Immunity-enhancing efficacy of the *lactobacillus spp* as probiotic, segregated from the gut of *Channa marulius* against *Aeromonas sp.*

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Abstract:

In present investigation study of Haemato-immunological parameters, significantly higher erythrocyte count, haemoglobin content, haematocrit value, leucocyte count and respiratory burst activity were observed in LBD-3 (fed with diet containing 0.70 probiotic than other treatment groups, and control showed significantly lower values. The leucocyte count of all the experimental groups differed significantly from each other, with highest count in LBD-1 and lowest in control. The number of adherent neutrophil was significantly higher in all the treated groups than the control. But there was no difference between the treatments. Significantly higher total protein content was found in the LBD-3 (fed with diet containing 0.70 probiotic. The percentage survival of fishes of the entire probiotic treated group after challenging with *Aeromonas Spp* was significantly higher than the control.

***Index Terms*:** *Channa marulius* , *Aeromonas* , Probiotic, Immune- enhancement

1. Introduction

The immunological system of fishes is physiologically similar to higher vertebrates with certain differences. In contrast to higher vertebrates, fish are free-living organisms from early embryonic stages of life and depend on their innate immune system for survival .

Immunity is referred to as the state of acquired or innate resistance or protection from a pathogenic microorganism or its products or the effect of toxic substances. The cells of the immune system consist of lymphocytes, specialized cells that capture and display microbial antigens, and effectors cells that eliminate pathogens.

Innate immunity is a basic defense way in fishes. Besides, it plays a key role in the acquired immune response and homeostasis through a system of receptor proteins. These receptor proteins identify molecular patterns that are typical of pathogenic microorganisms, including polysaccharides, lipopolysaccharide (LPS), peptidoglycan bacterial DNA, viral RNA and other molecules that are not normally on the surface of multicellular organisms. This response is divided into physical barriers and cellular and humoral immune responses.

The immune system in fishes is playing the role of destroying pathogens by acquired and innate mechanisms, with humoral and cellular levels to prevent the occurrence of diseases. Fish are predisposed to viral, bacterial, fungal and parasite agents, yet, fish can resist microbial attack by specific and innate mechanisms. The unspecific mechanisms which are the production of numerous antibacterial compounds, proteins of inflammation acute phase, complement activated by the alternative pathway, cytokines, phagocytosis and inflammation. Recently, there has been an increasing practice of managing bacterial fish diseases by using probiotic strains to control populations of potential pathogens, either by competitive inhibition, enhancement of fish immunity or by the microbial enhancement of the environment. They are usually incorporated into the fish feed. Keeping all such factors in mind, in this study, an attempt was made to evaluate the immunity-enhancing efficacy of the probiotic lactobacillus spp. isolated from the gut of *Channa marulius* against *Aeromonas spp.* Infection.

2. Materials and Methods:

2.1: Sample collection: Healthy *Channa marulius* fingerlings were collected from Godavari River Nanded District (MS) (19.1383° N, 77.3210° E). All the fishes were transported to the wet laboratory and fishes were randomly divided into stock in the circular plastic tank (200 L) filled with fresh water with continuous aeration. During this period, the fishes were fed with commercial pellets (5% body weight) twice a day. Fishes were acclimatized to these conditions for 10 days before the experiments. During the experiment, the water temperature was maintained at 28°C±0.6, pH 8.1±0.5, salinity 28±3 ppt, and dissolved oxygen concentration were 5.7±0.8 (mg/l). The ammonia and nitrite content in the water was maintained at the permissible levels.

2.2: Experimental groups: The different experimental groups were as follows: Control(C): fed with the control diet **,**Treatment 0 (B0) (LBDO) Served As Control.Treatment 1 (B1): fed with a diet containing 0.25 probiotics (LBD1),Treatment 2 (B2): fed with a diet containing 0.50 probiotics (LBD2),Treatment 3 (B3): fed with a diet containing 0.70 probiotics (LBD3),Treatment 4 (B4) fed with a diet containing 1.00 probiotics (LBD4).

