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Increase in growth and haematological parameters by *Bacillus licheniformis* dietary supplementation to climbing perch, *Anabas testudineus* (Bloch, 1792)

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Abstract
An experiment was conducted for 60 days to assess the probiotic potential of *Bacillus licheniformis*, a Gram-positive, aerobic, spore forming bacterium in climbing perch, *Anabas testudineus* juvenile having average weight of 10±2 g. fifteen rectangular fiber tanks of 100 liters capacity were taken for study and each tank was stocked randomly with 12 fishes. Treatment groups T_1, T_2, T_3 and T_4 were fed with four different diet containing (1.0×10^7 cfu g^{-1}, 0.5×10^8 cfu g^{-1}, 1.0×10^8 cfu g^{-1}, and 0.5×10^9 cfu g^{-1} diet respectively) *B. licheniformis* for 60 days and the control group (C) was fed with diet without *B. licheniformis* for the same period. The growth, haematological and serum parameters were monitored at fortnight interval. In treatment groups the weight gain (WG) and specific growth rate (SGR) were significantly high ($p<0.05$) in comparision to control. Fishes fed with diet containing 1.0×10^8 cfu g^{-1} *B. licheniformis* showed highest weight gain (87.47±2.98%) and SGR (1.47±0.03). The TEC and haemoglobin content increased significantly in treatment groups in comparision to control group. The highest TEC (2.08±0.04 x $10^6/mm^3$) and haemoglobin content (7.47±0.02 g%) was observed in T_4. The present study suggests that *B. licheniformis* at 1.0×10^8 cfu g^{-1} feed could be used in climbing perch farming to enhance growth and haematological parameters.

Keywords: Haematological parameters, *Anabas testudineus*, *Bacillus licheniformis*, probiotic, Growth,

Food conversion ratio.

1. Introduction
The fast growth of aquaculture in recent years has led to emergence of disease problems in fishes. These diseases are the major limiting factors in intensive fish culture. In recent years, probiotics is becoming an attractive approach to control disease occurrences in fishes [4]. The use of probiotics in the culture of aquatic organisms is increasing with the demand for more environment-friendly aquaculture practices [8]. Probiotics are the beneficial microorganisms, which protect the host from diseases. Fuller [7] defined probiotics as "the live microbial feed supplement which beneficially affect the host by imposing its intestinal microbial balance. Probiotic protection can be due to different mechanisms such as nutritional competition or production of antibacterial substances [13, 14].
Bacillus spores are being used as probiotics for human and animal consumption due to their immunostimulatory properties on the gastrointestinal immune system [3]. *Bacillus* when used as dietary supplementation significantly increases growth rate [21, 2, 19, 12]. *Bacillus* secretes many enzymes that degrade slime and biofilms and allow their antibiotics to penetrate slime layer around Gram negative bacteria. *Bacillus licheniformis* is a Gram-positive, oxidase-positive and catalase-positive endospore forming non-pathogenic bacterium [3, 15]. It produces wide range of extracellular enzymes and has been reported as a probiotic bacterium for terrestrial animals [1, 9, 6]. Though research has already begun, but there is an urgent requirement to test this bacterium for their probiotic potential on different aquatic animals. The *B. licheniformis* along with *B. subtilis* mixture as feed additive tried for rainbow trout and found that it enhances resistance in fish against infection with *Yersinia ruckeri* [16]. Kumar *et al*. [12] demonstrated that dietary supplementation of *B. licheniformis* improves growth, feed efficiency and enhance protection against bacterial infection by increasing response in *M. rosenbergii*. *B. licheniformis* isolated from *Chitala chitala* exhibited proteolytic and lipolytic activity [15].

However, climbing perch *Anabas testudineus* is one of the promising candidate species for freshwater aquaculture and its culture is gaining momentum in eastern part of India [11, 10]. This fish also encounters disease problems when cultured in high density. Therefore, present study was aimed to assess the effect of *B. licheniformis* on growth and haematological parameters of *A. testudineus.*

2. Material and methods

2.1 Experimental animals

Animals used for experimental purpose were advanced fingerlings of climbing perch, *Anabas testudineus* with an average weight of 10±2 g. The fishes were procured from local market, Gaya and transported in a big circular container (500 L) with sufficient aeration to the laboratory. Fishes were acclimatized and maintained on normal diet for two weeks. The physico-chemical characteristics of the water were as follows: temperature, 25-28 °C; hardness, 78 mg L^{-1} (as $CaCO_3$); alkalinity, 91 mg L^{-1}; pH, 7.6; and dissolved oxygen concentration, 5.6 mg L^{-1}.

