, *Pangasianodon hypophthalmus,* with *Aeromonas hydrophila* A) Control, B) 10^3, C) 10^5, D) 10^7, E) 10^9 CFU/g of *Lactobacillus acidophilus* supplemented diets. No histopathology was observed among treatments showing intact hepatic architecture hepatocytes (H); acini of exocrine pancreas (AEP) with centro acinar cells (CAC) and portal vein (PV).

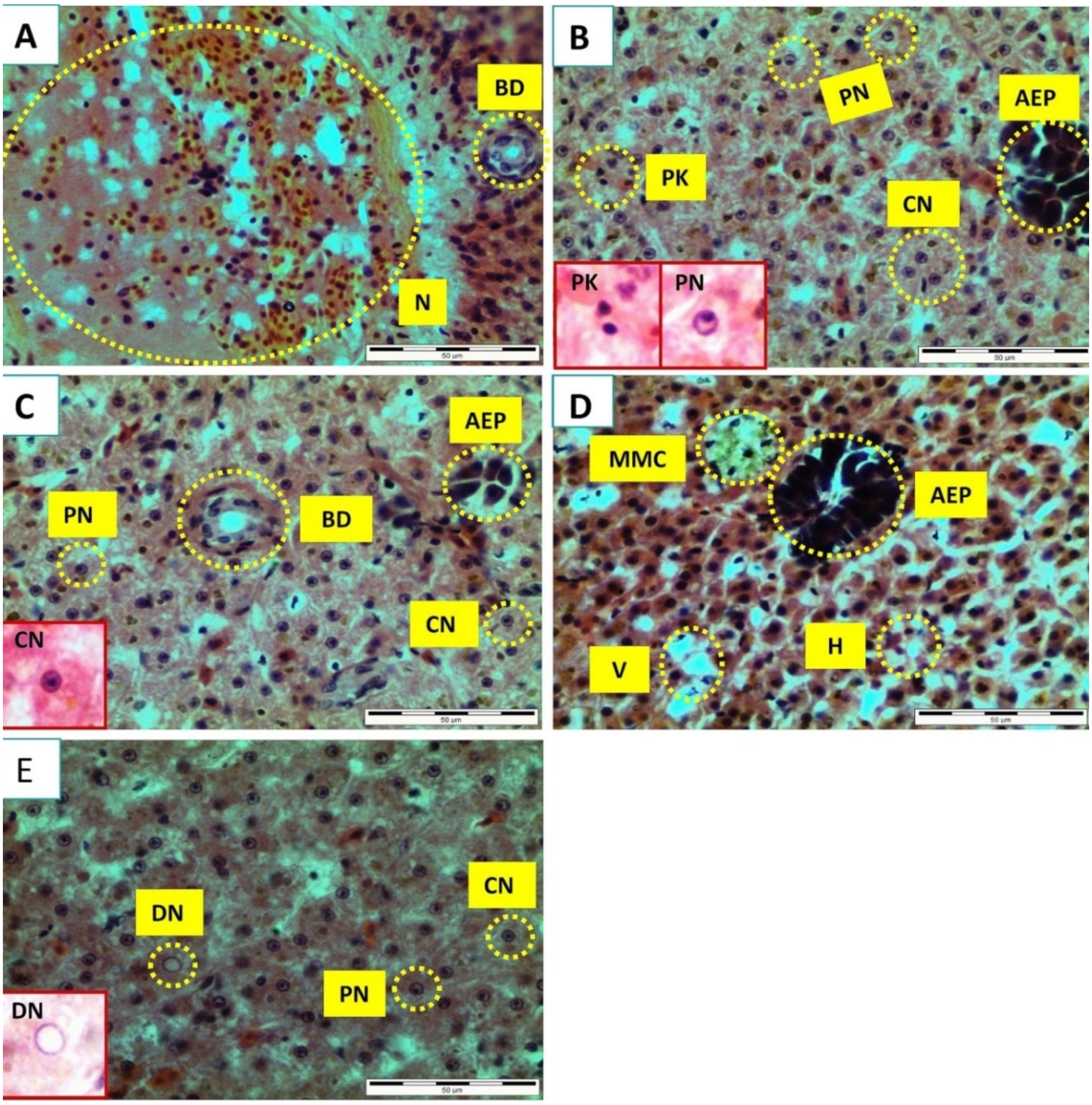

Figure 5 Liver stained with haematoxylin and eosin, bar 50 µm; sample collection at 2-week post infected striped catfish, *Pangasianodon hypophthalmus,* with *Aeromonas hydrophila* A) Control diet showing massive necrotic areas (N) near the bile duct (BD); B) 10^3 CFU/g of *Lactobacillus acidophilus* diet with nucleoli displayed to the nuclear periphery (PN), normal hepatocyte with central nucleoli (CN), acini of exocrine pancreas (AEP), and pyknotic nuclei (PK); C) 10^5 CFU/g diet with lower number of swelling hepatocytes (H) with central nucleoli (CN), nucleoli displayed to the nuclear periphery (PN), bile duct (BD), and acini of exocrine pancreas (AEP); D) 10^7 CFU/g diet hepatocytes (H) with cytoplasm vacualization (V); malanomacrophage center (MMC) deposition near the acini of exocrine pancreas (AEP), and E) 10^9 CFU/g diet showing hepatocyte with periphery nucleoli (PN), central nucleoli (CN), and complete disappearance of nucleoli (DN).