2.3: Feeding trial:

The fishes were fed with prepared pelleted diets at a rate of 5% of the bodyweight every day. Two times feeding trials were implemented the first feeding trial was feed at 9 AM and 6 PM. Ingredients of the present diet were shown in table 1

2.4 Immunological and blood parameter studies:

2.4 .I: Collection of Blood: At the end of the experimental period, fishes from each treatment group were anesthetized with clove oil ($50\mu lL^{-1}$) and blood was collected from the caudal vein using a medical syringe, which was previously rinsed with 2.7% Ethylene Diamine Tetra Acetic Acid (EDTA) solution. The blood was then transferred immediately to a test tube containing a pinch of EDTA powder (as an anticoagulant) and shaken gently to prevent haemolysis of blood. The blood was used for determination of Haemoglobin content, total erythrocyte and leukocyte count, Haematocrit value**,** nitroblue tetrazolium activity (NBT) and neutrophil adherence/ NBT assay.

2.4. II: (RBC) Total Erythrocyte Count: Blood (20 µl) was mixed with 3980 µl of RBC diluting fluid in a clean test tube. The mixture was shaken well to suspend the cells uniformly in the solution. A small drop of this mixture was charged to Neubauer's counting chamber of a haemocytometer. Care was taken to avoid air bubbles trapped. The red blood cells were counted in five groups of squares. All the cells lying inside the five small squares are counted under high power (40X) of the light microscope. The following formula is used to calculate the number of RBC per µl of the blood sample:

$$\text{Number of RBC } \mu l^{-1} = \frac{\text{N x dilution}}{\text{Area counted x Depth of fluid}}$$

$$= (N \times 200)/ (0.2 \times 0.1)$$

$$= N \times 10000 \times 10^3$$

Where N is the total number of red blood cells counted in 5 squares of the hemocytometer.

2.4. III: (WBC) Total Leukocyte Count:: Twenty microliters of blood were mixed with 3980 µl of WBC diluting fluid in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. Care was taken that there were no air bubbles trapped. The numbers of cells were counted in four big squares under high power (40X) magnification of the light microscope. The number of WBC per µl of the blood sample was calculated using the following formula:

$$\text{Number of WBC } \mu l^{-1} = \frac{\text{N x dilution}}{\text{Area counted x Depth of fluid}}$$

$$= 3\,(N \times 200)/\,(4 \times 0.1)$$

$$= 3\,N\,X\,500$$

Where N is the total number of white blood cells counted in 4 squares of the hemocytometer.

2.4. IV: (Hb) Haemoglobin Content: The Haemoglobin level of blood was analyzed by the cyanme tHaemoglobin method using Drabkins Fluid (Qualigens). Blood (20 µl) was mixed with 5 ml of Drabkin's working solution. The absorbance was measured using a spectrophotometer at a wavelength of 540 nm. The final concentration was calculated by comparing it with the standard cyanmet Haemoglobin (Qualigens Diagnostics). The haemoglobin concentration was then calculated by using the following formula:

$$\text{Haemoglobin } (gdL^{-1}) = [OD\,(T)/OD(S)] \times [251/1000] \times 60$$

Where, OD (T) = Absorbance of test OD (S) = Absorbance of standard

$$\text{Number of WBC } \mu l^{-1} = \frac{\text{N x dilution}}{\text{Area counted x Depth of fluid}}$$

$$= (N \times 200)/\,(4 \times 0.1)$$

$$= N\,X\,500$$

Where N denotes the total number of white blood cells counted in 4 squares of the hemocytometer.

2.4. V: level of Haematocrit: : For estimating the Haematocrit, level Haematocrit capillary tubes were two-third filled with the whole blood and centrifuged for 5min and the percentage of the packed cell-volume was determined by the Haematocrit tube reader.

2.4. VI: (NBT) Nitroblue tetrazolium assay:: Nitroblue tetrazolium assay was done by the method of Secombes (1990) 0.05ml of blood was placed into the wells of flat bottom microtitre plate and incubated at 37^0C for 1hr to facilitate adhesion of cells. The supernatant was removed and the loaded wells were washed three times with PBS. Then 0.05 ml of 0.2% NBT was added and the plate was incubated for further 1hr. The cells were then fixed in 100% methanol for 2-3 minutes and again washed thrice with 70% methanol and allowed to air dry. 0.12ml of 2N potassium hydroxide and 0.14 ml dimethyl sulphoxide were added into the wells to dissolve the formazone blue precipitate formed. The optical density of the colored solution was then read at 620 nm in an ELISA plate reader (iMark Microplate Reader S/N 10489, Bio-Rad).