2.2 Culture of *Bacillus licheniformis*

The bacterial isolate of *Bacillus licheniformis* was inoculated into test tube containing Nutrient broth and kept in incubator for 24 hours at 30 °C. After which a loopful of the bacterial culture was streaked on Nutrient Agar (Himedia). The bacterial colonies, which grew on the Nutrient agar, after 24 hours of incubation period at 30 °C, were either circular, irregular, wrinkled or mucoid in appearance. The colonies were confirmed as pure isolate of *Bacillus licheniformis* by performing the essential biochemical tests for confirmation. The pure culture of *Bacillus licheniformis* was inoculated into nutrient broth containing conical flask (250 ml), and incubated at 28 ^{0}C for 24 hrs in a shaker incubator. The culture was centrifuged at 12,000 rpm for 15 min at 4 ^{0}C. The supernatant was discarded, while the pellet was re-suspended in Phosphate buffer saline (pH 7.2). The suspension was similarly washed and centrifuged for 3 times and then quantified by spread plate technique.

2.3 Formulation and Preparation of Experimental Diets

Commercial ingredients such as rice bran, oil cake, fish meal, soya meal, wheat flour, corn flour, sunflower oil, vitamin and mineral mixture (Agrimin), vitamin C (Roche) and vitamin B complex (Becosules) were taken for feed formulation (Table 1). Five diets were prepared of which four contained *B. licheniformis* in different concentrations. The diets were C (control), T_1 (1.0 x 10^7 cfu g^{-1}), T_2 (0.5 x 10^8 cfu g^{-1}), T_3 (1.0 x 10^8 cfu g^{-1}) and T_4 (0.5 x 10^9 cfu g^{-1}).

All the ingredients were weighed properly as per the requirement and kept in a big plastic container. The required mixed ingredients were then mixed to form dough with the addition of the necessary quantity of water. When the dough was formed, the calculated concentration of the oils were incorporated in it and mixed well. The dough was then transferred to an aluminum container, which was then placed in a pressure cooker for cooking/ steaming. The steaming was done for half an hour. The pressure cooker was then removed from the flame and kept aside for cooling. The steamed dough was taken out and was cooled further. When the steamed dough was completely cooled, vitamins and minerals along with the *B. licheniformis* were added so as to prevent their loss. After incorporation of these elements, the dough was mixed properly and pressed through a hand pelletizer to get uniform sized pellets. These pellets were spread on a sheet of paper and initially fan dried. After that the feed was transferred to trays and kept in sunlight for complete drying. After drying, pellets were packed in polythene bags, sealed airtight and labeled according to the treatments.

Table 1: Composition of experimental diet

Ingredients	Percentage (%)
Rice bran	28
Mustered oil cake	25
Fish meal	25
Wheat flour	15
Sunflower oil	4
Vitamin and minerals	2
CMC	1

2.4 Experimental Design

Fifteen rectangular fiber tanks of 100 liters capacity were arranged in the lab of Department of Zoology with aeration facilities. The tanks were filled with 60 liters of water, 2 days prior to the experiment. A total of 12 fishes were stocked in each fiber tank. Three tanks were kept as control, while the other twelve were treatment tanks. Feed was given at 3% of body weight twice daily at 08:00 and 16:00 h. fecal matter and uneaten feed were siphoned out daily with about 50% water exchange. Treatment groups T_1, T_2, T_3 and T_4 were fed with four different concentrations (1.0 x 10^7 cfu g^{-1}, 0.5 x 10^8 cfu $g^{-}1$, 1.0 x 10^8 cfu g^{-1}, and 0.5 x 10^9 cfu g^{-1} respectively) of *Bacillus licheniformis* for 60 days and the control groups (C) were fed with feed without *B. licheniformis* for the same period. On 0^{th}, 15^{th}, 30^{th}, 45^{th} and 60^{th} day samples were collected for different studies.

2.5 Growth parameters

The weight of each fish in an experimental group was recorded individually at every time of sampling and then biomass was determined to adjust the amount of feed for each experimental group. These data were used in the calculation of specific growth rate and feed conversion ratio. At the end of feeding trial, weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) for the experimental group was calculated.

2.5.1 Weight gain (WG)

Samplings were done at intervals of 15 days to assess the body weight of the fishes. Fishes were starved overnight before taking the weight. The weight was taken in an electric balance.