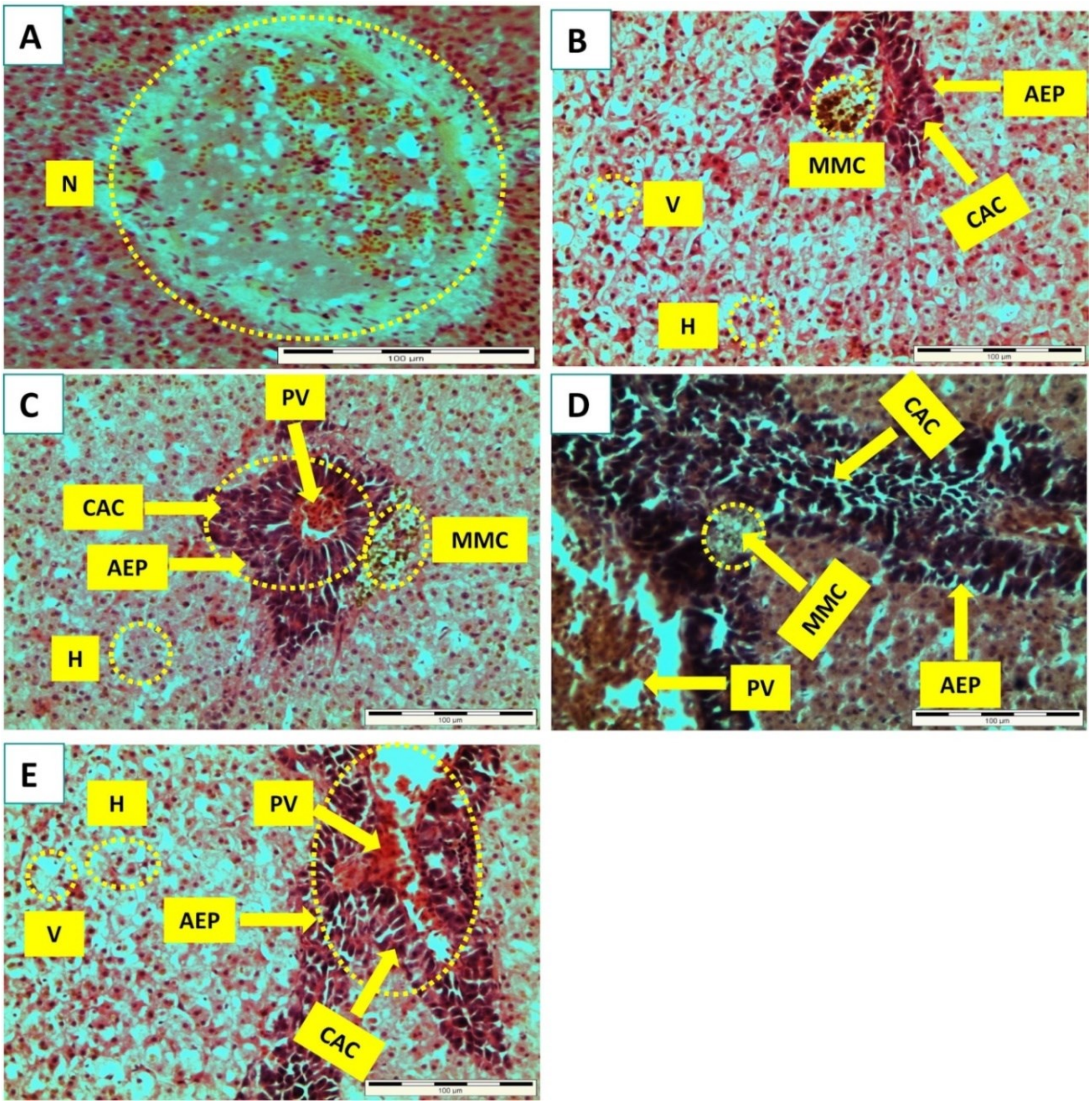

Figure 6 Liver stained with haematoxylin and eosin, bar 100 µm; sample collection at 3-week post-challenged striped catfish, *Pangasianodon hypophthalmus,* with *Aeromonas hydrophila* A) Control diet showing severe necrosis (N); B) 10^3 CFU/g of *Lactobacillus acidophilus* diet with slight swelling of hepatocytes (H) by cytoplasm vacuolization (V), melanomacrophage centre (MMC) deposited near acini of exocrine pancreas (AEP) and centroacinar cells (CAC); C) 10^5 CFU/g diet with lower number of swelling hepatocytes (H), regular shaped acini of exocrine pancreas (AEP) with portal vein (PV) and centroacinar cells (CAC), and malanomacrophage centre (MMC); D) 10^7 CFU/g diet with ruptured centroacinar cells (CAC) in the acini of exocrine pancreas (AEP), portal vein (PV), and melanomacrophage centre (MMC), and E) 10^9 CFU/g diet shows hepatocyte (H) with some vacuolization (V), acini of exocrine pancreas (AEP) with portal vein (PV) and centroacinar cells (CAC).

Discussion

An understanding of the haematological parameters can be used as an effective indicator to evaluate physiological and pathological abnormalities in fish to verify its health status [26]. Evidence has shown that probiotics have a direct influence on both the innate and the acquired immune system [27-30]. Among the haematological parameters, leukocyte count is considered the most important parameter to evaluate the fish health status, as the nonspecific or innate immunity of fish is primarily dependent [31,32] on the leukocyte levels in the fish blood [7]. In addition, phagocytosis of the invading pathogen through macrophage is performed by the different types of leukocyte [33] and there is also evidence that leukocytes (B lymphocyte) are able to induce the production of antibodies which play a key role in the acquired immune system.