2.4. VII: Adherence of Neutrophil Assay:: The NBT-glass adherent assay was performed by placing 0.1 ml of blood on a glass coverslip and incubating for 30 min at room temperature. The coverslip was then gently washed with phosphate-buffered saline (PBS). Then 0.1 ml of 0.2% NBT was placed on a microscope slide and covered by the coverslip with the adhering cells and incubated at room temperature for 30 min with the NBT solution. The activated neutrophils were then counted under a microscope (x400) **(Anderson, et al. 1992).** [2]

A/G ratio was calculated by dividing albumin values by globulin values.

2.6: Challenge study with *Aeromonas spp.*

Aeromonas spp. was grown on nutrient broth for 24 hours at 28°C. After 24 hours the bacterial culture was centrifuged at 3000 rpm for 10 minutes. The pellet was washed three times in PBS (pH – 7.4) and resuspended in PBS and the final concentration was adjusted to 1.6×10^7 CFU ml^{-1}. After 8 weeks experimental period, fishes in all the experimental groups were injected intraperitoneally with 0.2ml of *Aeromonas spp.* suspension. The mortality of fishes in all groups was observed for 7 days. The relative percentage of survival was calculated using the following formula.

$$\text{Relative percentage survival} = \frac{\text{No. of survived fish after challenge} \times 100}{\text{No. of fish injected with bacteria}}$$

3: RESULTS AND DISCUSSION:

3.1. I: (RBC) Total Erythrocyte count:

The result of different probiotic levels of a diet containing *lactobacillus spp*. on the total Erythrocyte count ($X10^6$ cells/µl) on *Channa marulius* fingerlings were shown in Table 3.2 and FIG.31. The RBC count significantly differed ($P\leq0.05$) in all the experimental groups, with the higher count in the B3 group which fed by a probiotic diet containing *lactobacillus spp.* In concentration with 0.70 Diet-3 (2.75 ± 0.15) followed by B1 (2.21 ± 0.10) fed with diet containing 0.25 probiotic, B2 (1.70 ± 0.10) fed with diet containing 0.50 probiotic B4 (1.69 ± 0.15) fed with diet containing 1.00 probiotic and control group B0 (1.22 ± 0.05) respectively.

3.1. II: Total Leukocyte count (WBC):

The effect of different probiotic treatments on the total leukocyte count ($X\ 10^3$ cells/ µl) of *Channa marulius* fingerlings is presented in Table 3.2 and FIG.3.2. The WBC count was significantly higher ($P\leq0.05$) in the B3 group (190 ± 2.1) which differed from all the other groups. The lower WBC count was observed in the control group (130 ± 1.68).

3.1. III: Haemoglobin content (Hb):

The effect of different probiotic treatments on the Haemoglobin content (g %) of *Channa marulius* fingerlings is presented in Table 3.2 and FIG.3 The Haemoglobin content showed a significant difference ($P\leq0.05$) between the different treatments and the control. Higher content was found in B3 (9.12 ± 0.24) fed with a diet containing 0.70% probiotic. Followed by B1 (8.4 ± 0.15) fed with diet containing 0.25 probiotic and B2 (7.40 ± 0.16) fed with diet containing 0.50 probiotic respectively, and lowest Hb was in control (6.51 ± 0.18).

3.1. IV: Haematocrit level:

Data related to the Haematocrit level (%) of *Channa marulius* fingerlings of different experimental groups are presented in Table 3.2 and FIG.3.4. The Haematocrit level of B3 group fishes (36.5± 1.50) fed with diet containing 0.70% probiotics was found to be significantly higher ($P\leq0.05$) than the other groups, followed by B1 and B2, B4 respectively. The control group fishes showed a lower Haematocrit level (15.5 ± 1.12).