The percentage weight gain was calculated using the following formula

$$\text{Weight gain (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

2.5.2 Specific growth rate (SGR)

The Specific growth rate was calculated by the following formula

$$\text{SGR (\%)} = \frac{\text{Log}_e \text{ final weight} - \text{Log}_e \text{ initial weight}}{\text{Number of days}} \times 100$$

2.5.3 Feed conversion ratio (FCR)

The Feed conversion ratio was calculated by the following

formula

$$FCR = \frac{\text{Feed given (dry weight)}}{\text{Body weight gain (wet weight)}}$$

2.6 Haematological Studies

Blood was drawn from the fish using a sterile syringe of 1 ml, which was rinsed first with 2.7% EDTA (Qualigens) solution. Blood was collected in small glass vials wherein 20 µl of 2.7% EDTA solution was coated by drying the vials in hot air oven (Newtronic). The amount of hemoglobin present in the blood was measured from 20 µl of blood using cyanmethemoglobin method. Another 20 µl of blood was used to determine the total erythrocyte and the total leucocyte counts. Blood smear for analysis of the differential leucocyte count was prepared from a drop of blood on a grease free slide.

2.6.1 Total erythrocyte count

For the total erythrocyte count (TEC, [17]) 20 µl of blood was mixed with 3980 µl of red blood cell (RBC) diluting fluid in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. The cells were counted using a hemocytometer (Feinoptik, Blakenberg, Germany) and expressed as:

$$\text{Number of RBC/mm}^3 = N \times 10000$$

Where N is the total number of red blood cells counted in five squares of the haemocytometer.
10,000 is the factor obtained after taking into consideration the initial dilution factor.

2.6.2 Total leukocyte count

For the total leukocyte count (TLC, [17]) 20 µl of blood was mixed with 3980 µl of white blood cell (WBC) diluting fluid in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. Then the cells were counted using a hemocytometer ans expressed as:

$$\text{Number of WBC/mm}^3 = N \times 500$$

Where, N denotes the total number of white blood cells counted in 4 squares of the hemocytometer. The factor obtained after taking into consideration the initial dilution factors was 500.

2.6.3 Haemoglobin content

Blood hemoglobin content (Hb) was analyzed following the Cyanmethemoglobin method using Darbkins fluid (Qualigens Diagnostic Kit, India). Twenty microlitres of blood was mixed with 5 ml of Darbkin's working solution. The absorbance was measured using a spectrophotometer at wavelength of 540 nm. The hemoglobin content (g dL^{-1}) was calculated as follows

$$\frac{\text{Test}}{\text{Standard}} \times \frac{251}{1000} \times 60$$

2.6.4 Differential leukocyte count

For differential leukocyte count (DLC), methanol fixed blood smears were prepared after collecting blood from each groups and then stained as per standard procedure using May-Grunwald Giemsa stain. Leukocytes were counted under microscope through many fields till it reached hundred cells per slide to find out the percentage. Similarly, the percentage of monocytes, granulocytes and lymphocytes in blood were determined using a blood cell counter.

2.7 Statistical Analysis

All the data were analysed by running one way ANOVA using SPSS V. 14 software (SPSS Inc., Chicago, Illinois, USA). The means were compared using Duncan's multiple range test (Duncan, 1955) to find the difference at 5% ($P< 0.05$) level.

3. Results and Discussion

Gross morphological examination showed that fish fed with probiotic treatment appeared more active and shiny as compared to control fish. The colonization of probiotic bacteria in the gut epithelium reduces the risk of pathogenic bacterial infection and hence fish can develop the capacity to protect themselves from various diseases. The present study was carried out to evaluate the effect of probiotic bacterium *B. licheniformis* on growth and haematological parameters.

Data pertaining to the WG and FCR of the experimental groups of climbing perch is given in table 2. The WG was significantly higher ($p<0.05$) in the treatment groups (T_1, T_2, T_3 & T_4) fed feed containing *B. licheniformis* in comparison to control group (C). Among the treatment groups T_3 (87.47±2.98) & T_4 (86.47±3.60) were significantly higher in comparison to T_1 (63.27±3.75) & T_2 (74.62±4.28). Food conversion ratio (FCR) was significantly low in treatment groups in comparison to control group. Among the treatment groups T_4 (1.76±0.04) showed lowest FCR followed by T_3 (1.78±0.03), T_2 (1.87±0.04) and T_1 (2.09±0.12). The SGR was significantly higher ($p<0.05$) in treatment groups in comparision to control group and highest SGR was recorded in fishes fed with diet containing 1.0 x 10^8 cfu $g^{-1}$of *B. licheniformis*. In the present study, significant increase in growth parameters (WG and SGR) and decrease in FCR were observed in the A. testudineus fed diet containing *B. licheniformis*. Kumar *et al.* [12] also reported an increase in the weight gain and SGR in *Macrobrachium rosenbergii* fed with diet containing *B. licheniformis.* Similarly, Kumar *et al.* [14] reported significant increase in weight gain in *Labeo rohita* fed diet supplemented with *Bacillus subtilis*. Probiotic when used with feed improve digestive activity by synthesis of vitamins, cofactors as well as enzymatic activity [8, 20, 18, 12]. These properties can enhance nutrient absorption resulting in better growth.