Based on the blood parameters obtained in this study, feeding fish with LAB based diets significantly improved the overall health status of the fish compared to the control fish. The better PCV and RBC values in response to probiotics were also reported for the African catfish [8], rainbow trout [7], snakehead [9,10] and for *L. rohita* fed with probiotic *B. subtilis* [34]. Elevated PCV values were also noted in tilapia when fed 2 microbial feed additives such as *Bacillus subtilis* and *Lactobacillus acidophilus* [18]. The decrease in ESR values, as a result of feeding a LAB based diet, might be an indicator of a lowered risk of infection or inflammation in fish [9]. Similar to the present study, a significant increase of lymphocyte count was also noted in beluga juvenile fed with oligofructose [35] and rainbow trout fed with inulin [36], suggesting an improvement in health status of striped catfish fed a probiotic LAB added diet, which is consistent with [37] the previous studies. The addition of LAB in the diet of the striped catfish had no effect on the haematological parameters including ESR, Hb, MCHC and WBC at 2-week post-challenged fish with *A. hydrophila.* Whereas, an increased PCV and RBC count were reported in the LAB supplemented fed groups than the control group, which might be a result of the haemopoitic stimulation because of probiotic feed [38]. This finding is consistent with those by Talpur *et al.* [9], who reported a significantly higher RBC count in snakehead fed with LAB diet after a 2-week challenge with *A. hydrophila*. A significantly lower level of MCV were also noted in the infected fish fed on the probiotic diets, compared to the control group, suggesting a positive influence on RBC during infection. An increase in MCV is reported to be due to the swelling of the RBC as a result of a hypoxic condition (osmotic stress) or macrocytic anaemia in fish exposed to stress [39] which possibly would increase the affinity for oxygen in the blood [40]. Although most of the haematological parameters were not influenced by the intake of LAB up to 3-week post-challenged, the more important haematological components, namely RBC and WBC, lymphocyte and granulocyte count, which are directly related to fish health and immunity, were significantly influenced. It is well documented that WBC of fish plays a crucial role in the cellular immunity and resistance to infectious diseases [41], whereas RBC linked to the oxygen transportation in the fish blood via haemoglobin [42]. Additionally, the significantly higher granulocyte count was observed in the group fed with LAB upto 10^7 CFU/g compared to those fish fed with the control diet, which indicated higher phagocytic activity of probiotic fed diets as granulocytes are involved in phagocytosis of invading pathogens. Generally, a reduction in PCV, RBC and Hb content were noted in 3-week post-challenged fish groups than the pre-challenged fish groups. Similar findings were also noted in *Labeo rohita* and *Channa striata* when fed with a probiotic diet [9,34]. The decrease in the RBC numbers and Hb content in the post-challenged state could be attributed to hypochromic microcytic anemia caused by *A. hydrophila* [43]. The cause of reduction in PCV, RBC and Hb content may be due to leukocytosis or erythroblastosis resulting from the induced pathogens due to stress caused by the adverse effects of *A. hydrophila* [9]. On the contrary, the reduction in the total RBC count after challenging with *A. hydrophila* was minimal in the case of LAB supplemented diets in comparison to the control and 10^3 CFU/g LAB diets, which showed a significant reduction in the post-challenged fish groups. Similar finding was reported in *L. rohita* fed with *B. subtilis* [34]. With the exception of 10^3 CFU/g of the LAB diet, a very significant increasing trend in WBC count was observed in the 3-week post-challenged fish group compared to the pre-infected fish group. The abrupt increased number of WBC count during infection [9] with pathogenic bacteria indicates the anti-infection properties of LAB, as WBC are believed to be the first line body defence mechanisms and may serve as a protective barrier against pathogenic organism [44]. The innate or non-specific immune system of fish is being considered to be the first line defence of fish against a wide range of invading pathogens, including both Gram-positive and Gram-negative bacteria [38,45-47], which can be influenced by the intake of probiotic. The major components of the immune system are comprised of cellular elements including macrophages, monocytes, granulocytes, and humoral elements, such as lysozymes, immunoglobulins [33,45,48]. These parameters could affect the inherent capability of fish to defend themselves against pathogens, before the specific immune response is developed and thus can be used as an immunological marker for resistance [47]. The specific immune response serves as the second line of defence which also has a significant role in protecting fish from different infectious diseases [38,49] which can be activated by the innate immune system [48]. Serum immunoglobulins are considered to be a major component of the humoral immune system, which is well recognized in providing disease protection in animal and human beings [50]. Several researchers have reported the positive effect of probiotic bacteria on the Ig production in fish [8,51,52]. Significantly increased total Ig content in the serum of snakehead fed with a probiotic LAB supplemented diet upon to challenge with *A. hydrophila* was reported by Talpur *et al.* [9]. In the present study, probiotic LAB added diets significantly increased total Ig levels in striped catfish after being challenged with *A. hydrophila* in comparison to the control diet, which indicated the activation of strong defence mechanisms against induced pathogens. Both pre- and post-challenged fish groups showed an increasing trend in total Ig content with increasing LAB supplementation up to 10^7 CFU/g, beyond which it showed a slight decreasing trend. This increased level of Ig in the LAB fed group after challenged could explain the higher survival rate obtained in the probiotic fed group.