3.1. V: Nitroblue tetrazolium (NBT) activity/ Respiratory burst activity:

The effect of different probiotic treatments on the nitroblue tetrazolium activity (OD at 620nm) of *Channa marulius* fingerlings is presented in Table 2 and FIG.5. The respiratory burst activity of all the probiotic treated groups was significantly higher ($P\leq0.05$) than the control group fishes. Group B3 was observed to be with the highest NBT activity (0.69 ± 0.04) fed with diet containing 0.70 probiotics (D3) whereas the control group fishes were with the lowest NBT activity (0.29^a±0.03).

3.1. VI: Neutrophil adherence/ NBT assay:

The effect of different probiotic treatments on the neutrophil adherence activity of *Channa marulius* fingerlings is presented in Table 3.2 and FIG.6. The number of adherent neutrophils was significantly higher ($P\leq0.05$) in all the probiotic treated groups compared to the control

(7.98 ± 0.48). There was no significant difference (P≥0.05) between the treatment groups.

3.3.5: Percentage survival after challenge study:

The percentage survival of *Channa marulius* fingerlings of different experimental groups after challenging with *A. hydrophila* graphically represented in FIG.A The percentage survival of fishes of the entire probiotic treated group was significantly higher (P≤0.05) than the control. There was no significant difference (P≥0.05) between the probiotic treated groups. The higher survival was in the B3 group (80.95 ± 2.4), followed by B1 (78.57 ± 4.1), B2, B3 (71.23 ± 4.1) and control (50 ± 4.1) respectively.

3.4.1: Haemato Immunological Parameters

The blood properties of fishes vary with seasons (Fange, 1992) and the number of teleost erythrocytes varies with species and is also affected by stress and environmental temperature, but they usually range between 1.05×10^6 μL^{-1} and 3×10^6 μL^{-1} (Roberts and Ellis, 2001). In the present investigation, the RBC count significantly differed in all the experimental groups, with the higher count in B3 fed with diet containing 0.70 probiotics (LBD3, followed by B1 (2.21 ± 0.10) fed with diet containing 0.25 probiotic, B2 (1.70 ± 0.10) fed with diet containing 0.50 probiotic B4 (1.69 ± 0.15) fed with diet containing 1.00 probiotic and control group B0 (1.22 ± 0.05) respectively. A similar result was shown by **Azarin et al., 2015** [3] he marks the fishes which were fed with probiotic diets shown to strengthen in the RBCs levels as compared with control.

Harikrishnan et al., 2010 [4] noted that the red blood cells and Haemoglobin content of probiotic treated *Cirrhinus mrigala* infected with *Aphanomyces invadans* did not show any change (P ≥ 0.05) compared to control group whereas in the infected untreated fish it was down significantly (P ≤ 0.05) over the control value for 30 days

As in other vertebrates, most teleosts have Haemoglobin in their erythrocytes [5] The content of Haemoglobin also showed a similar pattern as that of erythrocytes

Hb showed a significant difference between the different treatments and with the control with higher content in B3 fed with diet containing 0.70 probiotics (LBD3 by Followed by B1 fed with diet containing 0.25 probiotic and B2 fed with diet containing 0.50 probiotic respectively and B4 fed with diet containing 1.00 probiotic (LBD4), and lowest Hb was in control (6.51 ± 0.18).

This may be attributed to the fact that in most teleosts the Haemoglobin is contained in the erythrocytes.

Some research had been reported increase in the Haemoglobin levels. In these reports fishes fed probiotic-diets were indicated to have improved health condition compared to those fed control diets *Lactococcus sporogenes* in *Clarius batrachus* (Indian magur) [6] and combined dosage of these probiotics *L. sporogenes*, *L. acidophilus*, *B. subtilis*, *B. licheniformis*, *Saccharomyces cervirial* in *Cirrihinus mrigal* [7].

In the present study the WBC count was found to be significantly higher in B3 group (fed with diet containing 0.70 probiotics (LBD3) which differed from all the other groups. The lower WBC count was observed in the control group. A similar result was obtained [8] who reported higher leukocyte count in rohu, treated with probiotic bacterium *B. subtilis*.