Table 2: Effect of dietary supplementation of *B. licheniformis* on WG, FCR and SGR of *A. testudineus*

Treatments	Weight Gain (%)	FCR	SGR
C	55.54^{d}±1.44	2.65^{a}±0.14	0.93^{d} ± 0.02
T_1	63.27^{c}±3.75	2.09^{b}±0.12	1.26^{c} ± 0.03
T_2	74.62^{b}±4.28	1.87^{c}±0.04	1.35^{bc} ± 0.01
T_3	87.47^{a}±2.98	1.78^{c}±0.03	1.47^{a} ± 0.03
T_4	86.63^{a}±3.60	1.76^{c}±0.04	1.38^{ab} ± 0.02

Data pertaining to the TEC, TLC and haemoglobin content of the experimental groups of climbing perch is given in table 3. The TEC was significantly higher ($p<0.05$) in treatment groups in comparison to control group. Among the *B. licheniformis* fed groups T_4 (2.08±0.04x10^6 mm^3) showed highest TEC. There was an increase in TLC level in the T_2

($2.71\pm0.20 \times 10^4$ mm^3), T_3 ($2.69\pm0.15 \times 10^4$ mm^3) and T_4 ($2.69\pm0.15 \times 10^4$ mm^3) but not at a significant level. The haemoglobin content also increased significantly ($p<0.05$) in all the treatment groups in comparison to control and among the treatment groups T_4 (7.47±0.02 g %) showed highest haemoglobin level. The TEC and haemoglobin content were significantly higher in treatment groups in comparision to control group. The increase in TEC and haemoglobin content in *B. licheniformis* fed group are an indication of improved health of the fish. Similar finding was reported by Kumar *et al*. [14] in *L. rohita* fed with *B. subtilis*. The TLC increased in fish fed diet containing *B. licheniformis* compared with fish fed diet without *B. licheniformis*. This indicated the heightened immune response in the fish fed diet containing *B. licheniformis,* probably due to its immunostimulatory effect.

Table 3: Effect of dietary supplementation of *B. licheniformis* on TEC, TLC and Hb of *A. testudineus*

Treatments	TEC ($x10^6$/mm^3)	TLC ($x10^4$/mm^3)	Hb (%)
C	$1.76^{d}\pm0.04$	$2.64^{b}\pm0.03$	$7.07^{d}\pm0.05$
T_1	$1.88^{c}\pm0.30$	$2.60^{b}\pm0.01$	$7.18^{c}\pm0.30$
T_2	$2.00^{b}\pm0.45$	$2.71^{a}\pm0.20$	$7.32^{b}\pm0.80$
T_3	$2.07^{a}\pm0.02$	$2.69^{a}\pm0.15$	$7.46^{a}\pm041$
T_4	$2.08^{a}\pm0.04$	$2.69^{a}\pm0.02$	$7.47^{a}\pm0.02$

The differential leukocyte counts of *A. testudineus* of the experimental groups are shown in Figure 1. The number of granulocytes, lymphocytes and Basophils increased in treatments groups in comparison to control group but not at significant level ($p<0.05$). The T_3 group showed highest lymphocyte (82.00±2.00) among all the treatment groups. It has been shown that *Bacillus* strains supplementation in diet could increase disease resistance in fish through the stimulation of both the cellular and humoral immune function [22]. In the present study increased lymphocyte counts indicated the heightened humoral response in fished fed with *B. licheniformis.*

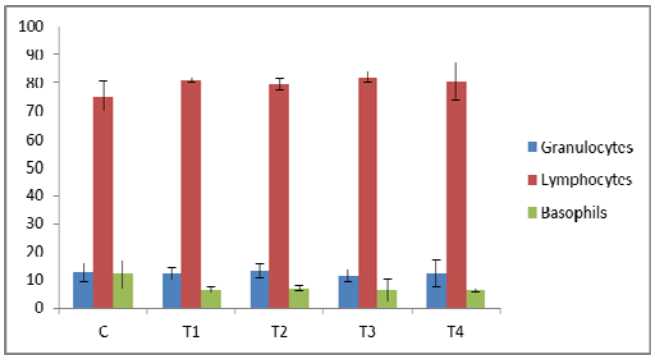

Fig 1: Effect of dietary supplementation of *B. licheniformis* on differential leukocyte counts of *A. testudineus*

4. Conclusion

The present study suggests that *B. licheniformis* at 1.0×10^8 cfu g^{-1} feed could be used in climbing perch farming to enhance growth and haematological parameters. However, further studies are required to assess the effect on immunological parameters and protection against the bacterial infection.

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