It is well known that lysozyme has a significant role in disease resistance through antibacterial activity against certain Gram-positive bacteria, and Gram-negative bacteria [16,53,54]. This enzyme, subjects the bacteria to lysis by attacking the β-1, 4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell walls [54]. Lysozyme activity was not influenced by the feeding of LAB in the uninfected fish groups as reported previously when rainbow trout were fed *L. plantarum* unlike *L. rhamnosus* JCM 1136 [16] and *L. casei* [55]; red sea bream fed with *L. rhamnosus* [56]; Nile tilapia fed with *Bacillus subtilis* endospores [57] and *Pangasias bocourti* fed with *B. aerius* B81e [58]. The variability of this result might be due to the physiological status of the fish and different rearing conditions [28]. However, a significantly higher lysozyme activity was noted in the 2-week infected fish group fed with LAB supplemented diets (except at 10^3 CFU/g) compared to the control group, which can be linked to the higher survival in probiotic fed group obtained in this current study. Previous studies mentioned that the lysozyme activity in fish is induced only after bacterial injection, and/or in response to bacterial infection [16,54], which may be due to an increase in the number of phagocytes secreting lysozymes [59]. However, the decreasing trend of lysozyme activity was noted at the end of 3-week post infection could be attributed to the fish recovering from the infection. Despite the fact that the exact mechanism of action of probiotics is still to be established in any animal, including fish, probiotics often showed host and strain specific differences in their activities [60]. The immunomodulatory activity of probiotics can be significantly influenced by various factors such as source, type, dose and duration of supplementation [60]. In the current study, the striped catfish fed with various levels of LAB diets for 12 weeks were challenged with *A. hydrophila*. The survival rate of probiotic fed diets showed significantly higher levels of protection against the test pathogen than the control fed group and supported by reports on snakehead [9,10], tilapia [14] and Pla-Mong fish [58]. Besides LAB, other *Lactobacillus* sp. also have the potential to increase the survival of fish after challenged with pathogenic bacteria [54,61-63]. The higher survival rate observed after feeding LAB supplemented diets could be due to the combined effects of the activation of the innate defences of the striped catfish, increased in serum lysozyme levels and total immunoglobulin contents, all of which subsequently improved its resistance to *A. hydrophila* infection [64]. Indeed, probiotic bacterial species, particularly lactic acid bacteria is capable of excluding some other bacteria for nutrients, space and/or through the production of antimicrobial compounds [64] or affording protection by preventing cellular damage [65]. The pre-challenged fish groups represented the normal histological features, by showing intact hepatic architecture hepatocytes (H), acini of the exocrine pancreas (AEP) with centro acinar cells (CAC) and portal vein (PV). This finding indicated absence of any pathological changes in liver function in the pre infected fish groups. Unlike, *Pangasias sanitwongsei* species [66], a lobular arrangement of hepatic tissue was not noticeable in the liver of striped catfish. Healthy fish hepatocytes are generally homogenous in structure which contain a single and centrally located spherical nucleus, with a clear, dark central nucleolus [66,67]. Like some other teleost fish species, the pancreatic tissue in striped catfish develops surrounding the portal vein and penetrate inside the liver tissues, which is commonly known as hepatopencrease [66-68]. The pancreatic? tissue can be distinguished from the hepatocyte by its acinar arrangement [67].

Like haematological parameters, histopathological observation of liver, which are considered to be a more vital organ for removing toxic substances and/or transforming them as less toxic material for excretion [69], displayed severe pathological abnormalities at 2- and 3-week post infection with *A. hydrophila*. Microscopic examination revealed that remarkable structural changes such as severe cellular and vacuolar degeneration of the liver was detected after challenged the experimental fish with *A. hydrophila.* These severe damages occurred after challenging fish with *A. hydrophila* in the hepatic cells might be due to the secretion of toxin by pathogenic bacteria, which also agreed with previous studies that reported the tissue death area in the liver of hybrid striped bass was observed when it was challenged with *A. salmonicida*, *Mycobacterium sp.* and *V. anguillarum* [70]. These pathological changes of the liver were severe in the fish fed the control diet, showing severe necrosis in the liver tissues compared to those fish maintained on LAB supplemented diets. This finding supports the hypothesis that LAB might be able to decrease pathological changes of the liver tissue after being challenged with *A. hydrophila* but the detail mechanism remains unknown.

Conclusions

In conclusion, it is evident from the results that the dietary supplementation of LAB had an immunostimulatory effect on the host, leading to enhance survival of juvenile striped catfish following challenged with the *A. hydrophila* infection. Supplementation with LAB is able to activate the immune response and resistance to *A. hydrophila* infection of striped catfish and can be effectively used as a feed additive for striped catfish (*P. hypophthalmus)* juveniles culture. To elevate the immune response of juvenile striped catfish, culture dietary supplementation of LAB at 10^5 CFU/g of diet can be used effectively as a feed additive.

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