In most teleosts, Haematocrit level ranges between 20-40% of blood [9]. Reduced Haematocrit value can indicate that fish are not eating or are suffering infections [10]. In the present study, the Haematocrit level (38.7 ± 1.53) of *Lactobacillus casei* treated group fishes was found to be significantly higher than the other three experimental groups, which indicates the efficiency of *L. casei* to improve the health status of the fish. The probiotic bacterium *L. casei* was found to induce the activation of the gut mucosal immune system through innate immunity when orally administered in mice [11,12] were observed that the level of

WBCs increased in Rainbow trout feed with the diet containing *B. subtilis.*

Respiratory bursts are produced by phagocytes to attack invasive pathogens during phagocytosis and have been widely used to evaluate the defense ability against pathogens. However, the excessive accumulation of reactive oxygen intermediates (ROIs) is extremely toxic to host cells [13]. The respiratory burst activity of all the probiotic treated groups was significantly higher than the control group fishes, with the highest NBT activity in group B3 (diet supplemented with *L. casei*). Salinas [14] reported increased respiratory burst activity on the incubation of seabream (*Sparus aurata*) leukocytes with 51M6, *L. delbrueckii subspp*. *lactis* and *B. subtilis*. All three bacteria gave increased values. A similar result was noticed [15] in *O. mykiss* when fed with *L. rhamnosus* at 7.9 $\times 10^4$ CFU g^{-1} feed for 2 weeks.

4.2: Percentage Survival after Challenge Study:

The percentage survival of fishes of the entire probiotic treated group after challenging with *Aeromonas spp.*. was observed to be significantly higher than the control, however, in between the treatment groups, there was no significant difference in the survival rate. This may be due to the protective effect of the probiotic bacteria against the pathogenic *Aeromonas spp*. Similar results were obtained by Aly et al., (2008a) when he evaluated the probiotic activity of two bacteria (*Bacillus subtilis* and *Lactobacillus acidophilus*) by its effect on the immune response of Nile tilapia (*Oreochromis niloticus*), and the in-vitro antimicrobial assay showed that *Bacillus subtilis* and *Lactobacillus acidophilus* inhibited the growth of *A. hydrophila*. Increased survival was also noticed .

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TABLES AND FIGURES: the data were shown separately

In tables and figures.

Table 1: Ingredient Content of Five Experimental Diets with Different Levels of Probiotics (g $100g^{-1}$ of Diet).

Ingredient	Experimental Diets				
	LBDO (control)	LBD1	LBD2	LBD3	LBD4
Groundnut oil cake	60.00	60.00	60.00	60.00	60.00
Rice bran	3.20	2.95	2.70	2.45	2.20
Wheat flour	3.20	3.20	3.20	3.20	3.20
Soybean	25.60	25.60	25.60	25.60	25.60
Chromic oxide (Cr_2O_3)	1.00	1.00	1.00	1.00	1.00
Beef Extract powder	7.00	7.00	7.00	7.00	7.00
Probiotics	-	0.25	0.50	0.70	1.00

Table 2: Immunological and Haematological Parameter of *Channa marulius* Fingerlings:

Experimental groups	Immunological/ Haematological Parameters					
	RBC*	WBC**	HB***	HCT****	NBT #	Adherence of Neutrophil*#
B1	2.21c±0.10	160c±1.2o	8.4c±0.15	28.5c±0.9o	0.44b±0.08	13b±0.67
B2	1.70b±0.10	144b±2.90	7.40b±0.16	20.3b±0.60	0.44b±0.03	11.50b±0.60
B3	2.75d±0.15	190d±2.1	9.12d±0.24	36.5d±1.50	0.69c±0.04	12.53b±0.82
B4	1.69b±0.10	142b±2.60	7.38b±0.13	21.2b±0.62	23.2b±0.73	10.30b±0.37
B0	1.22a±0.05	130a±1.68	6.51a±0.18	15.5a±1.12	0.29a±0.03	7.98a±0.48

The mean values bearing dissimilar superscript vary significantly (P≤0.05).

*($\times 10^6 \mu l^{-1}$), ** ($\times 10^3 \mu l^{-1}$), *** (gdL^{-1}), **** (%), # (OD at 620nm),*# (cells/ field)

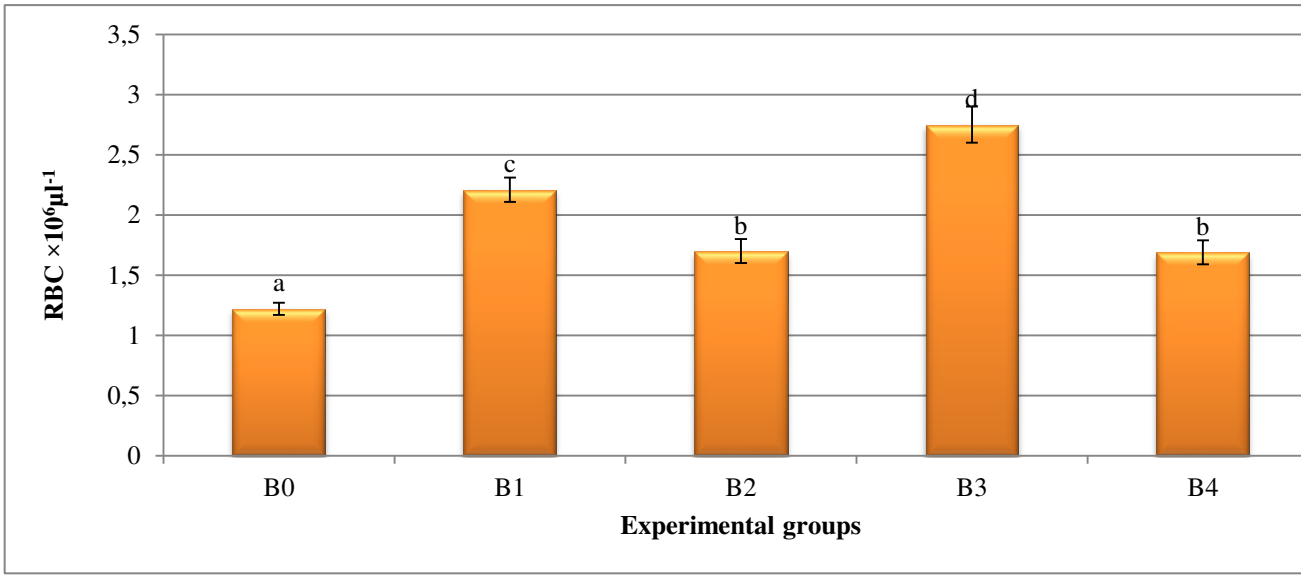

FIG.1: Total erythrocyte count (RBC) content of *Channa marulius* fingerlings of different experimental groups against *Aeromonas spp*.

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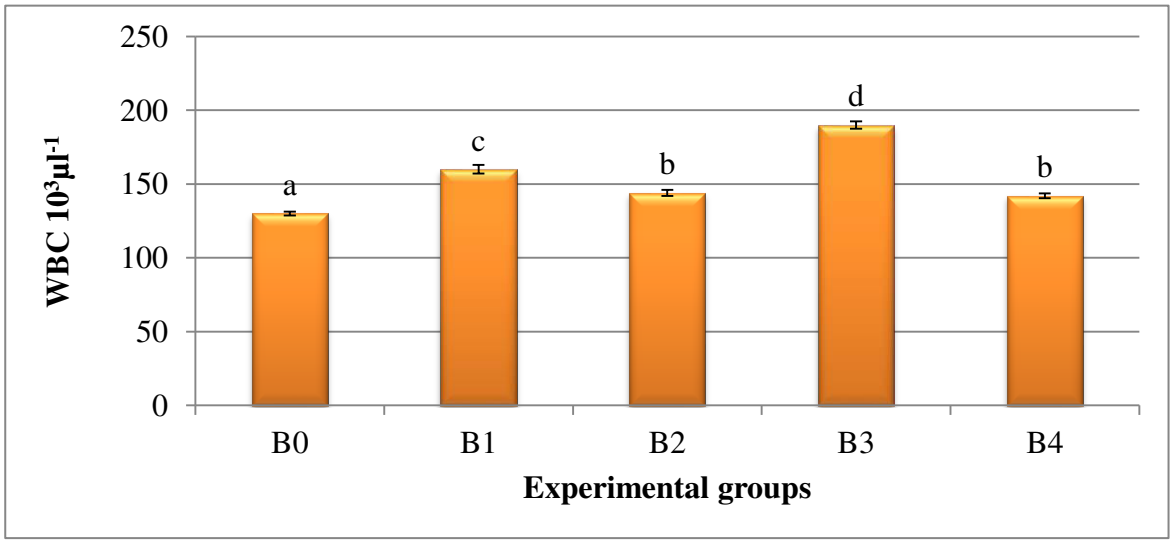

FIG. 2: WBC count of *Channa marulius* fingerlings of different experimental groups against *Aeromonas spp*

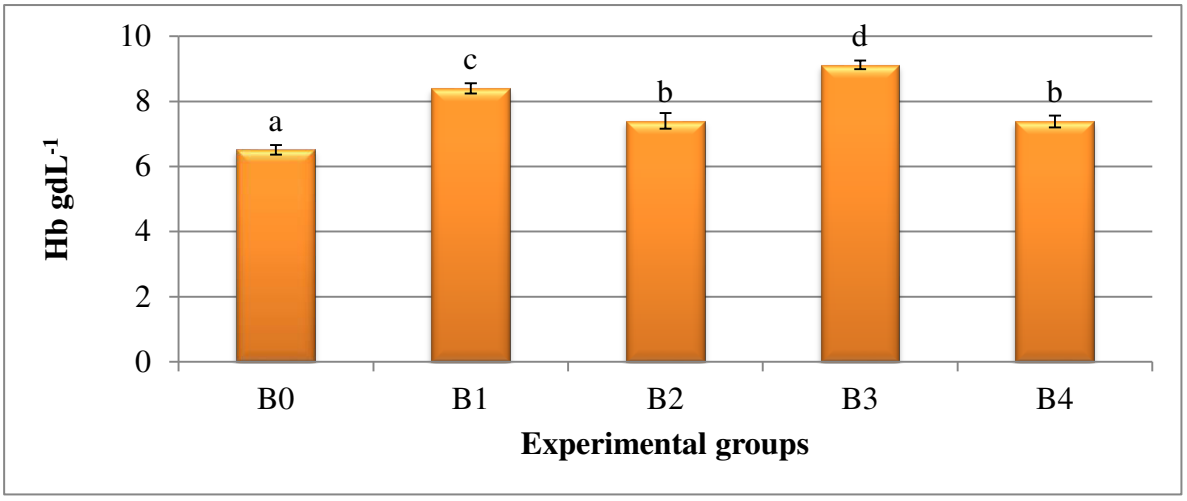

FIG.3: Haemoglobin content (gdL^{-1}) of *Channa marulius* fingerlings of different experimental groups against *Aeromonas spp*.

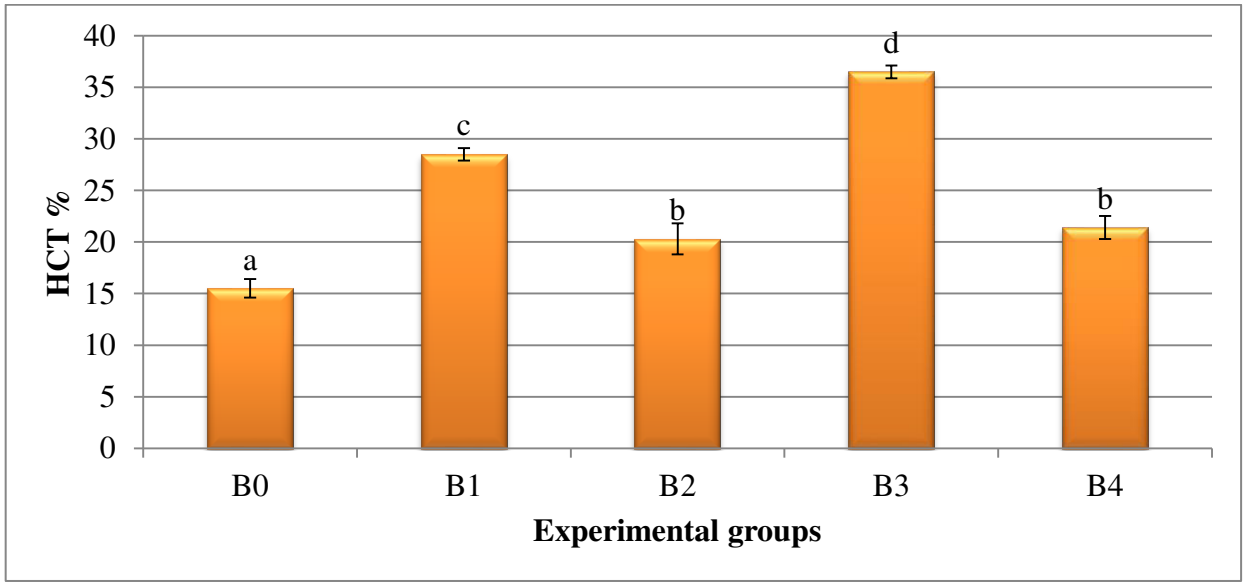

FIG.4: Haematocrit level (HCT) of *Channa marulius* fingerlings of different experimental groups against *Aeromonas spp*.

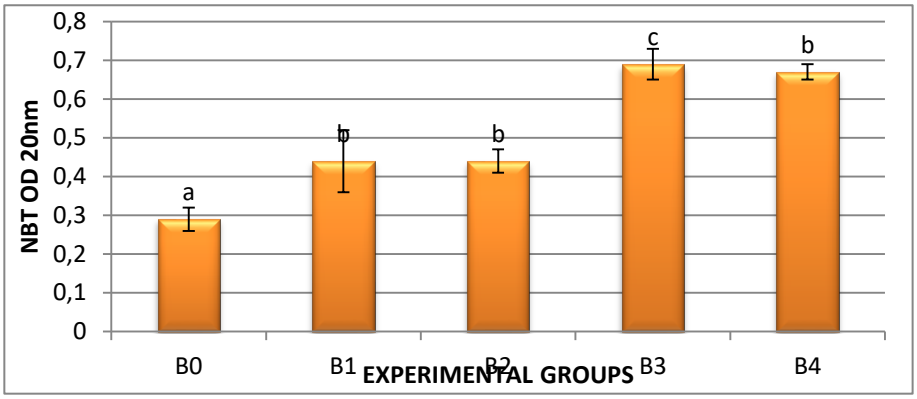

FIG.5: Respiratory burst activity (NBT-OD/620nm) of *Channa marulius* fingerlings of different experimental groups against *Aeromonas spp*.

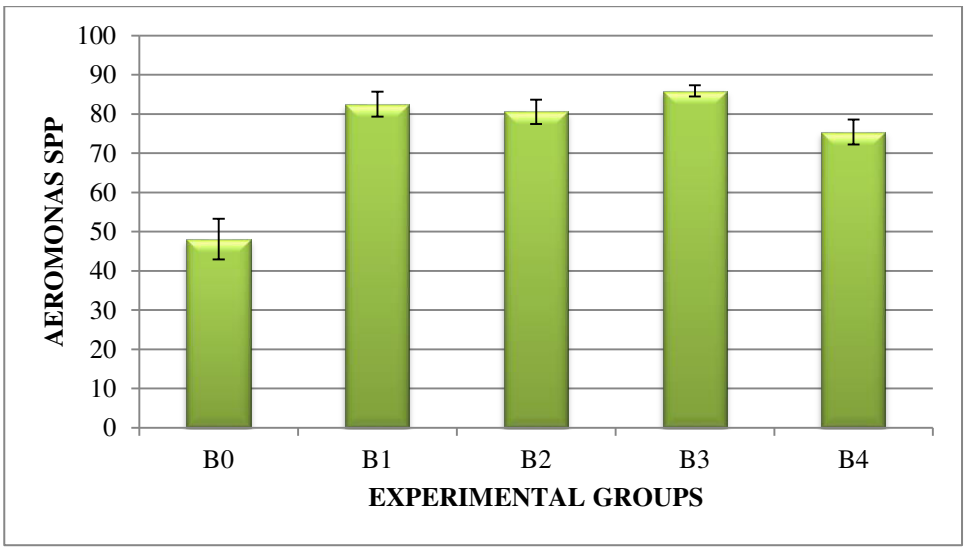

FIG 6 : Percentage survival after challenge study with *Aeromonas spp.*